

# Autophagy is required for toll-like receptor-mediated interleukin-8 production in intestinal epithelial cells

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**Abstract.** Autophagy is an evolutionarily conserved process that maintains cellular homeostasis via synthesis, degradation, and subsequent recycling of cellular products under various physiological conditions. However, the link between autophagy and the innate immune system remains unknown. In the present study, we evaluated Toll-like receptor (TLR)-mediated autophagy induction in intestinal epithelial cells (IECs) and its relationship to interleukin (IL)-8 production. IEC-6, HCT-15, RAW264.7, and THP-1 cells were cultured with or without various TLR ligands, followed by evaluation of the expressions of pro-inflammatory cytokines [IL-8, cytokine-induced neutrophil chemoattractants (CINC)-2 $\beta$ , macrophage inflammatory protein (MIP)-2] by real-time PCR and ELISA. To reveal the status of autophagy in IECs and macrophages, light chain 3 (LC3)-II expression was examined using Western blotting and immunofluorescence with confocal microscopy. Also, to evaluate the influence of TLR ligands on autophagy-mediated innate-immune responses, autophagy-related gene (Atg)7 specific siRNA was transfected into intestinal epithelial cells and IL-8 expression was determined following exposure to various TLR ligands. Cells treated with the TLR ligands produced considerable amounts of pro-inflammatory cytokines (IL-8, CINC-2 $\beta$ , MIP-2). Furthermore, the basal levels of LC3-II were markedly higher in IECs as compared to those in macrophages. Our findings indicated that autophagy induction following TLR ligand stimulation was not significantly evident in IECs as compared to macrophages. In addition, Atg7 gene

expression silencing led to down-regulation of TLR-mediated IL-8 expression in IECs, which indicates a potential role of autophagy in generating innate-immune responses. In conclusion, autophagy may be an important intracellular machinery for inducing the innate immune system in IECs.

## Introduction

In mammalian systems, autophagy, a process that degrades cell components through the lysosomal machinery, thus helping to maintain a balance between synthesis, degradation, and subsequent recycling of cellular products is evolutionarily conserved (1). The process of autophagy is characterized by formation of double-membrane vesicles known as autophagosomes around a targeted region of the cell, mediated by the autophagy-related gene (Atg)12-Atg5-Atg16 complex and by microtubule-associated protein light chain 3 (LC3)-phospholipid conjugates (LC3-II) (2,3). The resultant vesicle then fuses with a lysosome and subsequently degrades the cellular contents (4,5). Although autophagy is recognized as a homeostatic process that enables eukaryotic cells to survive during starvation, recent studies have also revealed a variety of roles for autophagy in the regulation of cell death, differentiation, and anti-microbial responses (6-8).

Innate immunity is triggered by pattern recognition receptors (PRRs) that sense pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS), flagellin, peptidoglycans, and bacterial DNA (9,10). The Toll-like receptor (TLR) family, an important class of PRRs, is well known to induce expression of various inflammatory genes in response to microbial components, which regulates the balance between activation and inhibition of the innate immune system (11). TLRs play essential roles in gut immunity, barrier function and healing during intestinal inflammation (12-16). In particular, the monolayer of intestinal epithelial cells (IECs) is endowed with a capacity for first-line defense against microbial pathogens, which contributes to the regulation of the gut innate immunity under physiological and pathological conditions (17-20).

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The role of autophagy in the innate immunity has been emphasized in several recent publications. The link between autophagy and the innate immune system was shown by the discovery that intracellular pathogens can be eliminated from cells via a TLR-induced autophagy pathway, which may help to maintain normal homeostasis during pathogen infection (21-24). Also, genome-wide association studies recently indicated that autophagy is an essential factor in a variety of disease states including inflammatory bowel disease (IBD) (25,26). Suppression of autophagy can lead to inflammation and tissue damage resembling Crohn's disease (CD), consistent with the identification of allelic loss of Atg16L1 and immunity-related GTPase M (IRGM) as risk factors of CD development (27-29). Although TLR-dependent induction of autophagy has been noted in numerous studies (21-24), the role of autophagy in the gut innate immune system remains largely unknown. To understand the pathogenesis of innate immune-related gut disorders, it is considered important to clarify the crosstalk that occurs between the TLR-mediated pathway and autophagy in IECs, which respond directly to luminal microbial components.

In the present study, we evaluated TLR-mediated autophagy induction in IECs and compared it to that in macrophages, as well as its relationship to the production of interleukin (IL)-8. Our results indicate that IECs have a high basal level of autophagy even without stimulation by various TLR ligands, while we were interested to note that expression of the autophagy system was not altered when stimulated with those TLR ligands. In addition, we also revealed that a deficiency of the autophagy pathway caused by transfection with Atg7 siRNA significantly decreased TLR-mediated IL-8 production in IECs. These are the first known results to show that autophagy may be an essential system for regulation of TLR-mediated IL-8 production in IECs.

