# Crucial role for autophagy in degranulation of mast cells

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**Background: Autophagy plays a crucial role in controlling various biological responses including starvation, homeostatic turnover of long-lived proteins, and invasion of bacteria. However, a role for autophagy in development and/or function of mast cells is unknown.**

**Objective: To investigate a role for autophagy in mast cells, we generated bone marrow–derived mast cells (BMMCs) from mice lacking autophagy related gene *(Atg) 7*, an essential enzyme for autophagy induction.**

**Methods: Bone marrow–derived mast cells were generated from bone marrow cells of control and IFN-inducible *Atg7*-deficient mice, and morphologic and functional analyses were performed.**

**Results: We found that conversion of type I to type II light chain (LC3)-II, a hallmark of autophagy, was constitutively induced in mast cells under full nutrient conditions, and LC3-II localized in secretory granules of mast cells. Although deletion of *Atg7* did not impair the development of BMMCs, $Atg7^{-/-}$ BMMCs showed severe impairment of degranulation, but not cytokine production on FcεRI cross-linking. Intriguingly, LC3-II but not LC3-I was co-localized with CD63, a secretory lysosomal marker, and was released extracellularly along with degranulation in $Atg7^{+/+}$ but not $Atg7^{-/-}$ BMMCs. Moreover, passive cutaneous anaphylaxis reactions were severely impaired in mast cell-deficient WBB6F1-$W/W^V$ mice reconstituted with $Atg7^{-/-}$ BMMCs compared with $Atg7^{+/+}$ BMMCs.**

**Conclusion: These results suggest that autophagy is not essential for the development but plays a crucial role in degranulation of mast cells. Thus, autophagy might be a potential target to treat allergic diseases in which mast cells are critically involved.**
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***Key words:*** *Mast cell, autophagy, CD63, degranulation, p62, light chain 3 (LC3)*

Autophagy is an evolutionarily conserved bulk degradation system in all eukaryotes that controls the clearance and reuse of intracellular constituents and is important for the maintenance of amino acid pools essential for survival.[1,2] This is initiated by the process in which organelles and cytosolic components are sequestered in double-membraned vesicles (so-called *autophagosomes*) and then delivered to lysosomes, where the autophagic cargo is degraded.[2] Yeast genetic screening studies have identified a variety of essential components of autophagy machinery, called autophagy related gene *(Atg)* genes, that are phylogenetically highly conserved. Among them, a mammalian counterpart of *Atg8*, light chain (LC)–3 localizes on autophagosomes through conversion of LC3-I to LC3-II by its C-terminal lipidification in an *Atg5*-dependent and *Atg7*-dependent fashion.[1,2] Although LC3-I diffusely distributes in cytoplasm, LC3-II shows a punctuate distribution. Accumulating studies using *Atg5*-deficient or *Atg7*-deficient mice have revealed that autophagy controls a variety of biological responses including aging, development, neurodegenerative diseases, intracellular bacterial infection, and cancer.[1,2] Intriguingly, autophagy has been shown to be critically involved in the packaging of antimicrobial peptides into granules in Paneth cells, thereby promoting efficient export of microbial peptides into the lumen of the gut.[3-5] Moreover, autophagy is also involved in the homeostasis of pancreatic β cells through degradation of toxic cytoplasmic components, such as damaged organelles and ubiquitinylated proteins.[6,7]

Mast cells express high-affinity receptors for IgE (FcεRI) on the cell surface and are able to release histamine, leukotriene $C_4$ ($LTC_4$), and other preformed chemical mediators on cross-linking of FcεRI receptors by polyvalent antigen, thereby playing a crucial role in allergic inflammatory reactions.[8,9] Mast cells are also involved in protection of the host from bacterial infection through production of TNF-α in response to bacteria components, such as LPS.[10-12] Most of these functions of mast cells are mediated by regulated exocytosis of specific secretory granules that are induced by antigen stimulation. Secretory granules in mast cells are considered to be secretory lysosomes that bridge the secretory and endocytic pathways. Selective transport of granular contents to granules of mast cells is considered to be regulated by synaptotagmins, mammalian homologue of Caenorhabditis elegans unc (Munc) 13-14, and rab27a.[13] However, the detailed mechanisms underlying sorting of granular contents to granules, and

