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## Review

## Autophagy, antiviral immunity, and viral countermeasures

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## ABSTRACT

The autophagy pathway likely evolved not only to maintain cellular and tissue homeostasis but also to protect cells against microbial attack. This conserved mechanism by which cytoplasmic cargo is delivered to the endolysosomal system is now recognized as a central player in coordinating the host response to diverse intracellular pathogens, including viruses. As an endolysosomal delivery system, autophagy functions in the transfer of viruses from the cytoplasm to the lysosome where they are degraded, in the transfer of viral nucleic acids to endosomal sensors for the activation of innate immunity, and in the transfer of endogenous viral antigens to MHC class II compartments for the activation of adaptive immunity. Viruses have, in turn, evolved different strategies to antagonize, and potentially, to exploit the host autophagic machinery. Moreover, through mechanisms not yet well understood, autophagy may dampen host innate immune and inflammatory responses to viral infection. This review highlights the roles of autophagy in antiviral immunity, viral strategies to evade autophagy, and potential negative feedback functions of autophagy in the host antiviral response.

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## 1. Introduction

Autophagy is a conserved pathway that functions in all eukaryotic organisms to maintain cellular homeostasis. The autophagy pathway targets for lysosomal degradation both cellular cytoplasmic constituents, such as damaged or surplus organelles, proteins and protein aggregates, as well as microbial invaders, including viruses [1,2]. During autophagy, an isolation membrane wraps portions of cytoplasm to form a double-membrane organelle (autophagosome), the autophagosome undergoes fusion with the endolysosomal system to form the autolysosome, and degradation of the engulfed material occurs inside the autolysosome. The first description of autophagy-like structures in virally-infected cells was decades ago; Palade et al. visualized poliovirus particles inside double-membraned vacuoles that resembled autophagosomes [3]. However, the significance of autophagy in virus infection, until recent years, has remained elusive. Recently, there has been an expansion of literature on autophagy and immunity, which in part, has helped elucidate the diverse functions of autophagy in virus infections. Although still controversial, the general concepts are that autophagy may function both as an antiviral pathway (that degrades viruses) or as a pro-viral pathway (that helps viruses replicate or exit from cells), as a pathway that facilitates or prevents viral pathogenesis, and as a pathway that regulates innate and adaptive immune responses to viral infections (Fig. 1). Moreover,

the genes that execute autophagy, called autophagy or *ATG* genes, may have cellular functions independent of autophagy, raising the possibility that the effects of some autophagy genes in viral infections may reflect the role of this machinery in alternate cellular processes. This review will summarize present knowledge and controversies about the complex relationships between autophagy genes, immunity, and virus infection.

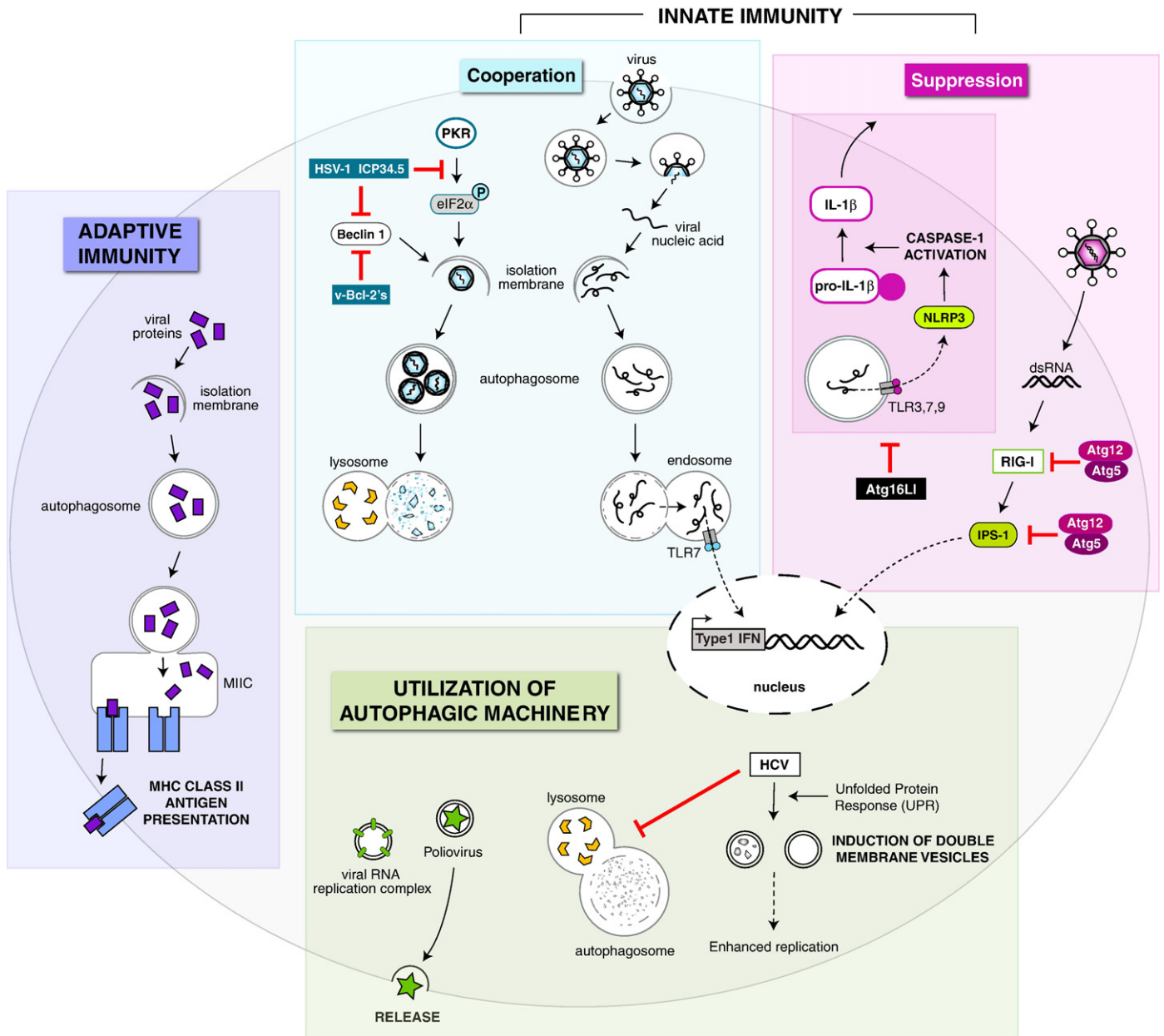
## 2. Autophagy and innate immunity in the host antiviral response