## Materials and methods

**Reagents.** The following reagents and antibodies (Abs) were used in our experiments: purified flagellin from *S. typhimurium* (InvivoGen, CA, USA), purified LPS, *E. coli* LPS (InvivoGen), purified Pam2CSK4 (InvivoGen), rapamycin (Sigma, St. Louis, MO, USA), Lipofectamine™ RNAiMAX (Invitrogen, CA, USA), a human IL-8 enzyme immune assay (EIA) (Invitrogen), a non-radioactive cell proliferation assay (CellTiter 96® AQueous) (Promega, Madison, WI, USA), Atg7 siRNA and control siRNA (Santa Cruz, CA, USA), anti-LC3 Ab (MBL, Nagoya, Japan), anti-p62 Ab (MBL), anti-β-actin Ab (Sigma), anti-rabbit IgG (Santa Cruz), FITC-conjugated anti-rabbit IgG (DAKO, Glostrup, Denmark), propidium iodide (Sigma), E64D (Sigma), and pepstatin A (Sigma).

**Cell cultures.** The human colorectal cancer cell line, HCT-15, the human monocytic leukemia cell line, THP-1, the rat small intestine epithelial cell line, IEC-6, and the mouse macrophage cell line, RAW264.7, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and grown in RPMI-1640 (Invitrogen) or Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific, Logan, UT, USA) and penicillin-streptomycin-amphotericin B (Invitrogen). The

cell lines were maintained at 37°C in 5% CO<sub>2</sub> in a humidified incubator.

**RNA extraction and real time-PCR.** Total RNA was extracted from each sample using Isogen (Nippon Gene, Japan), equal amounts of RNA were then reverse transcribed into cDNA using a QPCR cDNA kit (Stratagene, CA, USA). All primers (Table I) utilized were flanked by intron-exon junctions using the NCBI BLAST tool and the Primer3 software. Quantitative real-time PCR was performed using a StepOne Real-Time PCR system with SYBR-Green PCR master mix (Applied Biosystems, CA, USA), according to the manufacturer's instructions. The levels of human IL-8, rat cytokine-induced neutrophil chemoattractants (CINC)-2β, and mouse macrophage inflammatory protein (MIP)-2 mRNA were normalized to that of β-actin using a sequence detector software (Applied Biosystems).

**Protein extraction and Western blotting.** Protein extraction and Western blotting assays were performed as previously described (30,31). Briefly, cells were harvested from cultured dishes and were lysed in RIPA lysis buffer. Protein concentration was determined using the BCA protein assay kit (Thermo Scientific). Total cell lysates (20 μg) were separated by Tris-glycine SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. After blocking with 10% skim milk (Difco, Detroit, MI, USA) in PBS (pH 7.4), the membrane was reacted with anti-LC3 (1:1000), anti-p62 (1:1000), or anti-β-actin Abs (1:5000) at room temperature for 1 h, then reacted with peroxidase-conjugated anti-rabbit (1:10000) or anti-mouse (1:5000) Abs at room temperature for 1 h. The resulting signals were imaged using an ECL (GE Healthcare, Buckinghamshire, UK). To inhibit degradation of LC3-II protein, E64D and pepstatin A were used for the cultured cells.

**Confocal microscopy.** After stimulation with TLR ligands for 16 h, cells were washed in PBS and then fixed with 4% paraformaldehyde (Sigma) for 10 min at room temperature. Fixed cells were washed with PBS, immersed in 100 mg/ml of Digitonin for 15 min at room temperature, washed in PBS, and blocked in blocking buffer (X909; DAKO, Carpinteria, CA, USA) for 1 h at room temperature. Cells were subsequently incubated with the anti-LC3 antibody (PM036, MBL) for 1 h at room temperature. After 3 washes in PBS, cells were incubated with FITC-anti-rabbit Ab (DAKO) for 30 min at room temperature and then washed 3 times in PBS and subsequently incubated with propidium iodide (Sigma). Cells were observed with a Laser Scanning Confocal Microscope (Olympus, FV300), photographed at a x120 magnification, and analyzed using the Olympus confocal microscope software.

**Enzyme immune assay (EIA).** Proteins extracted from HCT-15 cells and culture supernatants were used for the assays. IL-8 contents were measured using an IL-8 EIA kit, following the manufacturer's protocol. Briefly, appropriate sample amounts were transferred by pipette into appropriate wells of anti-human IL-8-coated microtiter strips, followed by addition of a second biotinylated monoclonal Ab, then incubation was performed at room temperature for 90 min. After removing the excess secondary Ab by washing, the samples

Table I. Primer sequences.

