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# Autophagy and antiviral immunity

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Autophagy is an ancient pathway designed to maintain cellular homeostasis by degrading long-lived proteins and organelles in the cytosol. Recent studies demonstrate that autophagy is utilized by the cells of the innate and adaptive immune systems to combat viral infections. Autophagy plays a key role in recognizing signatures of viral infection, and represents a critical effector mechanism to restrict viral replication. On the other hand, autophagosomes have been exploited by certain viruses as a niche for viral replication. Furthermore, autophagy can be used to deliver endogenous viral antigens to the MHC class II loading compartment, allowing activation of CD4 T cells. In this review, we describe recent advances in the field of autophagy as it relates to innate and adaptive antiviral immune responses.

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## Introduction

Synthesis and degradation of intracellular proteins and organelles is required to maintain cellular homeostasis. The principal pathways responsible for the degradation of proteins in eukaryotes are the proteasome pathway and autophagy. The ubiquitin–proteasome system is involved in the constitutive degradation of soluble short-lived proteins that undergo continual turnover in living cells [1]. Autophagy, which was originally characterized as a starvation-induced response, delivers long-lived proteins and entire organelles for lysosomal degradation [2]. There are three types of autophagy: microautophagy, chaperon-mediated autophagy (CMA) and macroautophagy [3]. Microautophagy is characterized by the uptake of cytoplasmic components at the lysosome outer membrane via budding into the lysosome, through an undefined molecular mechanism. The CMA pathway is only conserved in higher eukaryotes and Ribonuclase A was identified as the first CMA substrate, which has a specific motif, KFERQ, necessary for degradation [4]. The chaperone protein Hsc70 recognizes this motif and interacts with lysosomal membrane protein (lamp) 2a, which is a peptide transporter [5–7]. Proteins are translocated into the lumen of lysosomes where they are subsequently degraded. Macroautophagy (hereafter as autophagy) is the major route of degradation of cytoplasmic constituents, which is mediated by the formation of the autophagosome, followed by its fusion with lysosomes. The autophagosome is a double membrane vesicle which forms via the elongation of a cup-shaped membrane and allows recycling of degraded proteins. A class of genes termed, autophagy-related genes (Atg), specifically regulate the process of autophagy. Yeast genetic studies have identified more than 20 Atg genes required for autophagy [8]. Two ubiquitin-like conjugation systems are necessary for autophagosome formation [9]. Once formed, the autophagosome fuses with the lysosome and, the cytoplasmic constituents are released into the lumen of the lysosomes and subsequently degraded by hydrolases, and are recycled through lysosomal transporters. The precise molecular mechanism of autophagy has been covered extensively by excellent reviews elsewhere [2,10,11].

In addition to the classical homeostatic function of autophagy described above, the importance of autophagy has now been defined in multiple biological processes including development, differentiation, and tissue remodeling [12]. Not surprisingly, autophagy is involved in both the prevention and pathogenesis of certain types of diseases [13]. Recent studies have shed light on how the immune system utilizes autophagy to fight microbial infection, while some pathogens have exploited this process for their own survival and replication [14,15]. In this review, we focus on the role of autophagy in antiviral innate and adaptive immune responses.

## Autophagy in viral pathogenesis

### The autophagosome as site of viral replication

Several viruses have been shown to subvert the autophagic machinery for their own replication and survival advantage. During poliovirus [16] and equine arteritis virus [17] infection (both positive stranded RNA viruses), the membranes that are induced resemble autophagosomes containing the characteristic double-membrane-bound morphology. The reduction of Atg12 and LC3 (two critical autophagy genes) by siRNA resulted not only in a reduction of extracellular poliovirus and Rhinovirus but also in decreased intracellular viral pools [18••]. It is proposed that the non-enveloped virus, poliovirus, uses the autophagic pathway as a nonlytic mechanism for viral