## 2.1. Autophagy in cooperation with innate immunity

When a virus invades a cell, the cell needs to recognize the virus immediately to evoke initial antiviral responses [4]. As sensors, pattern recognition receptors (PRRs) recognize viruses and trigger signaling cascades that induce antiviral mediators such as type I IFNs and pro-inflammatory cytokines. One family of PRRs, the endosomal Toll-like receptors (TLRs), is reported to have direct links with the autophagy pathway, at least in certain cultured cells. For example, the stimulation of TLR7 by single-stranded RNA (ssRNA) induces autophagy in a mouse macrophage cell line [5] and this autophagy induction is diminished by knockdown of TLR7, or MyD88, a myeloid differentiation factor 88 that mediates TLR7 signaling. Thus, since ssRNA viruses are recognized by TLR7 and TLR7 induces autophagy, TLR7 likely mediates autophagy induction by this class of viruses. Furthermore, TLR3, which recognizes dsRNA, a common viral replication intermediate, also induces autophagy [6]. It is not yet known whether TLRs that recognize other forms of viral nucleic acids,

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**Fig. 1.** Schematic overview of the relationships between autophagy (or autophagy proteins) and viral infection. *Adaptive immunity* (purple shaded region): Autophagy delivers endogenously synthesized viral proteins to MHC class II-loading compartments (MIIC), where they are loaded onto MHC class II molecules for presentation to CD4<sup>+</sup> T cells. *Cooperation with innate immunity* (blue shaded region): After virus infection, dsRNA structures activate the interferon-inducible antiviral molecule, PKR, which induces autophagy. Virions are engulfed in autophagosomes and degraded within the autolysosome. The HSV-1 virulence protein, ICP34.5, antagonizes both PKR activity and the autophagy protein Beclin 1 while viral Bcl-2 family members antagonize Beclin 1 to suppress autophagy induction. Viral nucleic acids are delivered by autophagosomes to endosomes containing toll like receptor (TLR7), which induces type 1 interferon (IFN) production. *Suppression of innate immunity* (pink shaded region): Recognition of viral nucleotides through TLR3, 7 and 9 may induce NLRP3 activation, which mediates caspase-1 activation. Active caspase-1 processes an IL-1 $\beta$  precursor into a mature cytokine, which is secreted. Atg16L1 blocks this process and Atg16L1 deletion results in increased IL-1 $\beta$  secretion. The Atg5-Atg12 conjugate suppresses the type I IFN response via retinoic acid-inducible gene I (RIG-I) and IFN- $\beta$  promoter stimulator-1 (IPS-1) inhibition. *Utilization of autophagy machinery* (green shaded region): Poliovirus infection induces double-membraned vesicles that contain autophagy marker proteins. Viral RNA synthesis occurs in association with these membrane vesicles, but autophagy proteins are not essential for poliovirus replication. Poliovirus may also utilize autophagosome-like structures to exit from the cell without lysis, via fusion of the double-membraned vesicle with the plasma membrane. Hepatitis C virus (HCV) induces double-membraned vesicles that contain autophagy markers through activation of the unfolded protein response (UPR) and blocks autophagosomal maturation. The double-membraned vesicles do not co-localize with HCV proteins, suggesting that HCV is neither targeted for autophagosomal degradation, nor uses autophagosome-like structures for viral replication or morphogenesis. Autophagy proteins are, however, required for enhanced HCV replication through mechanisms that are not yet defined. Throughout figure, dotted lines represent cross-talk that is either speculative or occurs by unknown, and possibly, indirect mechanisms. See text for further details.