Gene (Accession no.)	Sequences (5'-3')
Human GAPDH (NM-002046)	
Forward	CCACATCGCTCAGACACCAT
Reverse	TGACCAGGCGCCAATA
Human IL-8 (NM-000584)	
Forward	TGTGTGTAAACATGACTTCCAAGCT
Reverse	TTAGCACTCCTTGGCAAAACTG
Human Atg7 (NM-001136031)	
Forward	GATCCGGGGATTTCTTTCACG
Reverse	CAGCAATGTAAGACCAGTCAAGT
Rat GAPDH (NM-017008)	
Forward	AAGATGGTGAAGGTCGGTGT
Reverse	GATCTCGCTCCTGGAAGATG
Rat CINC-2 $\beta$ (NM-138522)	
Forward	GAGACGGGAATGCAATTTGTTT
Reverse	GGTCTGCTAGGAATGTTGTCGAT
Mouse $\beta$ -actin (NM_007393)	
Forward	GATTACTGCTCTGGCTCCTAGC
Reverse	GACTCATCGTACTCCTGCTTGC
Mouse MIP-2 (NM_009140)	
Forward	TGTC AATGCCTGAAGACCCTGCC
Reverse	AACTTTTTGACCGCCCTTGAGAGTGG

were incubated with streptavidin-peroxidase, after which a substrate solution was added to produce color that was directly proportional to the concentration of human IL-8 present in the sample. Quantitative results were obtained from a standard curve produced from the experimental findings.

**RNA interference.** HCT-15 cells were grown in 24-well plates ( $5 \times 10^4$  cells/well), then custom siRNAs (Santa Cruz) targeting the human Atg7 gene or control siRNAs were transfected (50 nM/well), according to the manufacturer's protocol. The efficiency of target gene knock-down was assessed by real-time PCR and the results were compared to those of the negative control siRNA-transfected condition. In addition, the efficacy of the Atg7 gene knockdown on autophagy induction was assessed by Western blotting for the detection of LC3-II and p62.

**Cell proliferation assay.** A non-radioactive cell proliferation assay kit was used to assess cell viability after treatment with Atg7 siRNA. HCT-15 cells were treated with control or Atg7 siRNA, then incubated with TLR ligands for 16 h, after which a cell proliferation assay was performed according to the manufacturer's protocol. The formation of formazan was determined with an EIA plate reader (Bio-Rad, Hercules, CA, USA) at 490 nm at 4 h after adding the PMS/MTS solution.

**Statistical analysis.** All data are expressed as the mean  $\pm$  standard error of the mean (SEM). Values were analyzed using the Student's t-test with the SPSS software version 10.1 (San Rafael, CA, USA). ANOVA was used for comparisons

of multiple values. P-values <0.05 were considered to be significant.

## Results

**TLR ligands stimulate production of pro-inflammatory cytokines in cultured IECs and macrophages.** Initially, we examined if IECs and macrophages can respond to TLR ligand stimulation (LPS for TLR4, flagellin for TLR5, Pam2CSK4 for TLR2). After 3 h of stimulation with the ligands, the gene expressions of IL-8, CINC-2 $\beta$  (rat counterpart of human IL-8), and MIP-2 (mouse counterpart of human IL-8) in cultured cells were examined by real-time RT-PCR. In both macrophage cell lines (RAW264.7, THP-1), all ligands tested in this study significantly induced the expressions of MIP-2 and IL-8 (Fig. 1A and B). On the other hand, though stimulation with flagellin and Pam2CSK4 markedly induced CINC-2 $\beta$  in IEC-6 cells and IL-8 in HCT-15 cells, stimulation with LPS in these cells resulted in relatively lower levels of the respective cytokines (Fig. 1C and D).

**TLR-mediated autophagy induction in IECs and macrophages.** The expression level of LC3-II, a mammalian Atg8 homologue widely used as an autophagosome marker and thought to be present on the membranes of autophagosomes, is generally correlated with the number of autophagosomes in a tested sample. In the present study, we assessed LC3-II protein levels in TLR-mediated IECs and macrophages using Western blotting and confocal microscopy. Rapamycin was used as a positive control for autophagy induction. As shown in Fig. 2A,

