

# Autophagy in Immune Cell Regulation and Dysregulation

Akanksha Chaturvedi, PhD, and Susan K. Pierce, PhD

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## Corresponding author

Akanksha Chaturvedi, PhD

Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Twinbrook II, 12441 Parklawn Drive, Room 213, MSC 8180, Rockville, MD 20852, USA.

E-mail: [achaturvedi@niaid.nih.gov](mailto:achaturvedi@niaid.nih.gov)

Current Allergy and Asthma Reports 2009, 9:341–346

Current Medicine Group LLC ISSN 1529-7322

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Autophagy is an ancient pathway required for cell and tissue homeostasis and differentiation. Initially thought to be a process leading to cell death, autophagy is currently viewed as a beneficial catabolic process that promotes cell survival under starvation conditions by sequestering components of the cytoplasm, including misfolded proteins, protein aggregates, and damaged organelles, and targeting them for lysosome-mediated degradation. In this way, autophagy plays a role in maintaining a balance between degradation and recycling of cellular material. The importance of autophagy is underscored by the fact that malfunctioning of this pathway results in neurodegeneration, cancer, susceptibility to microbial infection, and premature aging. Autophagy occurs in almost all cell types, including immune cells. Recent advances in the field suggest that autophagy plays a central role in regulating the immune system at multiple levels. In this review, we focus on recent developments in the area of autophagy-mediated modulation of immune responses.

## Introduction

Autophagy is a complex, highly conserved cellular process that functions in the lysosomal-mediated degradation and turnover of intracellular macromolecules, including long-lived proteins and damaged organelles [1–3]. The term, which means “self-eating,” was first coined by de Duve in describing cells undergoing various forms of autolysis [4]. It was initially thought to be simply a mechanism of cellular self-destruction. Recent advances, however, provide evidence for autophagy’s roles in promoting cellular homeostasis, tissue remodeling, and the overall health of the organism [1]. Deficiencies in autophagy are

linked to susceptibility to severe microbial infections, neurodegenerative disorders, cancer, and premature aging. Many autophagy-related diseases are characterized by an accumulation of aberrant, aggregation-prone proteins [5]. Although almost all cell types display constitutive autophagic activity under nutrient-rich conditions, autophagy is strongly induced in cells under various stress conditions (eg, starvation, heat, oxidative stress, accumulation of damaged organelles, and infection). Under stress conditions, autophagy functions as a catabolic pathway, degrading unnecessary cellular components, which results in the recycling of nutrients necessary for cell survival.

A central component of the most common autophagy process, macroautophagy, is a double-membraned vesicle 300 to 900 nm in diameter that is termed an *autophagosome*. Autophagosome formation is initiated by the induction of a double-membraned, cup-shaped structure—termed an *isolation membrane*—that sequesters cytoplasmic cargo [6]. The contents of the autophagosomes are then delivered to the lysosomes through direct fusion of the autophagosome with late endosomes or lysosomes, forming amphisomes and autolysosomes, respectively. The inner membrane of the autophagosome is degraded along with the sequestered cargo, and the resulting macromolecular products are released back into the cytosol [6]. The origin of the isolation membrane is not known but may involve the trans-Golgi network, endoplasmic reticulum cisternae, or mitochondria [7–9]. Isolation membranes present in the cytosol of resting mammalian cells were observed to form autophagosomes upon induction of autophagy [10]. At least 31 genes essential for autophagy—termed *ATG*—were identified in yeast [11]. Orthologs of many of the yeast *ATG* genes were identified in higher eukaryotes; their individual roles in the formation of autophagosomes were reviewed elsewhere [11]. Autophagosome formation requires two evolutionary conserved ubiquitin-like conjugation systems: the *Atg12–Atg5* and the *Atg8* (LC3)–phosphatidylethanolamine systems. One of the crucial components of autophagosome formation is *Becclin-1*, the mammalian homologue of yeast *Atg6*, which is a subunit of the class III phosphatidylinositol 3-kinase (III PI3K) complex. Activation of PI3K, one of the first steps in autophagosome formation, serves to localize other components of the autophagy pathway to the pre-autophagosomal membrane. Inhibitors of

III PI3K, both 3-methyl adenine (3-MA) and wortmannin, are widely used to block autophagosome formation.