such as TLR9 that recognizes viral DNA [4], or whether cytoplasmic viral nucleic acid sensors, such as the retinoic acid-inducible gene helicases (RLHs), retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene-5 (MDA-5), and laboratory of genetics and physiology 2 (LGP2) [4], function similarly in autophagy activation. Another important question is whether TLR induction of autophagy occurs during the context of a natural viral infection *in*

*vivo*; Saitoh et al. recently found that TLR agonists previously shown to induce autophagy in macrophage cell lines failed to induce autophagy in primary macrophages [7], suggesting that it may be difficult to extrapolate from *in vitro* studies to more physiological contexts.

The potential roles of TLR-mediated autophagy induction during viral infection are unknown. One speculation is that autophagy induction by TLRs may promote the capture and digestion of incoming

or newly replicating viruses inside the host cell. The signaling cascade by which TLRs induce autophagy also is not yet clear, but recent evidence suggests that certain TLR-signaling adaptor molecules may directly intersect with components of the autophagic machinery. For example, MyD88 and Trif, two signaling adaptors for TLRs, interact with the autophagy protein Beclin 1 in a manner that is enhanced by TLR signaling, and shRNA knockdown of MyD88, Trif, or Beclin 1 inhibits TLR-induced autophagy [6]. The interaction between TLR signal adaptors and Beclin 1 reduces the binding of Beclin 1 to the autophagy inhibitor, Bcl-2. Based on these findings, the authors postulate that TLR signaling leads to autophagy via adaptor-mediated recruitment of the autophagy protein, Beclin 1, which is part of the Class III phosphatidylinositol 3-kinase (PI3K) complex that functions at the stage of autophagosome membrane nucleation. It is not yet known whether TLR adaptor recruitment activates Beclin 1-associated Class III PI3K activity, which is necessary for autophagosome formation, and it is not yet known whether TLR adaptors interact with any other Atg proteins.

The interaction between TLRs and the autophagy pathway may be multidirectional. While TLRs likely function in autophagy induction during viral infection, there is also evidence that the autophagic machinery may function in the delivery of viral nucleic acids to endosomal TLRs. In plasmacytoid dendritic cells (pDCs) infected with vesicular stomatitis virus (VSV) or Sendai virus (SV), it has been shown that an autophagy protein is required for the delivery of viral nucleic acids to endosomes for detection by TLR7, and consequent production of the type I IFN, IFN- $\alpha$  [8]. pDCs that lack Atg5 fail to secrete IFN- $\alpha$  in response to VSV infection *in vitro* and *in vivo* in reconstituted lethally irradiated mice suggesting an important role for Atg5, a critical autophagy protein, in innate immune signaling.

Since Atg5 is reported to have autophagy-independent functions [9,10], it will be important to determine whether this function of Atg5 is through autophagy or some other pathway. Studies examining the role of other Atg proteins in type I IFN production stimulated by TLRs may be helpful in this regard. Also, it is not yet known how the autophagy pathway may specifically identify and capture VSV, and potentially other viral nucleic acids, for delivery to endosomal TLR7. A fascinating related question is how non-self versus self ssRNA may be targeted by autophagy for endosomal delivery. It is also not known whether DNA viruses that are recognized by TLR9 can be delivered to the endosome via autophagy.

One other convergent signal in antiviral immunity and autophagy regulation is the IFN-inducible gene, double-stranded RNA-dependent protein kinase (PKR). PKR, a key mediator of the antiviral action of IFN, was originally discovered in the context of its ability to reverse virus-induced host translational arrest mediated by eIF2 $\alpha$  phosphorylation [11]. However, it is now known that PKR, as well as other stress-induced eIF2 $\alpha$  kinases, are essential for autophagy induction in response to viral infection and other forms of cellular stress [12,13]. For example, PKR and the serine phosphorylation site of eIF2 $\alpha$  are required for herpes simplex virus-induced autophagy in mouse embryonic fibroblasts (MEFs) and primary neurons [13,14], and PERK, the PKR-like ER kinase is required for autophagy induced by ER stress in MEFs [15]. The mechanisms by which PKR/eIF2 $\alpha$  regulates autophagy are presently unknown. Nonetheless, studies with herpes simplex virus type 1 (HSV-1) suggest a potentially important role for PKR-dependent regulation of autophagy in viral pathogenesis; a mutant HSV-1 strain that lacks the ability to antagonize the autophagy protein Beclin 1 is highly neuroattenuated in wild-type mice but has full virulence in *pkrr*<sup>-/-</sup> mice [16]. Thus, the autophagy-stimulatory function of PKR may be important in mediating some of its antiviral effects, at least in HSV-1 encephalitis. Further studies are required to dissect the relative contributions of translational regulation and autophagy regulation in the antiviral effects of PKR in other viral infections.

