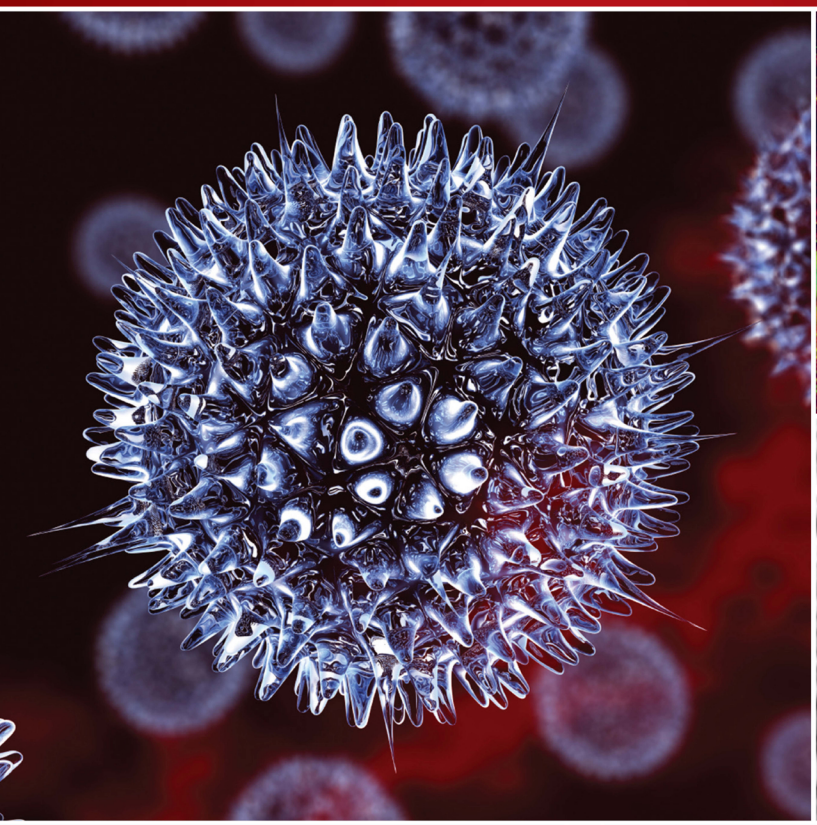
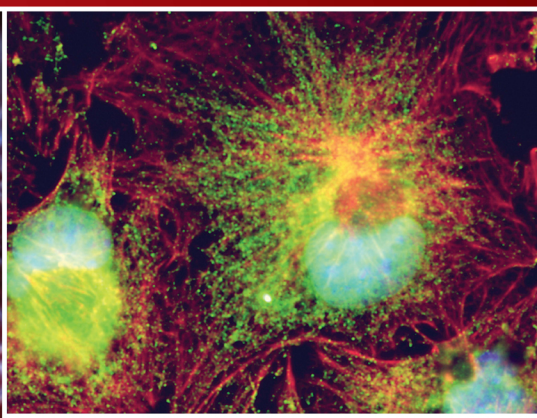
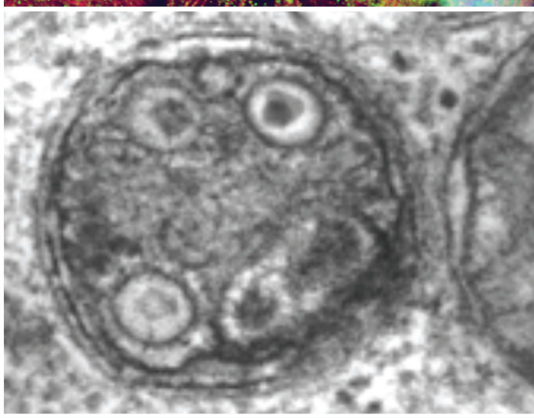
AUTOPHAGY, INFECTION, AND THE IMMUNE RESPONSE