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*Abbreviations used*

*Atg*: Autophagy related gene
BM: Bone marrow
BMMC: Bone marrow–derived mast cell
DC: Dendritic cell
FcεRI: High affinity receptor for IgE
GM-CSF: Granulocyte macrophage colony-stimulating factor
GFP: Green fluorescence protein
LC3: Light chain 3
LC3-I: Type I light chain 3
LC3-II: Type II light chain 3
$LTC_4$: Leukotriene $C_4$
M-CSF: Macrophage colony-stimulating factor
MFI: Mean fluorescence intensity
MVBs: Multivesicular bodies
PCA: Passive cutaneous anaphylaxis
PMA: Phorbol 12-myristate 13-acetate
Poly I:C: Polyinosinic-polycytidylic acid
SCF: Stem cell factor
siRNA: Small interfering RNA
SNARE: Soluble N-ethylmaleimide–sensitive factor attachment protein receptor

regulated exocytosis of granular contents by specific soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE), are not fully understood.[14] Because we found that conversion of LC3-I to LC3-II was constitutively induced in bone marrow (BM)–derived mast cells (BMMCs) under full nutrient conditions, we investigate a contribution of autophagy to the development and/or function of mast cells by using IFN-inducible *Atg7*-deficient mice.

## METHODS

### Mice

*Atg7*$^{Flox\ (F)/Flox\ (F)}$, *Atg7*$^{F/F}$*:Mx*, *Atg7*$^{F/F}$*:Mx:p62*$^{+/-}$, and *Atg7*$^{F/F}$*:Mx:p62*$^{-/-}$ mice were described previously.[15,16] Green fluorescence protein *(Gfp)-Lc3* transgenic mice were described elsewhere.[17] WBB6F1-+/+, WBB6F1-*W/W*$^{V}$, and C57BL/6 mice were purchased from Japan Clea (Tokyo, Japan). To induce deletion of *Atg7* gene, 8-week-old to 12-week-old *Atg7*$^{F/F}$*:Mx*, *Atg7*$^{F/F}$*:Mx:p62*$^{+/-}$, and *Atg7*$^{F/F}$*:Mx:p62*$^{-/-}$ mice were injected with 300 μg polyinosinic-polycytidylic acid (poly I:C; Sigma-Aldrich, Tokyo, Japan) 3 times every other day. At 2 weeks after the first poly I:C injection, BM cells from indicated genotyped mice were prepared as described and used for induction of BMMCs. In parallel experiments, *Mx-cre* negative flox mice were similarly injected with poly I:C, and BM cells were used to generate control BMMCs. Deletion of floxed alleles of *Atg7* genes was confirmed by PCR using BM cells or BMMCs. All experiments were performed according to the guidelines approved by the Institutional Animal Experiments Committee of Juntendo University School of Medicine.

### Antibodies

Anti-GFP (Medical and Biological Laboratories [MBL], Nagoya, Japan), anti-LC3 (MBL), anti–β-actin (Sigma-Aldrich), anti-CD63 (MBL), antiubiquitin (MBL), anti–c-kit (BD Bioscience, Franklin Lakes, NJ), anti-FcεRI (eBioscience, San Diego, Calif), and anti-p62 (Stressgen, Brussels, Belgium) were purchased from indicated sources. Rabbit anti-LC3 antibody was generated and described previously.[15]