Clearly, many important questions remain regarding the cooperation between autophagy and TLRs, RLHs and type I IFN signaling

in innate immunity. Nonetheless, the newly discovered links do suggest critical points of intersection, in terms of TLR-mediated autophagy induction, autophagy-mediated delivery of viral nucleic acids to TLRs, and regulation of autophagy by a key antiviral IFN-inducible molecule. This intersection seems likely to underlie critical, but as yet, incompletely understood roles of autophagy in innate antiviral immunity.

## 2.2. Autophagy in the suppression of innate immunity

Two recent lines of evidence suggest that autophagy may not only serve to activate innate immunity, but may also serve as a “brake” on the magnitude of the host innate antiviral response. The first line of evidence stems from observations suggesting that the autophagy proteins, Atg5 and Atg12, down-regulate another group of PRRs, the cytoplasmic viral nucleic acid sensors, the RLHs [17]. Jounai et al. found that type I IFN production is enhanced in *Atg5*<sup>-/-</sup> and *Atg7*<sup>-/-</sup> MEFs in response to VSV infection or immunostimulatory RNA. The increase in type I IFN production in *Atg5*<sup>-/-</sup> MEFs is associated with decreased viral replication; however, it is not yet known whether this decrease is an indirect result of enhanced IFN production or related to a more direct role for the autophagic machinery in promoting viral replication, as has been reported for other RNA viruses (see below). Interestingly, Jounai et al. also found that the Atg5–Atg12 conjugate can bind to RIG-I and the IFN- $\beta$  promoter stimulator 1 (IPS-1), an adaptor protein, to suppress the activity of RLHs in stimulating type I IFN production. While these findings suggest a plausible mechanism by which the autophagic machinery may suppress innate immune signaling, i.e. through direct inhibitory interactions with RLHs and their adaptor proteins, it is not yet known whether this process, or other, as yet unidentified mechanisms, may explain the increased type I IFN production observed in VSV-infected *Atg5*<sup>-/-</sup> cells.

A second line of recent evidence suggests that autophagy may also function as a “brake” on the pro-inflammatory response regulated by nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) proteins [7]. The NLR protein, cyroprylin/NALP/NLRP3, is reported to recognize ssRNA and dsRNA in the cytoplasm [18] and forms a complex known as the inflammasome, containing ASC (apoptosis-associated speck-like protein containing a caspase-activating and recruitment domain) and caspase-1 that is responsible for the processing of pro-IL-1 $\beta$  to its mature, secreted form [18,19]. Saitoh et al. found that hematopoietic cells from mice lacking the autophagy gene, *Atg16l1*, have increased production of the pro-inflammatory cytokines, IFN- $\beta$  and IL-18, following endotoxin stimulation of TLR4 [7]. Although not yet proven, it is reasonable to predict that viral stimulation of this inflammatory signaling pathway may also be negatively regulated by autophagy. A critical question, both with respect to autophagy-dependent negative regulation of type I IFN production and IL-1 $\beta$  production, is whether autophagy functions primarily as an immunosuppressive pathway that increases host susceptibility to viral infection, or rather, merely provides negative feedback to dampen immune/inflammatory signaling so as to avoid excessive and potentially harmful inflammatory host responses.

## 3. Autophagy and adaptive immunity in the host antiviral response

Autophagy may also be involved in adaptive immunity to viral infections [20,21]. The initial interest in autophagy and adaptive immunity was stimulated by the discovery that a majority of peptides presented by MHC Class II molecules were derived from cytosolic “self” proteins [22,23]. Paludan et al. were the first to use genetic approaches to demonstrate a role for the autophagy machinery in MHC class II presentation of an endogenously synthesized viral antigen [24]. They found that siRNA-mediated silencing of the autophagy gene, *Atg12*, inhibits the intracellular processing of the



Epstein Barr virus (EBV) nuclear antigen 1 (EBNA1), decreasing EBNA1-specific CD4<sup>+</sup> T cell responses. Subsequently, these authors showed that in dendritic cells, B cells, and epithelial cells, autophagosomes are constitutively formed and fuse with multivesicular MHC class II-loading compartments (MCIIs) [25]. Furthermore, the specific targeting of influenza virus matrix protein to autophagosomes via fusion to the autophagy protein, LC3, enhances MHC class II presentation to CD4<sup>+</sup> T cells.