Edited by William T. Jackson and Michele S. Swanson







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PREFACE

Since the discovery nearly 20 years ago that pathogenic bacteria and viruses intimately associate with autophagosomal membranes, scientists have determined that autophagy is a critical component of innate and acquired immunity. Of course, as with all aspects of the host immune response, some pathogens have turned autophagy to their advantage. For this volume, experts in the fields of bacteriology, virology, mycology, parasitology, immunology, and cell biology describe the cellular mechanisms of autophagosome formation and maturation, its contribution to host defenses, and the mechanisms pathogenic microbes have acquired to overcome and subvert this formidable barrier to infection. In addition, specialists discuss current efforts to exploit knowledge of the autophagy pathway to improve vaccine design. Accordingly, this thorough examination of an extraordinary cellular battleground between host and pathogen can stimulate ongoing research to understand and to manipulate autophagy to improve human health.

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Michele S. Swanson

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William T. Jackson
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AUTOPHAGY AND IMMUNITY

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1.1 INTRODUCTION

Autophagy is a highly controlled process in which cellular components are self-degraded and subsequently recycled. This pathway in part plays a "house cleaning" role in the cell, directing numerous cargoes to the lysosome (or the vacuole in yeast and plants) for degradation. Depending on the specific conditions, the cargoes include random portions of cytoplasm, protein aggregates, and damaged or superfluous organelles such as mitochondria and peroxisomes. Dysfunction of autophagy is linked with many pathologies, including cancer, diabetes, myopathies, heart, liver and lung diseases, and certain types of neurodegenerative disease (Castets et al., 2013; Gonzalez et al., 2011; Klionsky and Codogno, 2013; Murrow and Debnath, 2013; Rubinsztein et al., 2012; Yang and Klionsky, 2010).

Emerging studies have revealed that autophagy plays important roles in immunity. In 2004, independent studies demonstrated for the first time that invading pathogens can be cargoes for autophagy (Gutierrez et al., 2004; Nakagawa et al., 2004). Today it is well accepted that autophagy can directly eliminate intracellular pathogens, including bacteria, fungal parasites, and viruses. Autophagy can also activate innate immune signaling cascades such as Toll-like receptor (TLR) signaling to attack invading pathogens (Lee et al., 2007; Xu et al., 2007). However, microbes constantly undergo strong selective pressure to develop strategies to block host defense mechanisms. Indeed, studies indicate that some adaptations that confer pathogenicity involve microbial inactivation or subversion of

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autophagy through distinct mechanisms (Deretic and Levine, 2009; Kuballa et al., 2012; Levine et al., 2011; Yuk et al., 2012; Zhou and Zhang, 2012).

Autophagy's role in immunity is not limited to controlling infection by direct elimination of pathogens. For example, autophagy facilitates MHC (major histocompatibility complex) antigen presentation, indicating that autophagy is involved in adaptive as well as innate immunity (English et al., 2009; Paludan et al., 2005). Moreover, defects in autophagy are associated with autoimmune diseases such as Crohn disease (Levine et al., 2011; Schroder and Tschopp, 2010; Shi et al., 2012). Thus, autophagy is an integral part of our response to infection and plays a key role in immunity. A comprehensive understanding of autophagy as it pertains to microbial infection and the molecular mechanisms that underlie the interplay between autophagy and immune signaling pathways may enable us to unravel the pathogenesis of many infectious and immune diseases, and develop more effective therapeutic strategies for their treatment.

1.2 AUTOPHAGY

1.2.1 Types of autophagy

There are three main types of autophagy: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy (Figure 1.1). CMA is a process where a cytosolic chaperone protein, HSPA8/HSC70, specifically recognizes its cargo proteins through a KFERQ-like