release (Figure 1). Replication complexes derived from the coronavirus, mouse hepatitis virus (MHV), a positive stranded RNA virus, colocalize with autophagy proteins LC3 and Atg12. In addition, MHV replication was significantly impaired in $Atg5^{-/-}$ embryonic stem cell lines and this replication defect was cured by expression of Atg5 in the $Atg5^{-/-}$ cells [19]. By contrast, a recent study demonstrated that MHV replication was not impaired in the absence of Atg5 in primary mouse embryonic fibroblasts (MEFs) and bone marrow derived macrophages [20]. These studies emphasize the potential cell-type specific roles of autophagy in viral pathogenesis and the importance of primary cell systems in assessing autophagy-related events.

Certain viruses induce autophagy to enhance their niche for replication. Rotavirus, a double-stranded RNA virus of the reovirus family, induces autophagy via the NSP4 protein and replicate in proximity to autophagosome membranes [21]. B19 parvovirus, an ssDNA virus, also induces autophagy as a means to prolong survival of infected cells [22]. In addition, open reading frame 1a of the equine arteritis virus was shown to induce the formation of the double membrane vesicles from ER membrane [17]. Poliovirus encodes 2BC and 3A, which are known to be sufficient to induce double-membraned vesicles [18••]. These studies collectively suggest that viruses have evolved the ability to commandeer the cellular autophagosomal machinery to facilitate virus replication. However, whether the double membrane vesicles used by these viruses represent true autophagosomes, and to what extent viral replication depends on autophagy still requires further investigation.

### The role of autophagy in virus-induced disease

Human immunodeficiency virus type I (HIV-1) infection induces the depletion of CD4 T cells, resulting in AIDS development. CD4 T cells are killed by direct HIV infection, but large numbers of uninfected CD4 T cells also die in HIV-infected individuals. Recently, autophagy has been shown to promote cell death in bystander CD4 T cells during HIV-1 infection, independently of HIV-1 replication [23•]. In this study, authors demonstrated that envelope glycoprotein (Env) of HIV-1 could kill uninfected CD4 T cells in autophagy-dependent manner. By culturing effector cells expressing Env with target cells expressing CD4 and CXC chemokine receptor 4 (CXCR4), they showed that CXCR4 engagement activated autophagic pathway (Beclin 1 accumulation). Such death required the expression of CD4 and CXCR4 in target cells and was accompanied by the accumulation of autophagic vesicles. Env-mediated death of CD4 T cells required machinery of autophagy, as siRNA knockdown of Beclin 1/Atg6 or Atg7 inhibited the apoptotic process. Interestingly, authors showed that stromal cell-derived factor 1 (SDF-1), the natural ligand for the CXCR4 receptor, which induces migration of CXCR4-expressing T cells to lymphoid tissues, was unable to induce autophagy, suggesting that Env binding to CXCR4 induces specific signaling leading to apoptosis. Thus, autophagic death in CD4 T cells may play an important role in the pathogenesis of AIDS. Whether autophagy-dependent cell death contributes to pathogenesis of other viral diseases remains to be determined.