The process of autophagy is divided into three general categories: microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA). Microautophagy and macroautophagy involve membrane rearrangements to sequester components of the cytoplasm that are not observed in CMA. In microautophagy, a portion of the cytoplasm is taken up directly at the lysosome surface via invagination of the limiting membrane of the lysosome. In contrast, as described previously, in macroautophagy, a cup-shaped, double-membraned vesicle is formed within the cytoplasm that sequesters cytoplasmic cargo. Cargo-containing vesicles subsequently form autophagosomes that ultimately fuse with lysosomes. CMA, on the other hand, involves the translocation of soluble proteins that contain a signal peptide to the lysosomal membrane by a mechanism dependent on the chaperone, Hsc70. The process of autophagy can be selective and nonselective. In nonselective autophagy, multiple cytoplasmic structures are removed by what seems like a random process, whereas a precise and exclusive elimination of particular structures is the hallmark of selective autophagy. Both microautophagy and macroautophagy function selectively and nonselectively, but CMA works only selectively. The selective form of autophagy directs the elimination of intracellular pathogens, including bacteria, viruses, and protozoans. In this review, we focus on macroautophagy, referred to simply as autophagy hereafter.

### Role of Autophagy in Immune Cells

It recently has been appreciated that the autophagic degradation pathway plays a role in regulation in the immune system. Autophagy plays a direct role in host defense by selectively eliminating intracellular pathogens. Indeed, autophagy is referred to as an innate immune mechanism that monitors cells for the presence of pathogens and engulfs them into the autophagosomes for their targeting to the lysosome—a process termed *xenophagy*. Increasing evidence suggests that autophagy plays a much broader role in the immune system beyond the direct elimination of pathogens. The primary function of the adaptive immune system is to search the body for foreign antigens derived from pathogens or transformed tumor cells that are present within cells and in extracellular spaces. For T cells, an elaborate mechanism has evolved to ensure that all intracellular and extracellular proteins are presented on major histocompatibility complex (MHC) class I and class II molecules during development in the thymus and subsequently during immune responses in the periphery. Autophagy was recently demonstrated to play an important role in ensuring that peptides derived from self- and nonself proteins gain access to MHC class I and class II molecules. The innate immune system also surveys the body for pathogens, searching for pathogen-associated molecular patterns (PAMPs). Autophagy appears to play

a role in delivering PAMPs containing molecules to the innate immune system receptors. In addition, as a process that has the capability of selectively degrading cellular components, autophagy is shown to influence development and survival in both B and T lymphocytes. The wide range of roles played by autophagy in immune regulation, from antigen processing to lymphocyte development and homeostasis, and in inducing immune tolerance makes autophagy an attractive target for therapeutics.

### Autophagy in Antigen Presentation

One of the hallmarks of adaptive immunity is the MHC class I- and class II-restricted presentation of antigenic peptides by target cells and professional antigen-presenting cells (APCs) for the activation of cytolytic and T-helper cells, respectively. MHC class I molecules are assembled with peptides in the endoplasmic reticulum for recognition by CD8<sup>+</sup> cytotoxic T cells. In contrast, MHC class II molecule-peptide complexes are formed in specialized endocytic compartments and transported to the cell surface for recognition by CD4<sup>+</sup> T-helper cells. Until recently, it was believed that MHC class II molecules exclusively presented exogenous antigens that entered the APC through the endocytic pathways. In contrast, endogenous intracellular antigenic peptides derived from viral, tumor, and self-proteins were presented exclusively on MHC class I molecules after transport of the peptides from their site of synthesis in the cytosol into the endoplasmic reticulum by a specialized peptide transporter system. However, studies over the past few years indicate that both MHC class I and class II molecules can acquire antigens from inside and outside the cell. It was shown that many intracellular proteins derived from endogenous cytoplasmic compartments or the nucleus are presented by MHC class II molecules. These findings posed the following questions: how do extracellular antigens cross membranes to enter the cytosol to be presented by class I molecules, and how do intracellular antigens cross membranes to enter into the endocytic compartments for presentation by MHC class II molecules? Activation of III PI3K is the initial step in the autophagic pathway, and inhibitors of III PI3K, 3-MA, and wortmannin are used extensively to block autophagy in APCs to ascertain the requirement for autophagy in MHC class II presentation. Nimmerjahn et al. [12] showed that MHC class II-restricted presentation of an endogenously expressed bacterial cytosolic protein neomycin phosphotransferase II (NeoR) was totally abrogated in cells treated with III PI3K inhibitors. The generation of the NeoR antigenic peptides was dependent on the lysosomal rather than the cytosolic proteases, suggesting that NeoR was sequestered in the cytosol in autophagosomes and subsequently delivered into endosomal/lysosomal compartments, where the protein was degraded and bound to MHC class II molecules. Similarly, Dorfel et al. [13] showed that MHC class II presentation of mucin-1 peptides in dendritic cells transfected with mucin-1 RNA was sensitive to the inhibitors of autophagy.