### Cells

Bone marrow–derived mast cells were generated as previously described.[10] Briefly, BM cells were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS, 100 μmol/L 2-mercaptoethanol, 10 μmol/L minimum essential medium-nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 ng/mL murine IL-3 (Peprotech, Rocky Hill, NJ), or IL-3 + 10 or 100 ng/mL murine stem cell factor (SCF; Peprotech), for 5 to 8 weeks. After 5 weeks of culture, more than 98% of cells that were positive for both c-kit and FcεRI on the cell surface by flow cytometry and Diff-Quick staining were considered to be mature mast cells. A human mast cell line, laboratory of allergic diseases (LAD)2 cells (provided by Dr Arnold Kirshenbaum), were maintained as described previously.[18] Generation of macrophage colony-stimulating factor (M-CSF)–induced macrophages, granulocyte macrophage colony-stimulating factor (GM-CSF)–induced dendritic cells (DCs), and Flt3-induced DCs was described previously.[19]

### Expression vectors

A lentiviral expression vector for Cherry fused to LC3 (Cherry-LC3) was generated as a standard method. Briefly, a full-length fragment of human LC3 was generated by PCR using pEGFP-LC3 (provided by Dr Isei Tanida) as a template, and subcloned into pmCherry-C1 (Clontech). Then, a Cherry-LC3 fragment was transferred into a lentiviral vector, CSII-EF-MCS (provided by Dr Hiroyuki Miyoshi), and designated plenti-Cherry-LC3. pCR-Cherry-LC3 was constructed by inserting a Cherry-LC3 fragment into pCR-3 (Invitrogen, Carlsbad, Calif). An expression vector for CD63-GFP was generated by subcloning murine CD63 into pEGFP-N1 (Clontech, Mountain View, Calif).

### Generation of BMMCs stably expressing Cherry-LC3

Production of *Lentivirus* and infection of cells with *Lentivirus* were described elsewhere.[20] Briefly, 293T cells were transiently transfected with plenti-mCherry-LC3 along with packaging vectors, pCAG-HIV-gp, and pCMV-VSV-G-RSV-REV. At 48 hours after transfection, culture supernatants were collected and used for infection. BMMCs were incubated with the culture supernatants containing *Lentivirus* for 48 hours and then used for experiments.

### Transient transfection of BMMCs

Bone marrow–derived mast cells were transiently transfected with expression vectors for pCR-Cherry-LC3 and pEGFP-CD63 by using a Nucleofector (Amaxa; Program Y-01, Solution V, Lonsa Group Ltd, Basel, Switzerland). At 24 hours after transfection, cells were used for subsequent experiments.

### Knockdown of *Atg12* by small interfering (si) RNA

Wild-type BMMCs were transfected with control or *Atg12* siRNA (Applied Biosystems Japan, Tokyo, Japan) by using the Nucleofector (Amaxa; Program T-30, Solution V). Gene silencing efficiency was determined by quantitative (q)PCR. BMMCs were used for experiments 24 hours after transfection.

### Degranulation of mast cells

Bone marrow–derived mast cells were sensitized with 1 μg/mL anti-trinitrophenol (TNP) IgE mAb (clone IgE-3; BD Biosciences) for 1 hour and then suspended at $1 \times 10^6$ cells/mL in Tyrode buffer containing 0.1% BSA. Cells were stimulated with indicated concentrations of anti-IgE antibody (BD Biosciences) or 1.6 nmol/L phorbol 12-myristate 13-acetate (PMA) plus 1 μmol/L ionomycin (Sigma-Aldrich) for 40 minutes at 37°C unless otherwise indicated. Degranulation of mast cells was determined by β-hexosaminidase and histamine release as described previously.[10] Degranulation was expressed as net percent release of β-hexosaminidase ([OD: stimulated – unstimulated/OD: total lysate – unstimulated] × 100%). There were no significant differences in the spontaneous release of β-hexosaminidase from *Atg7*$^{F/F}$-BMMCs and *Atg7*$^{\Delta BM}$-BMMCs (results; means ± SD; 10.007 ± 2.105% and 10.649 ± 1.262%, respectively). Concentrations of histamine in the same supernatants were determined by histamine competitive ELISA kit (Oxford Biomedical Research, Inc, Rochester Hills, Mich) according to the manufacturer's instructions, and degranulation was expressed as percent release of histamine as described in β-hexosaminidase.