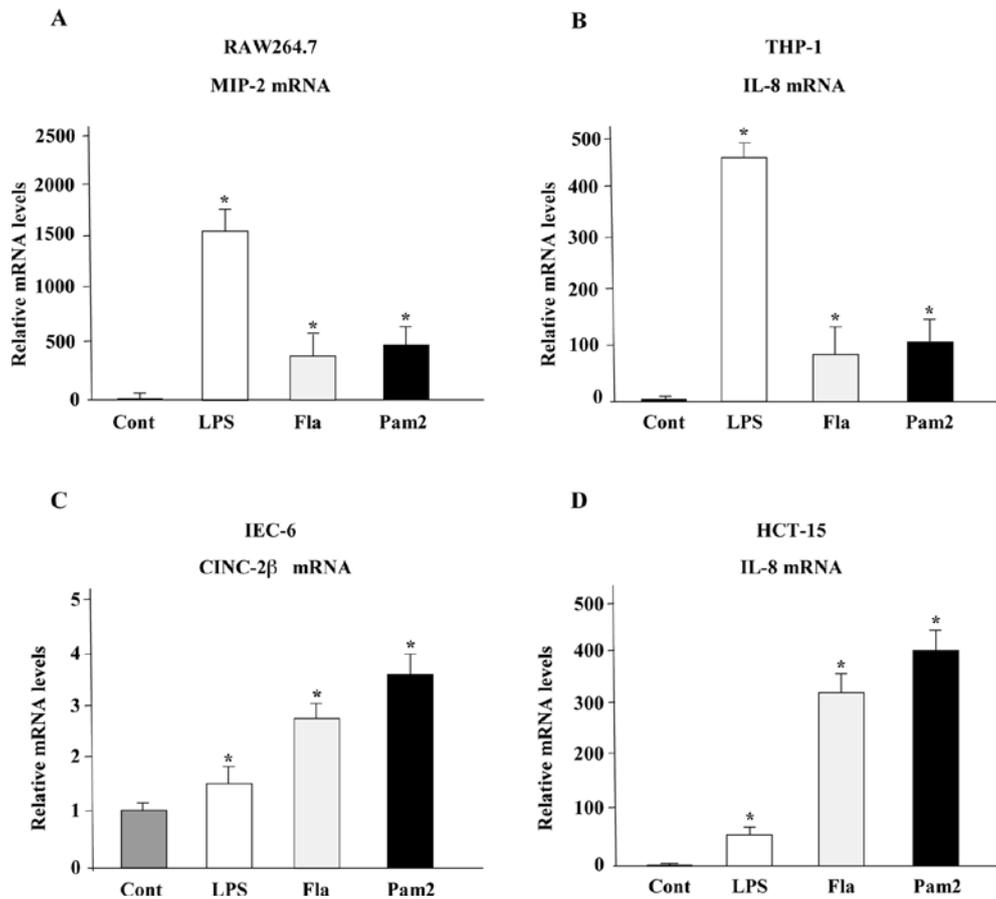


Figure 1. TLR ligand-induced production of pro-inflammatory cytokines in macrophages and IECs. RAW264.7 (A), THP-1 (B), IEC-6 (C), and HCT-15 (D) cells were incubated with various TLR ligands [LPS, 100 ng/ml; flagellin (Fla), 100 ng/ml; Pam2CSK4 (Pam2), 100 ng/ml] for 3 h. After ligand stimulation, total RNA was extracted from each sample and the expressions of IL-8, CINC-2 $\beta$  and MIP-2 were examined by real-time RT-PCR. All signals were standardized to the intensity of GAPDH. Error bars indicate the standard error of mean values obtained from 4 independent experiments. \* $P < 0.01$  vs. non-stimulated cells.

TLR4 (LPS) and TLR2 (Pam2CSK4) induced abundant levels of LC3-II in macrophages (RAW264.7, THP-1). In contrast, HCT-15 and IEC-6 cells have high basal levels of LC3-II protein in the absence of TLR ligand incubation, which were not changed even following stimulation with the TLR ligands (Fig. 2A). Furthermore, confocal microscopy revealed significant LC3-II immunoreactive signals indicating the presence of autophagosomes in the cytoplasm (green dots). The representative confocal microscopic images shown in Fig. 2B clearly show the results of Western blotting (Fig. 2B), indicating that IECs have a high basal level of LC3-II protein in the absence of TLR ligand incubation.

*Effects of Atg7 siRNA on autophagy induction and p62 protein levels in HCT-15 cells.* As shown in our experiments, IECs have a high basal level of LC3-II protein even after stimulation with TLR ligands, which was different from the basal autophagy levels observed in macrophages. Based on these findings, we focused on IECs (HCT-15 cells) and investigated the role of autophagy in TLR-stimulated IECs by using a gene knockdown method with an siRNA targeting Atg7. The siRNA used in this study showed a high gene knockdown efficacy, since basal Atg7 gene expression was decreased by 85-90% (Fig. 3A). The results of Western blotting clearly showed that knockdown of the Atg7 gene decreased LC3-II protein levels in both basal and rapamycin-treated conditions

(Fig. 3B), confirming that RNA interference by Atg7 can inhibit autophagy induction in IECs. In addition, to clearly reveal the siRNA-induced deficiency of autophagy function, we examined p62 levels after treatment with Atg7 or control siRNA by Western blotting. The p62 protein is a selective substrate for autophagy that binds ubiquitin and LC3, which regulates the formation of protein aggregates in the cytoplasm. Once autophagy is induced in cells, p62 protein is immediately degraded. As shown in Fig. 3C, the cellular p62 protein level was increased after treatment with Atg7 siRNA, suggesting that Atg7 siRNA-mediated autophagy deficiency leads to an accumulation of p62 protein in the cytoplasm.

*Knockdown of the Atg7 gene does not influence viability of HCT-15 cells in the presence of TLR ligands.* After confirming the efficacy of Atg7 siRNA in autophagy induction, we next examined whether siRNA-induced autophagy deficiency influences cell viability before and after stimulation with TLR ligands in serum. The results of our cell proliferation assay clearly show that transfection of Atg7 siRNA does not influence cell viability in the serum-treated condition (Fig. 4A). In addition, treatment with the autophagy inducer rapamycin had no influence on cell viability (Fig. 4B).