Thus, it seems likely that the autophagic delivery of certain endogenously synthesized viral antigens to MHC class II-loading compartments functions in adaptive antiviral immunity. A dual role for autophagosomes in degrading viruses and in the delivery of viral peptides generated by this degradation process to MHC class II-loading compartments could represent a very effective “two-pronged” mode of host antiviral defense. An important question is whether autophagy also plays a role in the more common exogenous pathway of MHC class II presentation of viral peptides, in which exogenous endocytosed antigens are presented to CD4<sup>+</sup> T cells. Even in the case of endogenously synthesized viral antigens, it is not yet clear how broadly the autophagy pathway is utilized for MHC Class II presentation; for example, in contrast to EBNA1, two other EBV-encoded nuclear antigens, EBNA2 and EBNA3C, are not presented by MHC class II molecules via an autophagy-dependent process [26]. Endogenous MHC class II presentation has been reported for other viral antigens, such as hepatitis C virus (HCV) core, influenza nucleoprotein and hemagglutinin, and human papillomavirus (HPV) 16 E7, but the involvement of autophagy has not yet been investigated in these examples [27–31]. Furthermore, it is not yet clear whether autophagic delivery of endogenous antigens for activation of adaptive immunity is important in the context of natural viral infections, as existing studies have been performed *in vitro* in cells ectopically expressing viral proteins or antigens. Regardless of these uncertainties, the prospects seem promising that the selective targeting of viral antigens to the autophagosome will significantly enhance the efficacy of viral antigen-based vaccines.

#### 4. Autophagy as a potential antiviral mechanism

The targeting of viral components (proteins or nucleic acids), assembled virions, or host factors required for viral replication for degradation via an autophagolysosomal pathway could in theory function as an innate antiviral mechanism. While it is not yet clear by which mechanism(s) autophagy restricts viral replication, two studies have demonstrated an inhibitory effect of autophagy genes on viral replication *in vivo*. One study involves plant infection by tobacco mosaic virus (TMV), a single-stranded RNA virus [32]. In the *Nicotiana benthamiana* plant, pathogen infection induces the hypersensitive response (HR), which is characterized by programmed cell death that is restricted to infected cells and prevents pathogen spread. In plants silenced for autophagy genes, including *BECLIN 1*, *VPS34*, *ATG3* or *ATG7*, TMV infection results in HR cell death that extends beyond the site of infection. Moreover, there is increased replication of TMV in infected leaves, suggesting that autophagy-related genes function both to prevent bystander cell death during the HR response and to limit virus replication. Another study involves mouse infection with Sindbis virus, a positive-strand RNA virus of the *Togaviridae* family that serves as an animal model of human arthropod-borne encephalitis [33]. With virus-driven ectopic neuronal expression of the Beclin 1 autophagy protein, there is decreased animal mortality, decreased virus-induced neuronal apoptosis, fewer Sindbis virus RNA-positive cells, and lower CNS viral titers. Although the role of endogenous autophagy genes in protection against Sindbis virus encephalitis has not yet been examined, this study demonstrates that enhanced autophagy can inhibit CNS viral replication.

Together, these studies suggest an evolutionarily conserved role for autophagy genes as an antiviral mechanism in both plants and

mammals. As discussed in more detail below, the impaired neuronal replication of an HSV-1 mutant virus that cannot inactivate Beclin 1 further supports this concept [16]. It is not yet known whether the antiviral effects of autophagy are restricted to the capture of newly synthesized virion components (as suggested by studies published to date) or whether viruses can also be targeted to autophagosomes during cellular entry. Furthermore, virtually nothing is known about the signals that target viral proteins to the autophagosome; it will be interesting to examine whether ubiquitination and the p62/SQSTM1 adaptor protein that function in “earmarking” misfolded or aggregated cellular proteins for autophagic degradation [34,35] also function similarly to earmark viral proteins. It will also be important to examine the role of viral pathogen-associated molecular patterns and pathogen recognition receptors in the delivery of viruses to the autophagolysosomal system for xenophagic degradation. In the case of intracellular bacterial infection with *Listeria* in *Drosophila*, the PRR molecule PGRP-LE (analogous to NLR in mammals) was shown to be important in recognizing a *Listeria* PAMP, resulting in autophagy induction and restriction of bacterial survival in primary hemocytes [36]. Further research is likely to reveal parallel roles for PAMPS and PRRs in autophagic control of viral replication.