However, mucin-1 appeared cleaved by lysosomal and cytosolic proteases, suggesting that mucin-1 was first processed into peptides in the cytosol, and the peptides were then transported to lysosomes through autophagy [13].

Paludan et al. [14] extended these findings by showing that peptides of the Epstein-Barr virus nuclear antigen-1 (EBNA-1) were presented by MHC class II molecules in the EBNA-1-expressing human B-cell line, B-LCL. Treatment of B-LCL to inhibit lysosomal acidification resulted in the accumulation of EBNA-1 in intracellular vesicles positive for both lysosomal-associated membrane protein (LAMP)-1 and monodansylcadaverine, a fluorescent dye that labels autophagosomes [14]. Moreover, using electron microscopy, EBNA-1 was directly visualized in autophagosomes and in developing isolation membranes. Blocking autophagy by 3-MA resulted in a 30% to 70% reduction in presentation of EBNA-1 peptides, suggesting that autophagy is the pathway by which nuclear EBNA-1 gains access to lysosomes for processing and presentation [14]. These authors also disrupted autophagy using small interfering RNA-mediated downregulation of *Atg12*, an important component of autophagosome formation, resulting in reduced MHC class II presentation of EBNA-1 [14]. Interestingly, these authors found that long-lived cytosolic and nuclear antigens are the prominent sources of natural MHC class II ligands, which suggests that autophagy may play a critical role in MHC class II-restricted CD4<sup>+</sup> T-cell recognition of long-lived intracellular viral and tumor antigens.

Dengjel et al. [15] characterized the peptides bound to cell surface MHC class II molecules in resting cells and in cells stimulated to increase autophagy. In resting cells, MHC class II molecules contained peptides derived from membrane and secreted proteins. In contrast, peptides derived from intracellular and lysosomal proteins were predominantly presented on MHC class II molecules following stimulation, indicating that autophagy was responsible for shuttling intracellular proteins into the endosomal/lysosomal compartments. Additional detail of the mechanism by which peptides derived from cytosolic and nuclear proteins gain access to MHC class II peptide loading compartments (MIICs) was provided by Schmid et al. [16••]. They showed that autophagosomes are constitutively formed in all APCs, including dendritic, epithelial, and B cells. LC3, a homologue of yeast *Atg8* and a marker for the autophagosomes, co-localized with markers of MIICs, including MHC class II molecules and LAMP-1 and LAMP-2 [16••]. Quantification of the co-localization data revealed that more than one half of the MIICs continuously fused with autophagosomes and received peptides from them. Moreover, targeting of the cytosolic protein influenza membrane protein-1 to autophagosomes by fusion with LC3 resulted in enhanced MHC class II presentation of influenza membrane protein-1-derived peptides [16••].