*Autophagy is required for TLR-mediated IL-8 secretion by HCT-15 cells.* After confirming the efficacy of siRNA, the

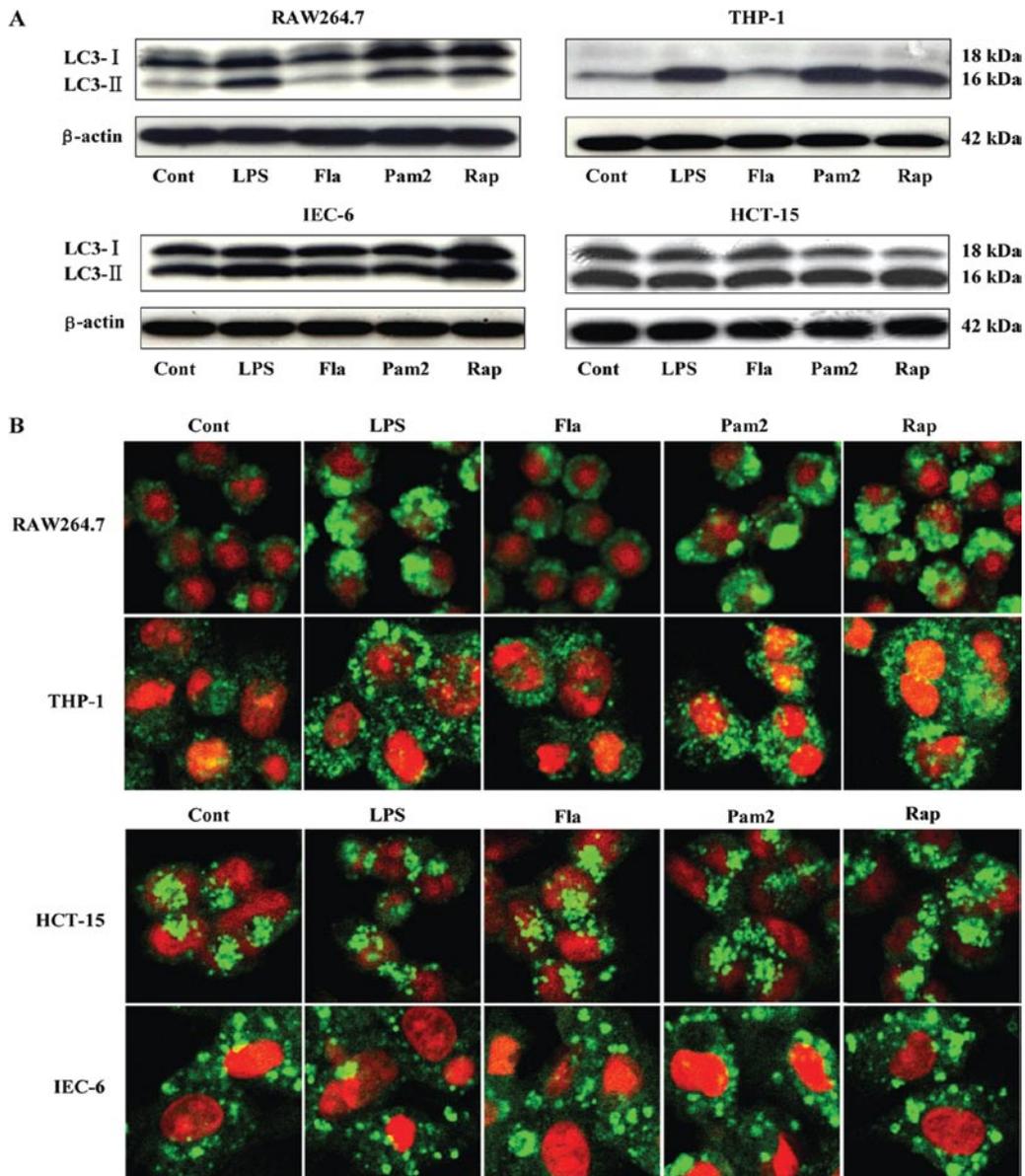


Figure 2. Representative images of TLR-induced autophagy-related protein LC3-II in IECs and macrophages. RAW264.7, THP-1, HCT-15, and IEC-6 cells were incubated with various TLR ligands [LPS, 100 ng/ml; flagellin (Fla), 100 ng/ml; Pam2CSK4 (Pam2), 100 ng/ml] for 16 h. Rapamycin was used as a positive control for autophagy induction. Total LC3-II protein levels and cellular localization were examined by Western blotting (A) and confocal microscopy (B), respectively. Immunoreactive signals (green dots) from LC3-II indicate the presence of autophagosomes in the cytoplasm (B).