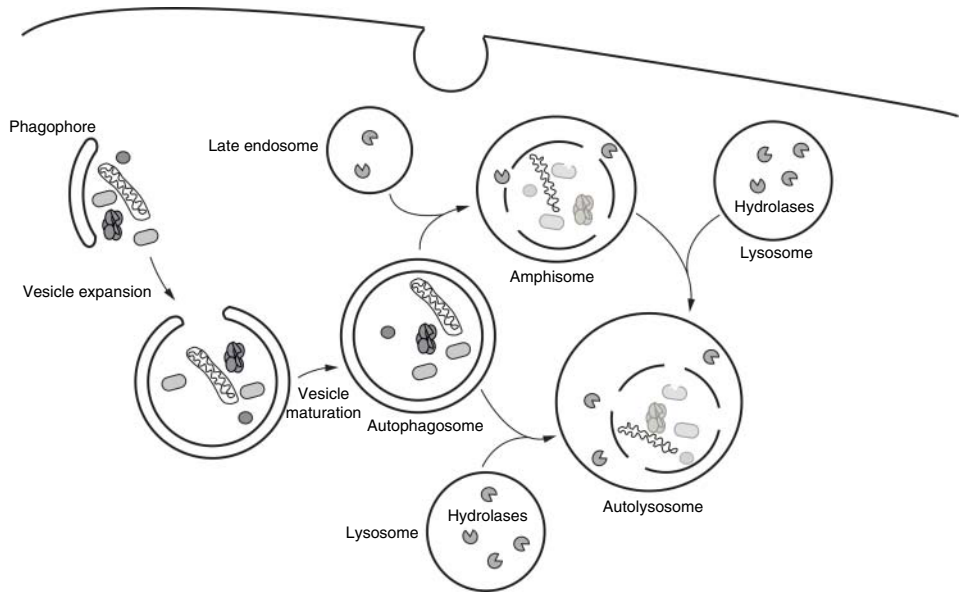

Figure 1.1. Schematic model of mammalian autophagy. Cargoes including cytosolic proteins, protein aggregates, and damaged organelles are sequestered by a phagophore, which will expand and mature to form a complete autophagosome. The outer membrane of the autophagosome fuses with either a late endosome (forming an amphisome, which then fuses with a lysosome) or lysosome, forming an autolysosome. Finally, the cargoes together with the inner membrane are degraded and the breakdown products are released back into the cytosol for reuse.

motif and facilitates their translocation directly across the lysosomal membrane for degradation (Dice, 2007; Kaushik and Cuervo, 2012). Microautophagy involves the uptake of portions of cytoplasm by the direct invagination or protrusion of the lysosomal or vacuolar membrane (Mijaljica et al., 2011). The third process, macroautophagy, hereafter referred to as autophagy, is the best characterized and will be the focus of this chapter.

1.2.2 Morphology

The morphological hallmark of autophagy involves the *de novo* formation of a double-membrane organelle named the autophagosome; however, this structure is essentially an end product of the sequestration process and as such is not really the primary functional unit of autophagy. Rather, the precursor to the autophagosome, the phagophore, is the dynamic membrane structure that is responsible for sequestering the cargos such as damaged organelles and invading pathogens (Figure 1.1). The phagophore expands with the addition of membrane, the sources of which are suggested to include almost every intracellular organelle. Upon completion, the phagophore seals and becomes a completed autophagosome. The autophagosome may fuse directly with a lysosome or, first, with a late endosome to form an intermediate amphisome. The subsequent fusion of the outer membrane of the autophagosome or the amphisome limiting membrane with a lysosome generates an autolysosome and exposes the cargoes to the degradative lysosomal enzymes. The degradation products, especially amino acids, are subsequently released back into the cytosol and are used in generating energy or as substrates for biosynthetic pathways.

1.2.3 Molecular machinery

Even though autophagosomes have been observed by electron microscopy as early as the 1950s, the molecular mechanisms of autophagy have been poorly studied until the past two decades (Stromhaug and Klionsky, 2001). The molecular machinery was first identified through studies in budding yeast, *Saccharomyces cerevisiae*, and to date more than 30 autophagy-related (*ATG*) genes have been identified as being involved in this process (Harding et al., 1995; Klionsky et al., 2003; Thumm et al., 1994; Tsukada and Ohsumi, 1993). Subsequent work with mammalian cells has revealed homologs of the core autophagy machinery (Xie and Klionsky, 2007), supporting the notion that autophagy is evolutionarily conserved. At the same time, there are also increasing numbers of ATG proteins being identified in mammals and other model systems such as *Caenorhabditis elegans* that lack yeast homologs, suggesting an increased complexity and diversity of function in higher eukaryotes (Klionsky and Codogno, 2013). For ease of discussion, the protein machinery of autophagy is subdivided into four major complexes in the following sections, and we focus on the mammalian autophagy machinery.

