The macroautophagy machinery in endo- and exocytosis

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Abstract

Macroautophagy and the autophagy related gene (Atg) machinery execute during their canonical function cytoplasmic constituent degradation in lysosomes. This canonical function contributes to pathogen restriction and intracellular antigen presentation on major histocompatibility complex (MHC) class II molecules to CD4⁺ T cells. However, in recent years it has become clear that the Atg machinery is also used for other membrane transport functions, including endocytosis and exocytosis. This review describes these non-canonical functions in the context of antigen presentation on MHC class I and II molecules to CD8⁺ and CD4⁺ T cells, respectively, and during viral replication. Future studies will need to address how the Atg machinery is modified for these non-canonical functions, gets recruited to the respective sites of membrane modification and recruits alternative Atg interactors to execute endo- and exocytosis instead of macroautophagy.

1. Introduction

23 years ago this year's nobel laureate Dr. Yoshinori Ohsumi discovered the first 15 molecular components of the machinery that generates and degrades autophagosomes for delivery of cytoplasmic components for lysosomal degradation ¹. These components, originally named apgs 1-15 (autophagy) by Dr. Ohsumi, enable yeast cells to react to nutrient deprivation with self-eating (autophagy) to survive times of starvation. Extending this original list of essential genes, today more than 30 autophagy related gene (atg) products are known that are essential for macroautophagy, one of at least three pathways that deliver cytosolic components to lysosomes, and constituting the molecular machinery of autophagosome formation and degradation that Dr. Ohsumi started to describe.

In its essence, however, this molecular machinery enables ubiquitin-like modifications of membranes, that allows their remodeling and the recruitment of proteins to these ubiquitin-like membrane tags. At the core of this machinery Atg8 is processed by the protease Atg4 to reveal a C-terminal glycine residue that is then coupled with the E1-like enzyme Atg7, the E2-like enzyme Atg3 and the E3-like enzyme Atg5/12/16L1 to phosphatidylethanolamine (PE) in membranes, reminiscent of the machinery that couples ubiquitin to proteins ². In higher eukaryotes, like human cells, Atg8 has been diversified to 6 orthologues, namely LC3A-C, GABARAP, and the GABARAP-like proteins 1 and 2 (GABARAPL1 and 2). Presumably, these different ubiquitin-like molecules fulfill slightly different functions, but except for LC3C, which recruits proteins via an altered recognition sequence to membranes ³, these functional differences have remained ill-defined. Atg8 and its orthologues mediate membrane fusion events that are thought to be crucial for the elongation and sealing of the double-membrane that engulfs cytoplasmic material during macroautophagy and gives rise to the double-membrane surrounded autophagosome ^{4; 5}. In addition, they recruit proteins via LC3 interacting regions (LIRs) ⁶. When this occurs at the inner autophagosomal membrane, it leads to substrate recruitment into autophagosomes. For example, anchor proteins, including p62/sequestosome 1,

NDP52, NBR1, optineurin and TAX1BP1, bind ubiquitinated proteins with their ubiquitin binding domain and recruit these via their LIR sequence to forming autophagosomes. This allows the degradation of ubiquitinated cargo via macroautophagy. Attachment of the Atg8 orthologues to the outer autophagosome membrane has been suggested to regulate the transport of autophagosomes along microtubules and facilitate their fusion with lysosomes ^{7; 8}. In these studies, NDP52 was shown to interact with LC3A, LC3B and GABARAPL2 as well as myosin VI, in order to facilitate autophagosome transport along the cytoskeleton and accelerated fusion with lysosomes ⁷. These roles of NDP52 in autophagosome transport were independent of its role in Salmonella targeting to autophagosomes, which was mainly mediated via LC3C. Furthermore, pleckstrin homology domain containing protein family member 1 (PLEKHM1) was found to contain a LIR sequence and binds to the homotypic fusion and protein sorting (HOPS) complex involved in Rab7 mediated fusion with lysosomes ⁸. Thus, Atg8 binding proteins might be recruited to the outer autophagosome membrane and facilitate transport to and fusion with lysosomes. Taken together, the molecular machinery of macroautophagy might be designed to mark membranes with Atg8 and its orthologues, leading to different biological outcomes already during autophagic membrane elongation as well as protein recruitment at the inner and outer autophagosomal membrane. In this review I will discuss further, how the same machinery might even modify membranes apart from autophagosomes and fulfill functions outside canonical autophagy, including endo- and exocytosis. These alternative uses of Atgs will be discussed during antigen processing for MHC presentation to T cells and during virus infections, two areas for which now strong evidence for non-canonical use of the macroautophagy machinery exists.