role of autophagy in TLR-mediated inflammatory responses in IECs was examined. Control or Atg7 siRNAs were transfected into HCT-15 cells, after which the cells were stimulated with various TLR ligands for 16 h and the IL-8 contents in the culture supernatants were measured using EIA. As shown in Fig. 5A, TLR ligand-induced IL-8 mRNA expression in Atg7 siRNA-treated cells was significantly lower than that in control siRNA-treated cells. In addition, Atg7 siRNA treatment significantly inhibited TLR-mediated IL-8 protein production (Fig. 5A). To further confirm the role of autophagy, we induced autophagy in IECs by treatment with rapamycin and also examined the TLR-mediated production of IL-8. After stimulation with the TLR ligands, IL-8 mRNA expression and IL-8 contents in the culture supernatants of rapamycin-treated cells were significantly increased as compared to the control cells (Fig. 5B).

## Discussion

In the present study, we found that IECs have a high basal level of autophagy, which did not increase following stimulation with various TLR ligands. In addition, our results clearly demonstrated that Atg7 siRNA-induced inhibition of basal autophagy significantly decreased TLR-mediated IL-8 production in IECs, suggesting that the presence of basal autophagy in IECs is essential for TLR-mediated inflammatory responses to maintain the innate immune system in the gut.

TLRs are sensors of microbial products that initiate host defense responses in various organs (9-12). There is abundant evidence indicating that TLRs play a vital role in maintaining intestinal epithelial homeostasis as well as regulating inflammation, while dysregulation of TLR-mediated signaling leads

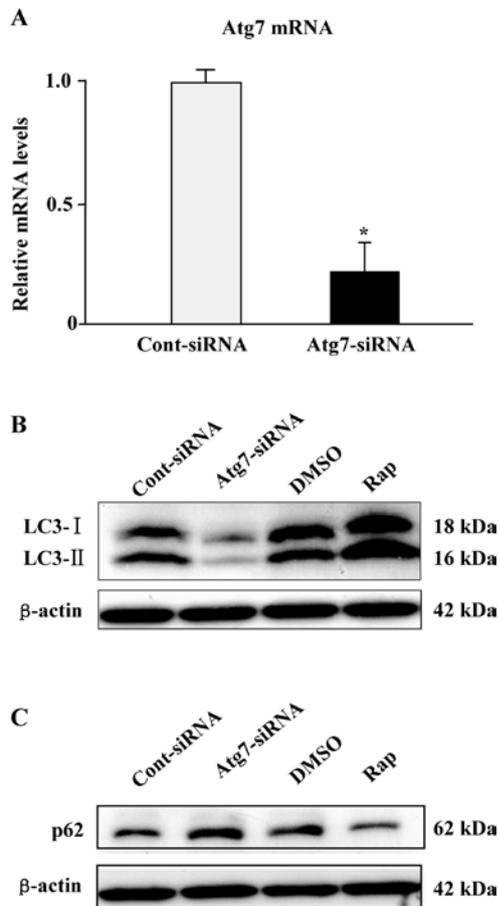


Figure 3. Efficacy of Atg7 siRNA on autophagy induction and p62 protein levels in HCT-15 cells. (A) The efficacy of Atg7 siRNA was evaluated by real-time PCR and compared to that of control siRNA. Error bars indicate the standard error of mean values obtained from 3 independent experiments. \* $P < 0.01$  vs. control siRNA. (B) and (C) Representative images of Western blotting showing LC3-II and p62 levels in HCT-15 cells treated with control siRNA, Atg7 siRNA, DMSO (vehicle), and rapamycin.

to development of several gut immune disorders (13-19). Autophagy is also an important physiological process that controls a variety of cellular functions closely associated with the innate immune system (21-24). Although recent findings suggest crosstalk between autophagy and TLR signaling in the intestinal tract, that process remains largely unknown. To investigate this issue, we performed several different *in vitro* experiments in the present study.

To investigate the effects of stimulation with TLR ligands on autophagy induction, we initially used macrophage cell lines in our *in vitro* experiments. Stimulation with LPS and Pam2CSK, but not flagellin, significantly increased cellular LC3-II protein levels in RAW264.7 and THP-1 cells, showing that TLR4 and TLR2 are essential sensors for autophagy induction in macrophages. Recently, Xu *et al* reported that LPS induced autophagy in RAW264.7 cells, which was found to be regulated through a pathway that is dependent on the Toll-IL-1 receptor domain containing adaptor-inducing interferon- $\beta$  (TRIF), while it was independent of the myeloid differentiation factor 88 (MyD88) (32). Delgado *et al* also demonstrated that activation of TLR4 and TLR7 was associated with autophagy induction in RAW264.7 cells. On the other hand, it has been reported that TLR5 is not a significant