ULK1/ULK2 complex Autophagy occurs at a basal level in cells under normal conditions. Upon stress or other stimuli, autophagy can be induced, and defects in regulation that prevent proper induction can lead to aberrant cell physiology; however, too much autophagy activity can also be detrimental to the cell. Thus, the level of autophagy must be tightly controlled. Accordingly, there are various factors that regulate autophagy induction, and studies have shown that the ULK1/ULK2 (unc-51 like autophagy activating kinase 1/2) complex functions in part in an early stage of autophagy regulation.

ULK1 and ULK2 are kinases and the other components of the complex include ATG13, RB1CC1/FIP200 (RB1-inducible coiled-coil 1), and ATG101. ATG13 directly interacts

with ULK1/ULK2 and RB1CC1 regardless of the nutrient availability (Hosokawa et al., 2009; Jung et al., 2009); however, the phosphorylation status of these proteins changes under different conditions. In nutrient-rich conditions, a key upstream negative regulator of autophagy, the mechanistic target of rapamycin complex 1 (MTORC1) interacts with the complex and phosphorylates ULK1/ULK2 and ATG13, inhibiting ULK1/ULK2 kinase activity. Upon starvation, MTORC1 is released from the complex. ULK1/ULK2 and ATG13 are then partially dephosphorylated, leading to activation of ULK1/ULK2 kinase activity, which in turn leads to phosphorylation of ATG13 (presumably on distinct sites from those used by MTORC1) and RB1CC1 to induce autophagy (Chan, 2009; Hara et al., 2008; Hosokawa et al., 2009). AMPK (AMP-activated protein kinase) also binds ULK1/ULK2 and positively regulates autophagy through phosphorylation upon glucose starvation; as expected, AMPK and MTORC1 phosphorylate ULK1 at different sites (Kim et al., 2011; Zhao and Klionsky, 2011).

Class III phosphatidylinositol 3-kinase complexes The class III phosphatidylinositol 3-kinase (PtdIns3K) is generally thought to act downstream of the ULK1/ULK2 complex, mediating formation of phosphatidylinositol-3-phosphate (PtdIns3P) on the phagophore membrane, an event essential for autophagy. PtdIns3P serves to recruit downstream factors such as WIPI1 (WD repeat domain, phosphoinositide interacting 1) and WIPI2, which are involved in the trafficking of ATG9 and promote autophagosome maturation (Polson et al., 2010). In mammals, there are multiple class III PtdIns3K complexes with the core components being PIK3C3/VPS34 (phosphatidylinositol 3-kinase, catalytic subunit type 3), BECN1/Beclin 1 (beclin 1, autophagy related), and PIK3R4/VPS15/p150 (phosphoinositide-3-kinase, regulatory subunit 4). BECN1 can interact with several proteins, including AMBRA1 (autophagy/beclin-1 regulator 1), ATG14/ATG14L/Barkor, UVRAG (UV radiation resistance associated), KIAA0226/Rubicon and BCL2 (B-cell CLL/lymphoma 2) to form distinct complexes (Furuya et al., 2005; Itakura et al., 2008; Matsunaga et al., 2009; Petiot, 2000). BECN1 was first identified as a BCL2 binding protein. The interaction between BECN1 and BCL2 inhibits the binding of the former with PIK3C3, thus inhibiting autophagy. The ATG14–BECN1–PIK3C3–PIK3R4–AMBRA1 complex is specific for autophagy; ATG14 may direct this complex to the phagophore to promote autophagosome biogenesis (Itakura et al., 2008; Matsunaga et al., 2009), whereas the SH3GLB1 (SH3-domain GRB2-like endophilin B1)–UVRAG–BECN1–PIK3C3–PIK3R4 complex functions at a later step to promote autophagosome maturation (Itakura et al., 2008). In contrast, the KIAA0226–UVRAG–BECN1–PIK3C3–PIK3R4 complex localizes to late endosomes and negatively regulates autophagosome maturation (Matsunaga et al., 2009).