## Autophagy in antiviral defense

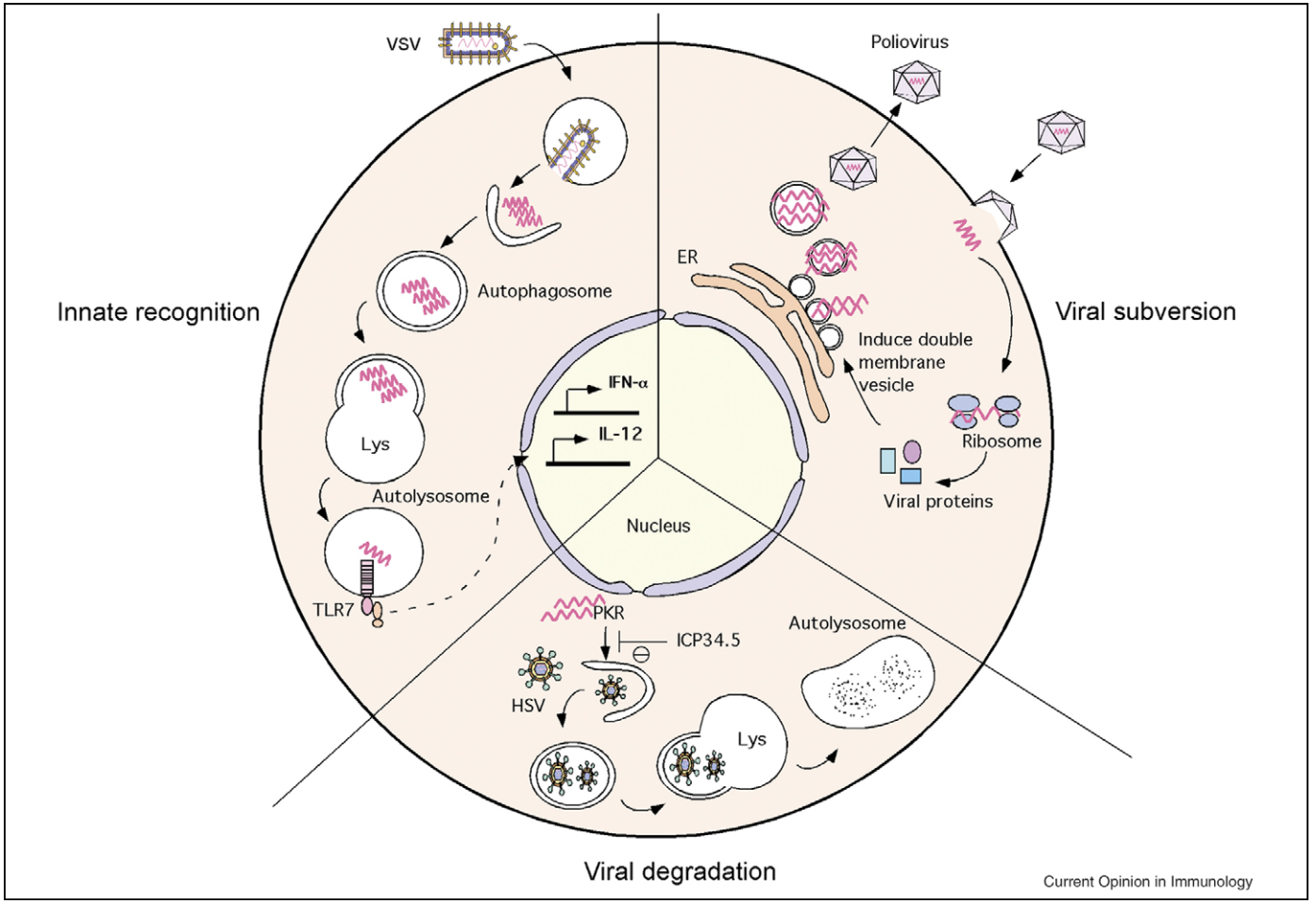
### Autophagy limits viral replication and pathogen-induced cell death

Autophagy plays an integral pro-survival role during starvation, stress and infection. Several studies indicate that viral replication and infection-induced cell death could be limited by autophagy. In plants, autophagy exerts antiviral effects against tobacco mosaic virus (TMV) by (i) restricting cell death to the infection site, and (ii) limiting replication and cell-to-cell movement of TMV [24••]. In mammalian hosts, encephalitis induced by Sindbis virus can be reduced by the forced expression of Beclin 1 in mice [25]. In this study, Beclin 1 protein was found to bind to bcl-2 and suppress virus-induced apoptosis in mouse brains, resulting in protection against lethal encephalitis. These studies revealed an important anti-apoptotic function of autophagy and therefore protection against virus-induced pathologies. While the precise mechanism(s) by which autophagy or autophagy-related genes prevent apoptosis upon viral infection are currently unknown, it will be interesting to determine whether such mechanism overlaps with those utilized under starvation conditions.