2. Intracellular antigen processing for MHC presentation by macroautophagy

T cells, which coordinate adaptive immunity and mediate most of the cytotoxic effector functions during cell-mediated immune responses, recognize antigen fragments which are displayed to them by major histocompatibility complex (MHC) molecules on the surface of cells. Helper CD4⁺ T cells recognize peptides with a nonameric core sequence, but often C- and N-terminal extensions, presented on MHC class II molecules. These peptides are generated by lysosomal proteolysis and loaded in late endosomes, so called MHC class II containing compartments (MIICs), onto MHC class II molecules⁹. Cytotoxic CD8⁺ T cells react to octa- or nonameric peptides presented on MHC class I molecules, which are usually generated by proteasomal processing before getting imported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). They are loaded onto MHC class I molecules in the ER before being transported to the cell surface. These considerations already suggest that the macroautophagy machinery, which delivers cytoplasmic constituents for lysosomal degradation would contribute to the MHC class II restricted antigen presentation to CD4⁺ T cells.

Indeed, 20-30% of MHC class II ligands originate from cytosolic and nuclear proteins and some of these are more efficiently presented on MHC class II molecules after macroautophagy stimulation¹⁰. Among these endogenous MHC class II ligands, peptides of LC3A, LC3B, GABARAP, GABARAPL2 and TAX1BP1^{10; 11} have been identified. These findings suggest that autophagy cargo gains access to MIICs and gets loaded onto MHC class II molecules. A pathogen derived antigen that seems to follow this antigen processing pathway is the nuclear antigen 1 of the Epstein Barr virus (EBNA1). It was found to be endogenously processed for MHC class II presentation to CD4⁺ T cells¹². Silencing of Atg7 or Atg12 compromised EBNA1 presentation on MHC class II molecules to CD4⁺ T cell clones and EBNA1 accumulated in autophagosomes upon lysosomal inhibition^{13; 14}. Furthermore, in vivo CD4⁺ T cell responses against herpes simplex virus (HSV) were compromised in the absence of Atg5 from dendritic cells (DCs)¹⁵. Moreover, loss of Atg5 in thymic epithelial cells compromised positive and

negative selection of CD4⁺, but not CD8⁺ T cells in mice ¹⁶. Complementing these loss of function experiments, conjugation of matrix protein 1 or hemagglutinin epitopes of influenza A virus, of the human immunodeficiency virus (HIV) antigen gag or of the tumor antigen NY-ESO1 to LC3B increased MHC class II presentation to CD4⁺ T cells in vitro ^{17; 18; 19; 20}. The same targeting to autophagosomes enhanced also MHC class II presentation of the simian immunodeficiency virus (SIV) antigen gag to CD4⁺ T cells in vivo ²¹ and was able to induce negative selection of CD4⁺ T cells when targeted to macroautophagy in thymic epithelial cells in mice ²². These studies document that endogenous antigens can gain access to MHC class II presentation via macroautophagy (Figure 1).

Possibly using a similar endosomal compartment, MHC class I molecules were also described to get loaded with autophagosome cargo, when the canonical MHC class I antigen processing pathway is blocked. Along these lines, many herpesviruses encode immune evasins that target MHC class I restricted antigen presentation, including TAP inhibitors ²³. Among these antigens of the α -herpesvirus HSV and the β -herpesvirus human cytomegalovirus (HCMV) have been shown to be processed for MHC class I presentation via macroautophagy late during infection, when TAP inhibition is in place ^{24; 25}. HCMV pUL138, HCMV pp65 and HSV gB seemed to be following this pathway, but only in TAP deficient or TAP inhibited cells, while the canonical pathway was dominant for antigen processing towards MHC class I presentation, when TAP was intact. These studies reveal a subdominant pathway for antigen processing towards MHC class I presentation, which can be utilized when viral immune evasins block the canonical pathway, and this alternative route then uses macroautophagy.