Although most studies focus on the role of autophagy in MHC class II presentation, recent reports suggest

that MHC class I-restricted presentation of exogenous antigens, a phenomenon termed *cross-presentation*, also involves autophagy. Ovalbumin (OVA)-expressing cells treated to induce autophagy showed enhanced presentation of OVA peptides on MHC class I molecules [17]. Conversely, treatment of cells with 3-MA to inhibit autophagy significantly decreased the presentation of OVA peptides on MHC class I molecules [17]. Induction of autophagy also enhanced the cross-presentation of a melanoma-produced tumor antigen gp100, whereas inhibition of autophagy reduced the cross-presentation of this antigen. Knockdown of *Beclin-1* or *Atg12* in antigen-expressing tumor cells significantly reduced cross-presentation [17]. Autophagosome formation was required for cross-presentation, and autophagosomes functioned as carriers of antigen from donor cells [17]. The mechanism by which autophagosomes deliver their content for MHC class I presentation is not known but would be of interest to determine if mechanisms similar to MIIC fusion with autophagosomes also play a role in MHC class I antigen presentation.

### Autophagy in Self-Antigen Presentation in the Thymus

To ensure that T cells that respond to self-tissues are eliminated, both positive and negative selection takes place in the thymus through a mechanism referred to as *central tolerance* [18,19]. Positive selection ensures that T cells have low affinity to self-MHC molecules, whereas negative selection eliminates T cells with high affinity to self-MHC molecules or self-peptides [18,19]. Using LC3-GFP transgenic mice, Mizushima et al. [20] demonstrated that autophagy is a constitutive process in epithelial cells in the thymus. Autophagy in thymic epithelial cells (TECs) from both the thymic cortex and medulla is constitutively highly active irrespective of nutrient conditions, as observed by confocal microscopy of LC3-GFP puncta formation that characterizes the autophagosome formation, and immunoelectron microscopy [20]. Such high autophagic activity was suggested to play an important role in the presentation of intracellular self-antigens to CD4<sup>+</sup> T cells during negative selection. However, it was recently shown that autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance [21••]. TECs and dendritic cells play a central role in T-cell selection by displaying all tissue-restricted self-antigens via a mechanism involving autoimmune regulator [19,22]. Although both TECs and dendritic cells constitutively express high levels of MHC class II molecules, TECs differ from dendritic cells in their inability to capture and process exogenous protein antigens. Therefore, TECs rely on the unconventional pathways (eg, autophagy) to provide intracellular self-peptides to MHC class II molecules. Nedjic et al. [21••] demonstrated that in autophagy-deficient mice, positive and negative selection of T cells is perturbed. In the absence of autophagy, TECs displayed altered

composition of peptide ligands on MHC class II molecules, leading to impaired positive selection of certain MHC class II-restricted T-cell receptor specificities [21••]. However, positive selection of MHC class I-restricted T-cell receptor specificities remained unchanged. Moreover, in the absence of autophagy, T cells lost self-tolerance, which led to autoimmunity [21••].

### Autophagy and Pattern-Recognition Receptors

A role for autophagy in the removal of intracellular bacteria, viruses, and parasites was studied extensively and reviewed previously [23]. This function of autophagy was recently implicated as playing a role in the transport of pathogens to pattern-recognition receptors (PRRs), the innate immune system's receptors responsible for recognizing PAMPs. Toll-like receptors (TLRs) are one of the best characterized subfamilies of PRRs. Ligand binding to TLRs initiates intracellular signaling pathways, leading to upregulation of costimulatory molecules and secretion of various cytokines and interferons. TLRs can be broadly divided into two categories on the basis of their location: cell surface TLRs, including TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, and endosomal TLRs, including TLR3, TLR7, TLR8, and TLR9. Independent studies suggest that TLR ligands induce autophagy in mouse macrophage cell line RAW 264.7 and in mouse and human primary macrophages. Xu et al. [24] showed that the TLR4 ligand lipopolysaccharide induced autophagy in primary human macrophages and in RAW 264.7 cells through the TLR signaling pathway involving the adaptor molecules TRIF and MyD88. Moreover, they found that lipopolysaccharide induced autophagy-enhanced intracellular mycobacteria co-localization with the autophagosomes [24]. Sanjuan et al. [25] showed that zymosan, a ligand for TLR2, resulted in the recruitment of LC3 and *Beclin-1* to the phagosomes in an *Atg5*- and *Atg7*-dependent manner. Recruitment of autophagic machinery to phagosomes was associated with the fusion of phagosomes with lysosomes, leading to rapid acidification and enhanced killing of the ingested bacteria [25]. In addition to TLR2 and TLR4, ligands for TLR3, TLR5, TLR7, TLR8, and TLR9 have been shown to induce autophagy [26•]. The most potent inducer of autophagy in this study was the TLR7 ligand single-stranded RNA (ssRNA). Stimulation of autophagy with ssRNA greatly reduced the viability of mycobacteria in infected macrophages [26•]. Although different TLRs induce autophagy, how the TLRs communicate with the autophagic system is not known. A recent report demonstrated that the TLR adaptor proteins, MyD88 and TRIF, form a complex with *Beclin-1* and affect *Beclin-1* and *Bcl-2* interaction upon TLR stimulation [27]. This result suggests that both MyD88 and TRIF serve as activators of autophagy and, together with *Beclin-1*, influence the cross-talk between autophagy and innate immune mechanisms.