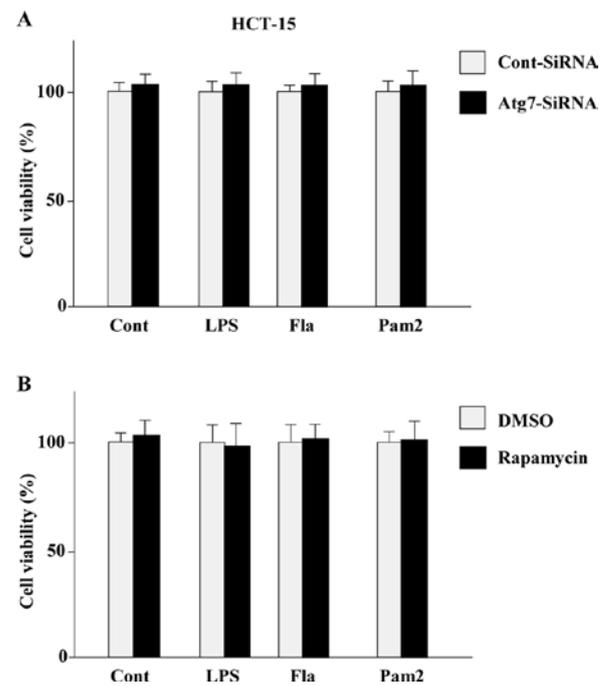


Figure 4. Results of cell proliferation assays. HCT-15 cells were treated with (A) siRNA and (B) rapamycin (Rap), then incubated with various TLR ligands [LPS, 100 ng/ml; flagellin (Fla), 100 ng/ml; Pam2CSK4 (Pam2), 100 ng/ml] for 16 h and cell viability was assessed. Transfection of Atg7 siRNA did not influence cell viability in the serum-treated condition. Error bars indicate the standard error of mean values obtained from 3 independent experiments.

sensor for autophagy induction in RAW264.7 cells or bone marrow macrophages (23). These recent findings support our results obtained with the macrophage cell lines.

In contrast to those findings, we found that HCT-15 and IEC-6 cells have high basal levels of autophagy, as determined by the presence of converted LC3-II protein in the cells. Several studies have shown that basal levels of LC3-II protein were detected in cultured IEC lines, including IEC-6, IEC-18, Colon-26, and HT-29 (33-35). Others have also reported that immune-reactive signals of LC3-II were observed in epithelial cells from mice and human intestinal tissue sections. Although various stimuli including serum starvation, glutamine treatment, and  $\gamma$ -irradiation induce significant autophagy in IECs (33-36), little is known regarding the effect of TLR activation in that process. In the present study, we found that Pam2CSK and flagellin markedly stimulated pro-inflammatory cytokines in HCT-15 and IEC-6 cells. However, these TLR ligands did not have an influence on autophagy induction in these cells. Lee *et al* previously indicated an inability to detect induction of autophagy in TLR7-mediated plasmacytoid dendritic cells (pDCs) (37). Furthermore, Schmid *et al* noted that a high level of constitutive autophagosome formation was observed in DCs, which was not increased after incubation with various immunological agonists (38). Also, a recent study revealed that TLR ligands including LPS did not influence autophagy induction in mice primary cultured macrophages isolated from the peritoneal cavity and bone marrow (27). Together, these findings suggest the possibility that basal autophagy and TLR-mediated autophagy are cell type- and TLR ligand-dependent.