ATG9 trafficking system The Atg9 trafficking system is best characterized in yeast, although even in that model organism there are many questions that remain to be answered. The current model is that the transmembrane protein Atg9 cycles between the phagophore assembly site (PAS) and peripheral (i.e., non-PAS) sites, and that this process is needed for the proper delivery of membrane from various donor organelles to the expanding phagophore (Noda et al., 2000; Reggiori et al., 2005). Atg23 and Atg27 interact with Atg9 and facilitate its anterograde traffic from the peripheral sites to the PAS, whereas Atg2–Atg18 and the Atg1–Atg13 complex (yeast homolog of the ULK1/ULK2 complex) are required for its retrograde transport from the PAS back to the peripheral sites (Guan et al., 2001; Reggiori et al., 2004; Wang et al., 2001; Yen et al., 2007).

In mammals, ATG9 localizes to the *trans*-Golgi network and endosomes in nutrient-rich conditions. A pool of ATG9 translocates to MAP1LC3 (microtubule-associated protein 1 light chain 3)/LC3-positive compartments upon starvation. This translocation is dependent on ULK1 and PIK3C3 kinase activity (Young et al., 2006). The dynamic movement between ATG9 and the phagophore membrane during autophagy suggests a conserved role for ATG9 in membrane movement during phagophore expansion. Similar to yeast, ATG9 retrieval from the phagophore membrane is dependent on WIPI2, a homolog of yeast Atg18, but movement in this direction is ULK1 kinase independent (Orsi et al., 2012).

Ubiquitin-like conjugation systems There are two ubiquitin-like (Ubl) conjugation systems, which involve the Ubl proteins ATG12 and LC3. These systems are quite well-studied, playing important roles in phagophore expansion and maturation (Ichimura et al., 2000; Mizushima et al., 1998, 2001). ATG12 is conjugated with ATG5 in a manner that is similar to canonical ubiquitination (Mizushima et al., 1998). The E1-like enzyme ATG7 activates ATG12 via a thioester bond (Tanida et al., 2001). ATG12 is then transferred to an E2-like enzyme, ATG10, before it is finally conjugated to an internal lysine of ATG5. ATG5 then noncovalently binds ATG16L1 (autophagy related 16-like 1 (*S. cerevisiae*)), which subsequently dimerizes. During autophagy, ATG5 directs the ATG12—ATG5–ATG16L1 complex to the phagophore (Mizushima, 2003).

The different isoforms of LC3 (and the related GABARAP (GABA(A) receptor-associated protein) subfamily proteins) are conjugated to the lipid phosphatidylethanolamine (PE), and this modification is required for association with the phagophore membrane (Kabeya et al., 2004; Tanida et al., 2003). Initially, the cysteine protease ATG4B removes the C-terminal amino acids of pro-LC3 to reveal a glycine residue, generating a cytosolic form named LC3-I. LC3-I is then sequentially activated by ATG7 and conjugated via the E2-like enzyme ATG3, resulting in the membrane-associated form, LC3-II (Tanida et al., 2001, 2002). The PE group can ultimately be cleaved by ATG4B in a deconjugation step, which is important for maintaining the proper level of autophagy activity (Tanida et al., 2006).

1.2.4 Physiological roles

Autophagy has many physiological roles. First, autophagy is a protective mechanism against cellular stress (Kuma et al., 2004; Yang and Klionsky, 2010). For example, autophagy's role in supplying essential building blocks or metabolic substrates such as amino acids under conditions of nutrient deprivation is critical for maintaining cell viability under adverse conditions; autophagic degradation and recycling enable cells to maintain the synthesis of essential proteins and to generate ATP.