However, aside from facilitating the degradation of intracellular pathogens, it remains unclear how TLR-

induced autophagy impacts immune cell function. At least for TLR7, it was shown that autophagy may play a role in transporting the TLR7 ligands, viral ssRNA, and replication intermediates from the cytosol to the TLR7-containing endosomal/lysosomal compartments, possibly functioning as an amplification mechanism [28••]. TLR7-mediated interferon (IFN)- $\alpha$  production following vesicular stomatitis virus (VSV) infection is dependent upon autophagy, as plasmacytoid dendritic cells from *Atg5*-deficient mice failed to secrete IFN- $\alpha$ . It is proposed that during the induction of autophagy, isolation membrane sequesters TLR ligands, including viral nucleic acids and their replication intermediates, to autophagosomes and subsequently delivers them to the TLRs in the endosomal/lysosomal compartments [28••]. Thus, autophagy may provide an elegant mechanism for the delivery of TLR ligands from the cytosols to their corresponding TLRs in the lumen of endosomal compartments. In B cells, we recently discovered that the B-cell receptor (BCR) dictated the subcellular movement of TLR9 to autophagosome-like compartments, leading to hyperresponses to DNA-containing antigens. We showed that TLR9 resides in early endosomal compartments in resting cells and is recruited to autophagosome-like compartments upon BCR cross-linking [29••]. Within these compartments, the BCR and TLR9 cooperate for enhanced signaling, which was implicated in autoantibody production in systemic autoimmune disease [30]. Moreover BCR-driven recruitment of TLR9 is dependent on III PI3K activity [29••].

A second subfamily of PRRs comprises the retinoic acid-inducible gene-I (*RIG-I*)-like receptors, encompassing *RIG-I*, melanoma differentiation-associated gene-5 (*MDA5*), and laboratory of genetics and physiology-2 (*LGP2*), which are expressed ubiquitously in the cytoplasm and may also play a role in autophagy. One report suggests that the *Atg5*-*Atg12* conjugate directly associates with *RIG-I* and adaptor protein IFN promoter stimulator-1 (*IPS-1*) and negatively regulates IFN production [31]. Moreover, VSV infection induces autophagy in mouse embryonic fibroblasts, providing a direct link between autophagy and *RIG-I* [31]. However, *Atg5*-deficient mouse embryonic fibroblasts were resistant to VSV replication, a process largely dependent on *RIG-I* [32]. Further studies are needed to establish a link between *RIG-I*-like receptors and autophagy.

### Role for Autophagy in Lymphocyte Development

Autophagy plays a crucial role during embryogenesis and development by mediating bulk protein and organelle turnover. An important contribution of autophagy in vertebrate development came from mutations targeting *Atg5* and *Atg7*. Mice lacking *Atg5*, which is essential for autophagosome formation, appear almost normal at birth but die of starvation within 1 day [33,34]. In nonsuckling *Atg5*<sup>-/-</sup> mice, plasma and tissue amino acid con-