#### 5. Evasion of autophagy by viruses

Since autophagy functions as an innate immune mechanism to eliminate viruses, it is not surprising that some viruses have evolved mechanisms to escape host autophagy. Thus far, viruses described to evade autophagy primarily fall within the double-stranded DNA viruses of the *Herpesviridae* family. One  $\alpha$ -*Herpesviridae* family member, HSV-1 has two strategies to block host cell autophagy, both of which are mediated by the viral protein, infected cell protein 34.5 (ICP34.5), which is an important viral neurovirulence factor [37,38]. ICP34.5 both antagonizes PKR-dependent induction of autophagy and also binds to and inhibits the autophagy protein Beclin 1 [13,14,16]. A mutant HSV-1 virus lacking the Beclin 1-binding domain of ICP34.5 fails to inhibit autophagy in neurons and fails to cause lethal encephalitis in wild-type mice but has restored neurovirulence in *pkr*<sup>-/-</sup> mice [16]. These data indicate that PKR lies genetically upstream of Beclin 1 *in vivo*, that Beclin 1-dependent autophagy is important for protection against HSV-1 encephalitis, and that viral evasion of Beclin 1-dependent autophagy is important for viral neurovirulence. It is not yet known whether this role of autophagy in viral protection and this role of viral evasion of autophagy in viral virulence is restricted to neurotropic infections or also occurs in other cell types and tissues. Since basal autophagy in the mouse nervous system is critical to ensure protein quality control and the prevention of neurodegenerative diseases [39,40], autophagy inhibition by viruses may contribute to neuronal death and organism mortality via mechanisms that are independent of stimulatory effects on viral replication. Furthermore, as vital postmitotic cells, non-cytolytic mechanisms for viral clearance, such as those involving xenophagy (i.e. autophagic degradation of pathogens), may be particularly important in antiviral host defense in neurons.

Beclin 1 is also the target of autophagy inhibition by viral Bcl-2-like proteins encoded by the  $\gamma$ -herpesviruses, including Kaposi's sarcoma-associated herpesvirus (KSHV) vBcl-2 [41,42] and the murine  $\gamma$ -herpesvirus 68-encoded protein, M11. At present, it is unknown whether autophagy evasion by vBcl-2 is important for  $\gamma$ -herpesviruses replication and pathogenesis, but it is interesting to note that these viruses are oncogenic and that Beclin 1 and the autophagy pathway function in tumor suppression [43]. A member of the  $\beta$ -*Herpesviridae* family, human cytomegalovirus (HCMV) can also down-regulate autophagy [44]; the mechanism by which this occurs is not yet fully defined but requires viral protein expression and may involve stimulation of the autophagy inhibitory mTOR signaling

pathway. The HCMV genome does not have equivalents for either ICP34.5 or viral Bcl-2 proteins, indicating that each family of herpesviruses (e.g. the  $\alpha$ ,  $\beta$ , and  $\gamma$ -herpesviruses) may have evolved distinct molecular strategies to evade host autophagy.

The common evolutionary pressure for different families of herpesviruses to evolve such strategies suggests a fundamental need for viral evasion of autophagy in the life cycle of herpesviruses. Further work needs to be done to investigate whether other families of viruses also possess mechanisms to evade host autophagy, and the role of such mechanisms in viral pathogenesis. As it is already known that numerous viruses regulate autophagy signaling pathways, such as the PKR/eIF2 $\alpha$  signaling and the Akt/mTOR signaling pathways [45,46], it seems likely that viral evasion of autophagy will be common to many virus families. The specific strategies that individual viruses use to disarm host autophagy may represent novel targets for antiviral therapy.

## 6. Viral utilization of the autophagic machinery for replication

Although autophagy plays a role in antiviral immunity, somewhat paradoxically, some viruses may utilize components of the autophagic machinery for their own benefit. Increasing evidence suggests that autophagy proteins may have autophagy-independent functions, including in other membrane trafficking events and immune signaling, and in most cases, it is not yet clear whether the utilization of components of the autophagic machinery for viral replication represents a true “subversion” of the autophagy process or alternate functions of autophagy proteins in membrane dynamics or immune regulation.

Certain positive-stranded RNA viruses and cytoplasmic DNA viruses induce the formation of cytoplasmic membranes to facilitate the replication of their genomes on such membranes [47]. The vesicles which certain positive-stranded RNA viruses induce display some hallmarks of cellular autophagosomes, including a double-membraned nature and positive staining for the autophagy protein, LC3, although virus-induced vesicles are generally smaller in diameter than classical autophagosomes. Early work with poliovirus demonstrating the association of viral replication complexes with double-membraned structures [48] stimulated investigations of the role of autophagy in the life cycle of several different RNA viruses and the cytoplasmic DNA virus, vaccinia virus. These studies have revealed certain common themes as well as distinct differences, even within members of the same virus family, with respect to the utilization of the autophagy pathway in viral replication.