Recent studies indicate that autophagy is also indispensible during development. One example of such a role is seen after oocyte fertilization in *C. elegans*, where autophagy is involved in the elimination of maternal mitochondria (Al Rawi et al., 2011; Sato and Sato, 2011); however, this does not appear to be the case in mammals (Luo et al., 2013). In addition, during embryonic development, clearance of apoptotic cells is achieved through autophagy (Qu et al., 2007). Autophagy is also implicated in life span extension; induction of autophagy increases longevity in several model organisms (Rubinsztein et al., 2011) and its role in clearing aggregate-prone proteins and damaged mitochondria might be relevant to its antiaging effects.

As autophagy acts to eliminate many harmful components in a cell, malfunction of autophagy has also been suggested to correlate with or be the cause of a variety of diseases, such as cancer, neurodegeneration, cardiovascular myopathies, and lysosomal storage disorders (Klionsky and Codogno, 2013). For example, the selective degradation of damaged mitochondria is suggested to underlie the tumor suppressive effects of autophagy, possibly through reducing oxidative stress and preventing DNA damage (Narendra et al., 2008). Several lines of evidence suggest that the role of autophagy in clearing toxic aggregate-prone proteins is critical to prevent certain types of neurodegeneration, including those associated with Huntington, Alzheimer, and Parkinson diseases (Bjørkøy et al., 2005; Ravikumar et al., 2002).

1.3 AUTOPHAGY AND IMMUNITY

1.3.1 Xenophagy: autophagic clearance of intracellular microorganisms

For decades, scientists have explored how our body fights against invading pathogens. Even though an understanding of our immune systems has steadily increased, a major problem, how a cell breaks down an intracellular pathogen without harming itself, has been overlooked or at least unanswered. Only recently have researchers realized that autophagy plays a vital role in this process. This specific type of autophagy is termed "xenophagy."

Autophagic degradation of bacteria and parasites Several independent studies have revealed that xenophagy acts to eliminate many different bacteria and other microbes (Levine et al., 2011; Yuk et al., 2012). A good example of parasite clearance is seen with *Toxoplasma gondii* (Andrade et al., 2006). This parasite is able to survive within macrophages by residing in parasitophorous vacuoles that are modified to avoid fusion with lysosomes. However, stimulation of *T. gondii*-infected macrophages with CD40 (CD40 molecule, TNF receptor superfamily member 5), a member of the TNF (tumor necrosis factor) receptor superfamily, causes colocalization of parasitophorous vacuoles and LC3. Conversely, treatment of infected cells with the autophagy inhibitor 3-methyladenine (3-MA) or knockdown of BECN1 blocks the fusion of parasitophorous vacuoles with lysosomal compartments (Andrade et al., 2006). Thus, these results suggest that phagophores capture parasites that are residing within these vacuoles and direct them to the lysosome for degradation.

As for bacterial clearance, evidence indicates that autophagosomes can sequester both bacteria that reside within membranous compartments and those present free within the cytosol, through mechanisms that are overlapping, but distinct (Figure 1.2) (Levine and Deretic, 2007). The clearance of *Mycobacterium tuberculosis* is a good example of engulfment of bacteria residing within phagosomes (Gutierrez et al., 2004). After entering the cell through endocytosis, *M. tuberculosis* can actively survive in a host cell and evade the host defense by inhibiting phagosomal maturation. However, if autophagy is induced by either nitrogen starvation or rapamycin treatment, the inhibition of phagosomal maturation by *M. tuberculosis* is suppressed and intracellular bacterial survival is significantly decreased. Also, a substantial colocalization of *M. tuberculosis*-containing phagosomes with autophagosomes is observed upon autophagy induction, supporting the idea that phagophores capture bacteria residing within phagosomes and target them to lysosomal compartments for degradation (Gutierrez et al., 2004).