### Xenophagy and viral evasion

Autophagosomes have been shown to engulf viruses or viral components and destroy them in a process known as xenophagy [15]. Certain viruses are known to encode genes that specifically interfere with this pathway. One of the best-studied antiviral effector molecules, dsRNA-activated protein kinase (PKR), inhibits most translation initiation. PKR accomplishes this by phosphorylating a translation-initiation factor, eIF2a, preventing recycling of the eIF2a–GDP complex. Interestingly, one of the downstream consequences of PKR activation is the induction of autophagy [26]. HSV-1 encodes a protein, ICP34.5, which antagonizes the function of PKR by dephosphorylating eIF2a. When MEFs were infected with HSV-1 that lacks the ICP34.5 gene, an increase in autophagy activity was detected. This effect was abolished in MEFs from PKR-deficient mice, indicating that PKR is responsible for driving the increase in autophagy caused by infection with ICP34.5-deleted HSV-1. In the case of wild-type HSV-1 virus infection, no increase in autophagy activity was seen, presumably because PKR-induced eIF2a phosphorylation was sufficiently inhibited by the ICP34.5 protein. These observations raise the possibility that one of the mechanisms by which PKR exerts antiviral effect is through the induction of

**Figure 1**




The role of autophagy in antiviral immune responses. An example of viral subversion, degradation and innate recognition is depicted. *Viral subversion*: The positive strand RNA of poliovirus is translated by ribosomes and viral proteins are synthesized. Some of these proteins (2BC and 3A) trigger the induction of double membrane vesicle from endoplasmic reticulum (ER) by exploiting cellular machinery for autophagy. Viral RNA synthesis occurs in the vicinity of these membrane vesicles. Newly formed virus is released from the cell by lysis or possibly by fusion of autophagosomal membrane with plasma membrane. *Viral degradation*: After HSV-1 infection, dsRNA structures activate PKR, which in turn induces autophagy. HSV-1 virions are engulfed in autophagosomal structures and are degraded within the autolysosome. ICP34.5, a virulence protein encoded by HSV-1, antagonizes PKR function to suppress the induction of autophagy. *Innate recognition*: In pDCs, cytosolic RNA replication intermediates of VSV infection are engulfed by autophagosome and are delivered to the lysosome upon fusion. TLR7 is activated in the lysosome by recognition of such replication intermediates and triggers the induction of antiviral genes such as IFN-$\alpha$ and IL-12.

autophagy. A later study by the same group revealed that, indeed, ICP34.5-deficient HSV-1 virions are engulfed in autophagosomal structures and degraded in a PKR-dependent manner [27], indicating the importance of xenophagy in degradation of viral pathogens (Figure 1). In a recent study, ICP34.5 was found to bind to Beclin 1 and inhibit autophagy [28••]. This study also revealed the *in vivo* importance of autophagy-mediated viral control by demonstrating that Beclin 1-binding deficient ICP34.5 mutant HSV-1 is neuroattenuated in mice. Moreover, neurovirulence of the ICP34.5 mutant virus was restored in $PKR^{-/-}$ mice, suggesting that PKR is required to induce autophagy upstream of Beclin 1. Thus, in addition to thwarting other known functions of PKR, viral genes encoded by HSV-1 (US11) [29], Avian reovirus (sA) [30], Influenza virus (NS1) [31], Epstein-Barr virus (SM) [32], HIV (TAT) [33] and Myxoma virus (M156R) [34], might function by inhibiting the induction of autophagy through inhibiting PKR. It will be important to examine the contribution of autophagy in PKR-mediated antiviral effector function in these and other viruses.