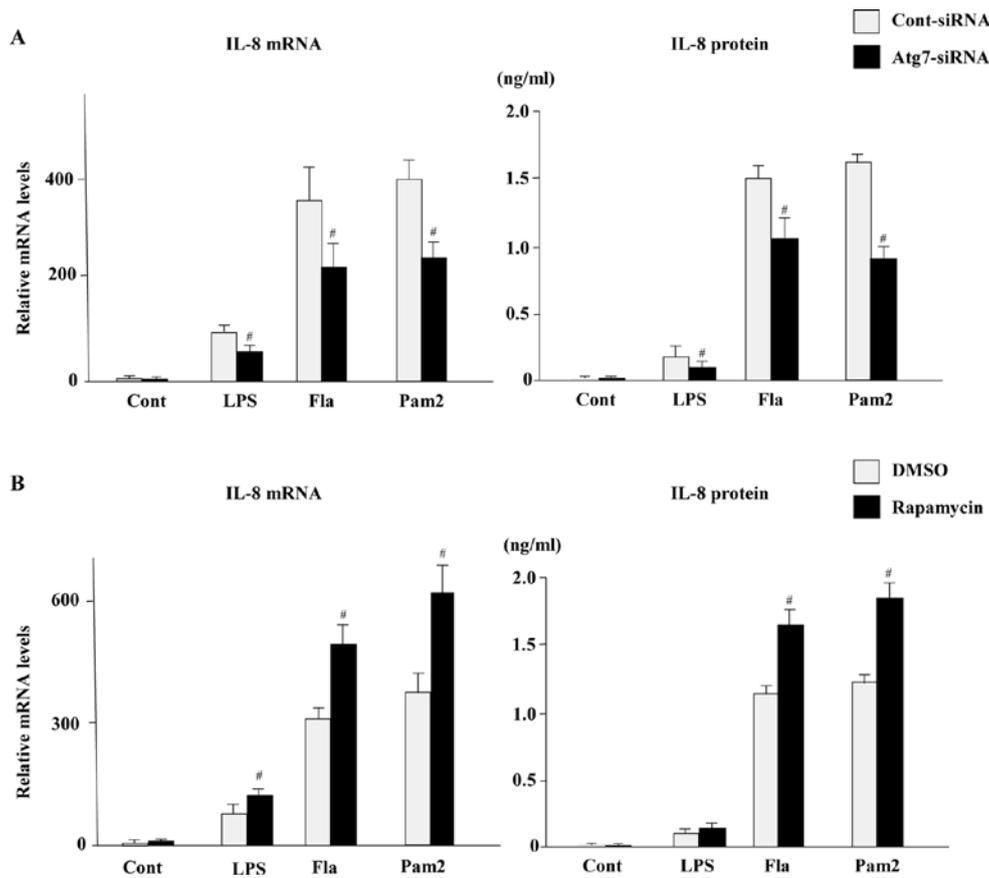


Figure 5. Effects of (A) Atg7 siRNA and (B) rapamycin (Rap) treatment on TLR-mediated IL-8 mRNA expression and IL-8 protein production in HCT-15 cells. HCT-15 cells were treated with siRNA or rapamycin, then incubated with TLR ligands [LPS, 100 ng/ml; flagellin (Fla), 100 ng/ml; Pam2CSK4 (Pam2), 100 ng/ml] for 3 h, after which IL-8 mRNA levels were evaluated by real-time PCR. All signals were standardized to the intensity of GAPDH. IL-8 protein contents in the culture supernatants at 16 h after treatment with TLR ligands were examined by EIA. Error bars indicate the standard error of mean values obtained from 3 independent experiments. #P<0.05 vs. control siRNA or DMSO.

Based on our results, we speculated that high basal levels of autophagy may have certain roles in TLR-mediated inflammatory responses in IECs. Thus, we employed an *in vitro* system using a gene knockdown method with an siRNA targeting Atg7, an essential gene for autophagy induction. Although gene knockdown of Atg7 did not influence cell viability, TLR ligand-induced IL-8 secretion by Atg7 siRNA-treated cells was significantly lower than that by control siRNA-treated HCT-15 cells. Sasaki *et al* demonstrated that the secretions of CCL2/monocyte chemoattractant protein-1 (MCP-1) and CX3CL1/Fractalkine in cultured biliary epithelial cells induced by various stress factors, such as oxidative stress and serum starvation, were significantly decreased by treatment with the autophagy inhibitor 3-MA (39). Furthermore, a recent study using peripheral blood macrophages clearly revealed that 3-MA or Atg5 siRNA-mediated inhibition of autophagy suppressed influenza virus-induced CXCL10 and interferon- $\alpha$  (IFN- $\alpha$ ) (40). These findings support our present results and suggest the possibility that autophagy is required for production of inflammatory cytokines in response to a variety of stimuli, including TLR ligands, in epithelial cells. The mucosal neutrophil response is induced when bacteria stimulate epithelial cells in various organs to secrete several chemokines such as IL-8. Hang *et al* also found that IL-8 receptor knockout mice have a dysfunctional migration of neutrophils leading to urinary tract infection, which results in

tissue destruction (41). Taken together, autophagy may have an essential contribution to maintain innate immune response by regulating inflammatory cytokine production in IECs.

Several studies of crosstalk between autophagy-related genes and intestinal inflammation have been recently reported. Saitoh *et al* demonstrated that mice lacking Atg16L1 in hematopoietic cells are highly susceptible to dextran sulphate sodium (DSS)-induced experimental acute colitis (27). They also found enhanced levels of LPS-induced IL-1 $\beta$  production in macrophages isolated from this mice strain, indicating that Atg16L1 is an important autophagy-related gene for the inhibition of TLR4-mediated inflammatory response. Apart from the present evidence regarding autophagy functions in macrophages, Cadwell *et al* found that Atg16L1 or Atg5 mutation leads to disruption of the Paneth cell granule exocytosis pathway, suggesting that abnormal Paneth cell functions may be associated with the pathogenesis of CD (42). Thus, we consider that autophagy has various essential roles in gut immunity, which may be dependent on the cell type and the type of autophagy-regulatory genes.

In summary, we investigated TLR-mediated autophagy induction in IECs, as well as the role of autophagy in IL-8 production in TLR-activated IECs. We have demonstrated that IECs have a high basal level of autophagy, which essentially regulates TLR-mediated IL-8 production in these cells. Our findings provide new insight into crosstalk that occurs

between autophagy and TLR signaling in IECs. Additional investigations will be necessary to elucidate the precise roles of autophagy in the pathogenesis of intestinal immune disorders.

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