In the case of HCV, a member of the flaviviridae family, two independent studies demonstrated that HCV induces the accumulation of autophagosomes (without inducing a complete autophagic response involving protein degradation) in human hepatocytes [49,50]. However, in both studies, the authors failed to observe colocalization of HCV proteins with autophagosomes, indicating that, unlike poliovirus, HCV replication complexes do not appear to be associated with autophagosome-like structures. Nonetheless, genetic knockdown of ER stress signaling molecules required for HCV-induced autophagy or of autophagy execution genes, such as LC3 or *Atg7*, reduced HCV RNA levels [49]. These data suggest that autophagy induction (or at least components of the autophagic machinery) somehow enhances HCV replication through an as-of-yet undefined mechanism that does not directly involve the utilization of autophagosomes for virion replication or morphogenesis. In light of recent data (discussed above) indicating that autophagy may participate in the suppression of innate immune signaling, one unexplored possibility is that the increased HCV replication observed in autophagy-deficient cells may be an indirect consequence of enhanced innate immune signaling. It is tempting to speculate that HCV, and other viruses that successfully establish persistent infections, may simultaneously activate autophagy to suppress innate

immune signaling, while simultaneously blocking the maturation of autophagosomes into autolysosomes that degrade virus. Given the absence of colocalization between HCV proteins and markers of even early autophagosomes (i.e. *Atg5*), it is also likely that HCV possesses a mechanism to block the initial targeting or sequestration of HCV proteins by the autophagosome.

Another important emerging theme is that components of the autophagic machinery may play a role in the exit of non-lytic viruses from infected cells. In poliovirus-infected cells, siRNA-mediated knockdown of LC3 and *Atg12* markedly inhibits the release of infectious virus while only minimally affecting viral replication [51], suggesting that the primary function of poliovirus's utilization of the autophagic machinery may be for viral release rather than, as previously speculated, to provide a membrane scaffold for RNA replication. It is not yet clear whether this function of the autophagic machinery is conserved for other picornaviruses. In Coxsackie B virus-infected cells, siRNA against the autophagy genes, *Atg7*, *beclin 1*, or *Vps34*, does decrease the levels of viral protein expression as well as extracellular viral titers, but the mechanism by which this occurs has not yet been explored [52]. Furthermore, in cells infected with human rhinovirus 2 or *Drosophila C* virus picornavirus (picorna-like virus), pharmacological or genetic inhibition of autophagy, respectively, does not affect viral replication [53,54]. Studies with other virus families that replicate in association with double-membraned structures, including the coronavirus, mouse hepatitis virus, and the poxvirus, vaccinia virus, have failed to reveal alterations in viral replication or morphogenesis in MEFs lacking *Atg5* or in embryonic cells lacking *beclin 1* [55–58].

Taken together, it appears that the components of the autophagic machinery are utilized to optimize viral yields of certain viruses, including HCV, Dengue virus, poliovirus, Coxsackie B virus, and potentially HIV (discussed in more detail below) [49–52,59–62]. The mechanisms by which this occurs may differ for different classes of viruses, as there is no evidence to date that flavivirus proteins colocalize with autophagy proteins whereas poliovirus proteins do colocalize with autophagy proteins. Therefore, certain picornaviruses may directly co-opt the autophagic machinery whereas the stimulatory effect of autophagy on viral replication may be more indirect for flavivirus replication. More detailed studies are needed to define the precise mechanisms by which components of the autophagic machinery contribute to, rather than inhibit, viral replication.

Another important related question is whether the observed roles of certain autophagy proteins in supporting viral replication reflects a role for the autophagy pathway per se, a role for autophagy proteins in the formation of alternative double-membraned cellular compartments involved in virion morphogenesis, or other as-of-yet undefined roles. An earlier study with the pestivirus, bovine viral diarrhea virus (BVDV), provides a provocative example of how an autophagy protein might benefit flavivirus replication in a manner that is independent of “classical autophagy” or double-membrane vesicle formation [63]. In most cases, cytopathogenic (cp) BVDV develops from non-cytopathogenic (noncp) BVDV by RNA recombination that can occur between the noncp BVDV genome and RNAs of either viral or cellular origin. A cp BVDV was isolated from an animal with lethal disease mucosal syndrome in which the recombination event that converted noncp BVDV into cp BVDV involved the insertion of the LC3 autophagy protein into the viral genome. This insertion induces an additional cleavage of the viral polyprotein downstream of the LC3-encoded sequence that is independent of the viral NS3 serine protease and contributes to the ability of the virus to replicate autonomously without a helper virus. Although not specifically investigated, the incorporation of LC3 presumably functions as a signal for specific processing of the viral polyprotein by the LC3-directed, cellular *Atg4* protease. Thus, this virus may use a proteolytic event of the autophagy machinery to process viral protein products to enhance its own replication. While the pathogenesis strategy of bovine pestivirus is

somewhat unique and one cannot necessarily extrapolate from findings observed with viral incorporation of an autophagy protein to effects of the cellular expressed autophagy protein, this study does highlight an interesting point. Autophagy proteins have complex biochemical functions, including the regulation of proteolytic processing and ubiquitin-like protein conjugations, and these biochemical functions could potentially directly impact viral replication in an autophagy-independent manner.