### Autophagy and viral recognition

Cells of the innate immune system encode a limited number of pattern-recognition receptors (PRRs), which serve to recognize viruses following detection of viral pathogen-associated molecular patterns (PAMPs) [35,36]. The Toll-like receptors (TLR) have been identified for their role in recognition of viral pathogens. Within the TLR family, TLR3, TLR7, TLR8 and TLR9 are known to recognize viral genomic nucleic acids. These TLRs are situated in the endosomal compartment and sense viral genomes that have been endocytosed by the host cells [37]. The recognition of viral RNA or DNA by TLR7 and TLR9 respectively induces type I IFN production by plasmacytoid dendritic cells (pDCs), a subset of DCs known for their ability to secret high amounts of IFN-α in response to viral infection [38]. Recently we demonstrated that this recognition pathway is not the only TLR-dependent mechanism by which pDCs detect presence of viruses. We found that IFN-α production following recognition of vesicular stomatitis virus (VSV) by TLR7 requires replicating virus, as UV-inactivated VSV failed to stimulate pDCs. This suggested that pDCs likely recognize replication intermediates in the cytosol rather than recognition of the viral genome by TLR7 in the endosome following endocytosis (Figure 1). However, it was unclear how cytosolic replication intermediates could gain access to the lumen of lysosomes where TLR7 recognition occurs. Our data demonstrated that cytosolic viral PAMP is recognized by TLR7 following transport into lysosome by the process of autophagy [39]. We found that pDCs that lack Atg5 failed to secrete IFN-α and IL-12p40 in response to VSV infection. Consequently, Atg5-deficient mice failed to mount IFN-α response following systemic infection with VSV. Autophagosome formation occurred constitutively in pDCs, which was demonstrated using pDCs from transgenic mice that express the autophagosome-essential protein LC3 fused to green fluorescent protein (GFP). About 10–15% of pDCs showed punctate GFP pattern indicating large autophagosome formation. In addition, $Atg5^{-/-}$ pDCs failed to produce IFN-α in response to HSV-1, a TLR9 ligand, while the IL-12 response remained intact in these cells [39]. These data suggest that the IFN-α signaling pathway is impaired in the absence of Atg5; however, the precise nature of the differential control of NF-κB versus IFN-α induction pathways in pDCs by autophagy remains to be determined. Our data indicate that, in addition to the viral genomes, cytosolic replicative intermediates serve as a signature of virus infection as detected by TLR7. This raises the question as to how TLR7 might discriminate between self and viral RNA. If autophagosomes directly deliver cytoplasmic viral RNA to the TLR compartment, then additional molecular mechanism must exist which allow for the discrimination of viral RNA from host RNA. Using synthetic or purified nucleic acids, TLR7 does not seem to distinguish viral or mammalian RNA [40], suggesting that secondary or tertiary structures of replication intermediates is likely important. Identification of the exact nature of the viral agonist for TLR7 would provide an important clue as to how self versus non-self recognition is mediated by this PRR.

Unlike pDCs, most cell types of the body utilize cytosolic sensors of viral replication such as retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (mda-5) [41–43]. Specifically, RIG-I recognizes single stranded viral genomic RNA bearing 5′ phosphates [44,45]. RIG-I and mda-5 were shown to be required for the recognition of infection by negative stranded ssRNA viruses [46] and picornaviruses [47,48], respectively, in non-pDCs. A recent report indicated that innate recognition of VSV in MEFs via RIG-I pathway is regulated by the Atg5–Atg12 conjugate [49•]. $Atg5^{-/-}$ and $Atg7^{-/-}$ MEFs produced enhanced type I interferons in response to VSV. Biochemical analyses indicated that the Atg5–Atg12 conjugate negatively regulates the type I IFN production pathway by direct association with both RIG-I and IFN-β promoter stimulator 1 (IPS-1) through the caspase recruitment domains. Thus, in contrast to pDCs, fibroblasts and perhaps other cell types that rely on cytosolic sensors of viral replication appear to utilize molecules involved in autophagy to repress type I IFN responses. The etiology behind this observation needs to be clarified by future studies.