centrations are reduced relative to those of wild-type mice. Tsukamoto et al. [35] recently showed that autophagy plays a critical role during preimplantation development of mouse embryos. Embryos derived from oocyte-specific *Atg5* knockout mice failed to develop beyond the four- and eight-cell stages if they were fertilized by *Atg5*-null sperm. This was caused by defects in the degradation of maternal proteins in oocytes after fertilization [35]. Similarly, neonatal mice deficient in *Atg7*, another integral component of autophagosome formation, display multiple cellular abnormalities, such as appearance of concentric membranous structure, deformed mitochondria, and accumulation of ubiquitin-positive aggregates. They also die of starvation [36]. Lineage-specific conditional deletion of *Atg5* and *Atg7* provided evidence that autophagy influences the regulation of the development and survival of T and B lymphocytes [37,38•,39,40]. T-lymphocyte lineage-specific deletion of *Atg5* resulted in decreased numbers of thymocytes and peripheral CD4<sup>+</sup> T-helper lymphocytes and CD8<sup>+</sup> T-cytotoxic lymphocytes, although T-cell maturation was not affected [37,38•]. Interestingly, *Atg5*-deficient CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes showed only limited proliferation upon T-cell receptor stimulation. Moreover, *Atg5*-deficient peripheral CD8<sup>+</sup> T lymphocytes showed dramatically higher apoptotic rates compared with CD4<sup>+</sup> T lymphocytes [37,38•]. Analyses of *Atg7*-deficient T lymphocytes provided evidence for autophagy in normal T-lymphocyte homeostasis [39]. It appears that the number of mitochondria is developmentally regulated in T lymphocytes, with thymocytes displaying large numbers of mitochondria, and mature T lymphocytes smaller numbers. Autophagy-deficient mature T lymphocytes failed to reduce their number of mitochondria in vivo, suggesting that autophagy functions to clear superfluous mitochondria in T lymphocytes during development. As a result of the failure to reduce the number of mitochondria, autophagy-deficient T cells have increased reactive oxygen species production, as well as an imbalance in expression of pro- and antiapoptotic proteins [39]. In addition, stimulation of T cells through the T-cell receptors or cytokine receptors and prolonged serum starvation induce autophagy in CD4<sup>+</sup> T lymphocytes in a c-Jun N-terminal kinase- and III PI3K-dependent manner [41]. Autophagy is induced at a much higher level in T-helper type 2 lymphocytes than in T-helper type 1 lymphocytes. By blocking autophagy by using chemical inhibitors or small interfering RNA knockdown of *Beclin-1* and *Atg7*, T-helper type 2 lymphocytes become more resistant to cell death induced upon growth factor withdrawal [41].

Similar to its role in T-lymphocyte development, autophagy plays an important role in B-lymphocyte development. Lineage-specific conditional deletion of *Atg5* in B lymphocytes resulted in significant defects at the pro- to pre-B-cell transition and resulted in dramatic reduction in the B1-B cells in the peritoneum but had no effect on B2-B cell development [40]. Autophagy also appears to play a role in promoting B-cell expansion

in response to antigen in germinal centers. Indeed, heterozygous disruption of *Beclin-1*, an ortholog of yeast *Atg6*, resulted in aberrant proliferation of B cells in germinal centers following immunization, which resulted in a significant increase in the number and size of germinal centers in the spleens of *Beclin-1*<sup>+/-</sup> as compared with *Beclin-1*<sup>+/+</sup> mice [42].

## Conclusions

Initially accepted as a mechanism involved in cell survival under stress conditions, autophagy was implicated as playing a much broader role in influencing adaptive and innate immune responses. As discussed in this review, autophagy modulates immunity by operating at multiple levels, including targeting intracellular pathogens for degradation, controlling lymphocyte development and homeostasis, facilitating presentation of endogenous antigens on MHC class II molecules and presentation of tumor antigens on MHC class I molecules, maintaining T-cell tolerance, and influencing innate immunity. However, several important questions still must be addressed. Many of the studies were carried out only in vitro. Whether similar mechanisms operate in vivo in immune response needs to be determined. Moreover, little is known about the cross-talk between autophagic pathways and immune receptor signaling pathways. A greater understanding of the candidates functioning at the intersection of autophagy and immune pathways will be useful in designing therapeutics.

## Disclosure

No potential conflicts of interest relevant to this article were reported.

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