## 7. Viral utilization of the autophagic machinery for pathogenesis

Recent studies with HIV-1 suggest that this virus may not only utilize components of the autophagic machinery for replication, but also utilize the autophagic machinery to induce a central feature of AIDS pathogenesis, the depletion of uninfected CD4<sup>+</sup> T lymphocytes. Several autophagy-related genes were identified in a genome-wide siRNA screen to identify host factors required for efficient HIV-1 replication, including *Atg7*, *Atg8* (*GABARAPL2*), *Atg12* and *Atg16L2* [64]. Since the screening was performed in HeLa cells, it will be important to investigate the role of these and other autophagy genes in HIV-1 replication in its natural target cells, such as lymphocytes and macrophages. Whether or not autophagy genes function to promote HIV-1 replication in natural target cells, there is strong *in vitro* evidence that HIV-1 induced bystander CD4<sup>+</sup> T cell death requires the autophagic machinery [60,61]. Espert et al. demonstrated that the binding of HIV-1 envelope protein to the CXCR4 receptor on uninfected CD4<sup>+</sup> T lymphocytes leads to cell death that is reversed by pharmacological autophagy inhibition (e.g. treatment with 3-methyladenine) or siRNA against two autophagy genes, *Atg7* and *beclin 1*. Future studies are required to determine whether this HIV-1 envelope protein triggered autophagy gene-dependent cell death contributes to the progressive decline in CD4<sup>+</sup> T cell numbers that occurs in patients with AIDS.

## 8. Autophagy as a cytoprotective mechanism during viral infection

In contrast to the role of autophagy genes in mediating cell death induced by HIV envelope glycoprotein in uninfected lymphocytes, other studies indicate that autophagy may play an important pro-survival or cytoprotective role during viral infections. As noted above, autophagy gene silencing results in extensive programmed cell death in uninfected bystander cells in tobacco mosaic virus-infected plants [31]. Thus, autophagy may protect uninfected plant cells against cytokine pro-death signals that are released during the antiviral innate immune response. It is tempting to speculate that a similar scenario may be operative in the brains of primates infected with simian immunodeficiency virus (SIV) or patients infected with HIV-1 where glutamate, TNF- $\alpha$ , and other cytokines are candidate neurotoxins [62]. Autophagy in neurons (which are not direct targets of SIV/HIV-1 infection) may protect these cells against toxic effects of cytokines released by SIV- or HIV-1 infected microglia. Furthermore, treatment with TNF- $\alpha$  or glutamate has been reported to reduce autophagy in neurons, and decreased autophagy (as measured by p62 mRNA expression levels) has been reported in the brains of non-human primates with SIV encephalitis and of patients with HIV dementia [62]. Therefore, decreased autophagy may be a mechanism underlying neurodegeneration in SIV and HIV-1 infection. It is also possible that neuronal autophagy inhibition by direct viral infection, in the case of HSV-1 encephalitis, may underlie some of the permanent neurologic dysfunction that occurs in this disease [65]. Finally, autophagy may also protect against virus-induced cell death in a cell autonomous fashion; Beclin 1 overexpression reduces Sindbis virus-induced neuronal apoptosis (as noted above) [32] and pharmacological inhibition of autophagy enhances parvovirus B19-induced erythroid cell death [66]. It is not yet clear how autophagy protects against virus-induced cell death and in the case of Sindbis virus

infection, it is not known whether this protection against cell death is merely a consequence of reduced levels of viral replication. Nonetheless, taken together, the accumulating evidence suggests that autophagy may play a critical role in reducing the cytopathology of both direct cellular targets of infection as well as neighboring cells that may be susceptible to toxic effects of cytokines released by virally-infected cells.

## 9. Conclusion

Throughout evolution, host organisms have developed increasingly complex defense systems against viruses, and in turn, viruses have evolved sophisticated mechanisms to evade host antiviral defense mechanisms. Although there are many unresolved questions regarding the role of autophagy in viral infection, the targeting of specific autophagy proteins by viral virulence factors underscores the likely importance of autophagy as a primordial mechanism of antiviral immunity. In its most primitive form, autophagy may merely be a mechanism by which individual cells (or unicellular organisms) protect themselves in a cell autonomous fashion by “eating” the viruses that attack them. With the evolution of metazoan organisms and more complex immune systems, the autophagy pathway proteins are likely to have acquired several different functions that help protect against virus infection at the organismal level: they function in the activation of innate immune signaling; they function in adaptive immunity; and they may function in protecting bystander cells from detrimental effects of cytokines released during viral infection. Even the recently reported role of autophagy proteins in the suppression of innate immunity may represent an adaptive mechanism that controls the magnitude of the host immune response during viral infection, thereby preventing detrimental host inflammation. Thus, autophagy proteins likely have evolved diverse functions to orchestrate an effective multi-pronged host defense against viral infection. In future studies, it will be important to more clearly define the precise molecular mechanisms by which autophagy proteins function in antiviral immunity and the precise strategies that viruses use to either evade or exploit host autophagy proteins for their own benefit. Such knowledge may empower us to outsmart the viruses that outsmart the host autophagy pathway.

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