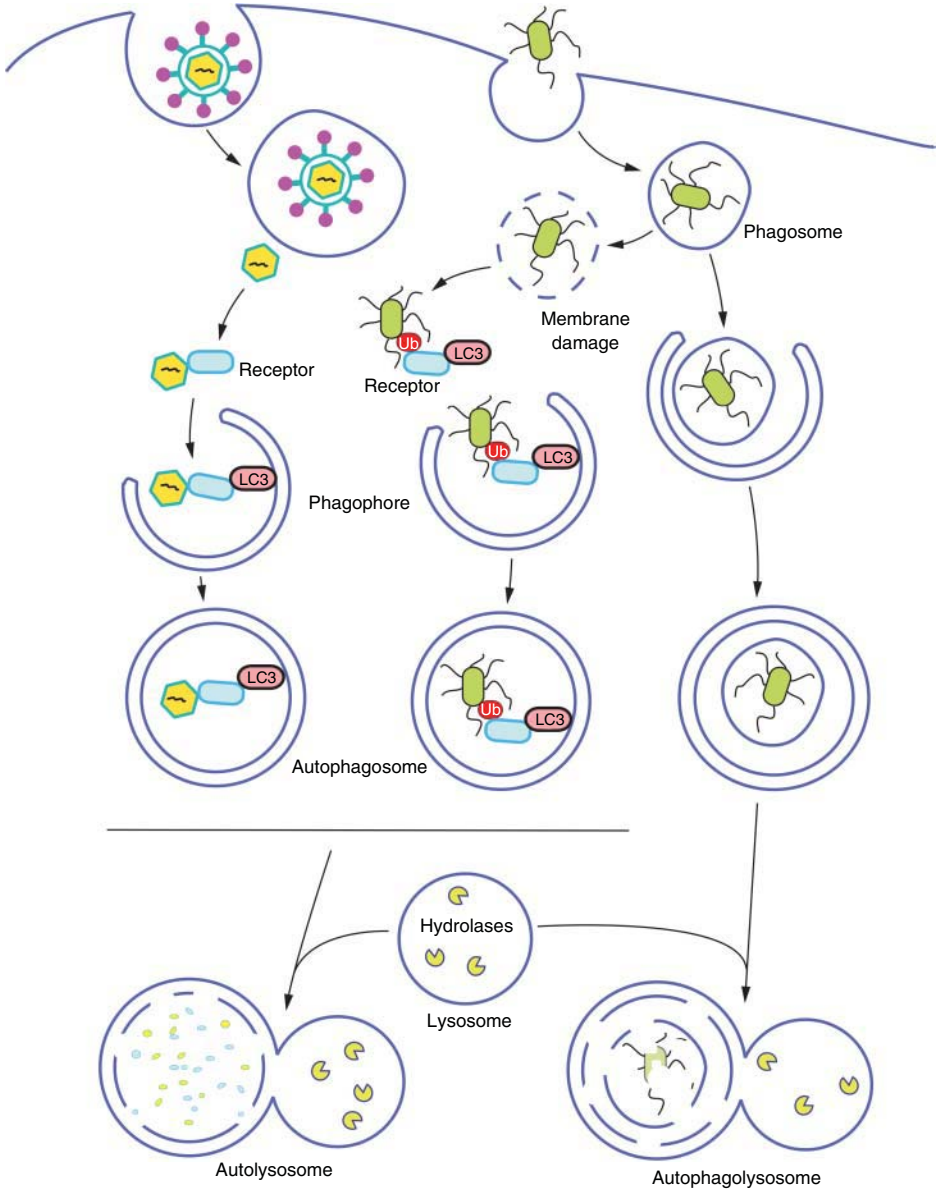

Figure 1.2. Models of autophagic elimination of invading pathogens. Intracellular virus proteins are recognized by autophagy receptors and recruited to autophagosomes by interaction between the receptors and LC3. Both bacteria within phagosomes and bacteria that have escaped from phagosomes can be degraded through autophagy. Bacteria residing in a phagosome can be engulfed by a phagophore; after completion of sequestration, the resulting autophagosome then fuses with a lysosome forming an autophagolysosome. (Note that we strongly recommend that this term be reserved to describe the compartment that results from the fusion of lysosomes with autophagosomes containing phagosomes, and not for the compartments that result from the fusion of other autophagosomes with lysosomes, which are termed autolysosomes.) Some bacteria are able to damage the phagosomal membrane and escape into the host cell cytoplasm. These cytosolic bacteria are polyubiquitinated and recognized by autophagy receptor proteins, directing their delivery to phagophores.