### Autophagy and viral antigen presentation

Dendritic cells are proficient antigen presenting cells capable of activating naive T cells [50]. Classically, it is known that intracellularly synthesized antigens are presented by MHC class I molecules to activate CD8 T cells whereas extracellular antigens are presented by the MHC

class II proteins to activate CD4 T cells. However, there are important exceptions to this paradigm. The cross-presentation pathway allows extracellular antigens to be processed and presented in the context of MHC class I proteins [51]. Conversely, intracellular antigens can be processed and presented on MHC class II [52]. Recent studies implicate autophagy in MHC class II processing of intracellular antigens. From starved EBV-transformed B cells, it was found that HLA-DR ligands from cytosolic and nuclear proteins were upregulated, while peptides from membrane and secreted proteins were unaffected [53•]. Starvation-induced autophagy was shown to promote peptide presentation on MHC II by decreasing the active cathepsins in the endocytic compartment [53•]. MHC class II presentation to CD4 T cells after autophagic degradation has been reported for several antigens [54–56,57••,58]. MHC class II presentation of peptides derived from overexpressed complement C5 protein in mouse macrophage and B cell lines was inhibited when treated with inhibitors of the class III PI3 kinase, including 3-methyladenine (3-MA) which are known to inhibit autophagy induction [54]. MHC class II presentation of peptides derived from a tumor antigen, Mucin gene 1, after transfection into DCs by RNA electroporation was inhibited when treated with 3-MA and Wortmannin [55]. Neomycin phosphotransferase II (NeoR) was processed for MHC class II presentation to CD4 T cells when NeoR was transfected into EBV-transformed human B cells or a renal cell carcinoma cell line. NeoR targeting to the lysosomes was blocked by 3-MA and Wortmannin [56]. Another report showed that the nuclear antigen 1 of EBV (EBNA1) is presented via autophagy in EBV-transformed B cell lines. EBNA1 accumulation in autophagosomes was visualized by electron microscopy. Further, siRNA-mediated silencing of Atg12, essential protein involved in autophagosome formation, inhibited intracellular EBNA1 processing for MHC II presentation [57••]. More recently, it was shown that targeting of a model antigen, influenza matrix protein 1 (MP1), to the autophagosome promoted MHC class II presentation [58]. Upon fusion of MP1 to the isolation membrane-associated LC3, delivery of the antigen for MHC class II presentation to MP1-specific CD4 T cells was enhanced by up to 20-fold, while MHC class I presentation of the same antigen to CD8 T cells was not affected. This effect was observed in transfected DCs, B cells, and epithelial cells. Furthermore, MP1-LC3 transport to the MHC class II compartment was inhibited by siRNA silencing of Atg12, demonstrating that cytosolic antigens can be delivered to the MHC class II compartment via autophagy for enhanced MHC class II presentation to CD4 T cells.

## Concluding remarks

Recent studies have demonstrated that autophagy is a fundamental process for anti-viral defense. It is becoming clear that autophagy plays a key role in innate recognition of viruses, innate effector function of viral destruction, and in the presentation of cytosolic viral antigens to CD4 T cells. Not surprisingly, viruses have developed strategies to use or subvert autophagy for their own benefit, and to prevent xenophagy by encoding inhibitors of this pathway. While evidence for autophagy as being an important pathway for antiviral immunity accumulates, very little is known with respect to the requirement for autophagy in antiviral defense during natural infections *in vivo*. As recent reports revealed the role of Atg5 in regulating survival of T cells [59], as well as the involvement of TLR4/TRIF pathway in mediating autophagy upon LPS stimulation [60], the list of contributions of autophagy to the immune system will certainly continue to grow. Since the various cell types of the immune system are likely to utilize autophagy in different manner, dissecting the cell type-specific role of autophagy in both innate and adaptive immunity will provide a more integrated picture of how this ancient pathway is utilized by the immune system to combat infections.

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