With regard to cytosolic bacteria, a major problem/challenge is that these microbes need to be specifically recognized and distinguished from other "self" endomembranes, including their endosymbiotic descendants, the mitochondria. Starvation-induced autophagy is usually nonselective, but there are also selective types of autophagy. Recent studies of selective autophagy reveal a common cargo–ligand–receptor–scaffold model (Mijaljica et al., 2012). A receptor protein recognizes ligands on cargoes and at the same time binds the scaffold protein of the autophagy machinery, selectively targeting cargoes into the autophagy pathway. Specific receptors have been identified that recognize intracellular bacteria during xenophagy, including SQSTM1/p62 (sequestome 1), NBR1 (neighbor of BRCA1 gene 1), CALCOCO2/NDP52 (calcium binding and coiled-coil domain 2) and OPTN (optineurin) (Kraft et al., 2010; Thurston et al., 2009; Wild et al., 2011; Zheng et al., 2009). Usually, cytosolic intracellular bacteria are coated with polyubiquitin, and these receptors are able to simultaneously bind the ubiquitinated bacteria and LC3, linking the cargo with the autophagy machinery. In this way, intracellular bacteria are specifically targeted for degradation.

Despite the utility of xenophagy in degrading intracellular bacteria, certain pathogens have been successful in developing strategies for evading autophagy. One example of such evasion is seen with *Listeria monocytogenes* (Birmingham et al., 2007). After infection of its host macrophages, a population of *L. monocytogenes* damages phagosomes and is released into the cytosol, where they will ultimately be recognized by autophagy. However, the expression of the virulence factor ActA triggers host cell actin polymerization. This provides the bacteria with actin-based motility, which allows cell-to-cell spread and avoidance of autophagic degradation.

Autophagic elimination of viruses The cargo of xenophagy is not restricted to protozoan parasites and bacteria; autophagy can also capture invading viruses. In general, the mechanism involved in the recognition of viruses and their sequestration by phagophores is conceptually similar to that of cytosolic bacteria (Figure 1.2). For example, after Sindbis virus infects the mouse central nervous system, SQSTM1 interacts with Sindbis virus capsid proteins, mediating their further degradation through autophagy (Orvedahl et al., 2010). This action significantly reduces virally-induced cell death.

Similar to bacteria, many viruses also act to inhibit autophagy to confer virulence. First, numerous viruses can either inhibit antiviral signaling pathways that induce autophagy or they can activate an autophagy inhibitory pathway. EIF2AK2/PKR (eukaryotic translation initiation factor 2-alpha kinase 2) is an interferon-inducible double-stranded RNA sensor that mediates overall downregulation of translation in host cells via phosphorylation of EIF2A (eukaryotic translation initiation factor 2A, 65 kDa). This signaling pathway also positively regulates virus-induced autophagy (Levine and Deretic, 2007). Viruses develop multiple strategies to block the EIF2AK2 pathway. For example, during infection herpes simplex virus type 1 (HSV-1) expresses the US11 protein to antagonize EIF2AK2-mediated phosphorylation of EIF2A by binding to the kinase, thus preventing autophagy induction (Lussignol et al., 2013). As discussed above, MTOR signaling is a negative regulator of autophagy. Upon infecting dendritic cells, human immunodeficiency virus-1 (HIV-1) downregulates autophagy by inducing MTOR and RPS6KB/p70 S6 kinase (ribosomal protein S6 kinase, 70 kDa) activation, thus promoting viral proliferation in host cells (Blanchet et al., 2010).

In addition, a virulence factor may also directly target the autophagy machinery to negatively regulate autophagy. For example, the HSV-1 protein ICP34.5 binds BECN1 to block autophagy, possibly through inhibiting PIK3C3 kinase activity (Orvedahl et al., 2007).