3. Extracellular antigen processing for MHC presentation by autophagy-related gene products

Already in the above mentioned studies, in which Atg5 was conditionally deleted in DCs, it became apparent that not only intracellular antigen processing for MHC class II presentation was compromised by Atg deletion, but that also extracellular antigen processing for CD4⁺ T cell stimulation was inhibited in the absence of Atg5¹⁵. Indeed prior to this study it was noted that the Atg8 orthologue LC3B can be coupled to the cytosolic side of phagosome membranes during a process called LC3 associated phagocytosis (LAP)²⁶. Depending on the cell type, LAP increases phagosome fusion with lysosomes in mouse macrophages²⁷, facilitates the fusion of phagosomes with toll like receptor (TLR) containing endosomal compartments in human plasmacytoid DCs²⁸ or preserved endocytosed antigens for prolonged time periods in human macrophages and DCs²⁹. These differences might originate from the ability of the respective cells to shed fusion inhibiting LC3 anchored Atg proteins from phagosomes prior to lysosome fusion³⁰, and their requirement to recruit microtubule motors and fusion machineries via LC3 for efficient lysosome fusion^{7; 8}. Along these lines, Fyco1 recruitment to LAP membranes was described to accelerate phagosome maturation³¹. However, in both mouse and man LAP supports MHC class II presentation of endocytosed antigens (Figure 1)^{29; 32}. Alternatively, MHC class II presentation of endocytosed antigens can also benefit from macroautophagy of phagosomes. This has been described upon KIM-1/TIM-1 mediated uptake of apoptotic bodies³³, while autophagosomes usually only engulf damaged endosomes^{34; 35}. Thus, Atgs might support extracellular antigen processing for MHC class II presentation by modifying phagosomal membranes and macroautophagy of damaged or specific receptor carrying endosomes. These two pathways can be distinguished by their molecular requirements. LC3 conjugation to the phagosome membrane requires NADPH oxidase 2 (NOX2) mediated reactive oxygen species (ROS) production at the phagosomal membrane^{27; 29; 36}, but not early components of autophagosome formation, which mediate nutrient status sensing²⁷. Nevertheless, the core

machinery of LC3 lipidation is required for both macroautophagy and LAP. In the absence of LAP, but not strictly macroautophagy-supporting components in macrophages, clearance of dying cells was compromised in mice, resulting in a hyperinflammatory response and autoantibody production, which is reminiscent of the systemic autoimmune disease systemic lupus erythematosus (SLE) ³⁷. Interestingly, loss of bona fide macroautophagy genes in macrophages led in lungs to a more pronounced inflammatory response than the deletion of components that are shared between LAP and macroautophagy ³⁸. This benefitted clearance of influenza virus infection with already early innate restriction of this virus in the absence of early macroautophagy components. Thus it will be interesting to investigate if LAP and macroautophagy curb inflammation preferentially at different anatomical sites and which role antigen processing for MHC class II presentation plays at these sites.

In addition to extracellular antigen processing for MHC class II presentation Atgs might also support cross-presentation, which describes MHC class I presentation of endocytosed antigens. Cross-presentation of both fungal *Aspergillus* and bacterial *Chlamydia* antigens has been reported to require Atgs ^{39; 40}. In these studies cross-presentation was inhibited by Atg6/Beclin-1 silencing or pharmacological macroautophagy inhibition. Furthermore, the mouse CD8⁺ DC population, which most efficiently performs cross-presentation has the highest steady-state macroautophagy flux ⁴¹. However, only soluble antigen cross-presentation seemed to require Atg7. Autophagy regulation during yellow fever vaccination was also associated with more efficient CD8⁺ T cell priming in humans ⁴². Similarly cross-presentation of the soluble HCMV pp65 protein by B cells was sensitive to Atg5 and/or Atg12 silencing ⁴³. Thus the molecular machinery of macroautophagy might modify phagosomal membranes or engulf endosomes in autophagosomes for more efficient antigen processing towards MHC class I and II presentation. It remains to be seen if similar vesicular compartments are targeted by Atgs for increased MHC class II presentation or cross-presentation of extracellular antigens.

4. Receptor regulation via the macroautophagy machinery

The above discussed studies suggest that the macroautophagy machinery can regulate endosome maturation. However, Atgs might regulate phagocytosis even earlier at the level of surface receptor expression. Along these lines the scavenger receptors MARCO and MSR1 were found to be expressed at higher levels on the surface of Atg7 deficient macrophages⁴⁴. This higher expression seemed to result from elevated transcription of these receptors in Atg deficient macrophages. Similarly, Atg16L1 deficient DCs were reported to display higher surface levels of the co-stimulatory molecules CD80 and CD86 due to increased transcription of the respective genes⁴⁵. Therefore, several surface receptors might be expressed at higher levels on Atg deficient antigen presenting cells and therefore phagocytose antigens as well as stimulate immune responses more efficiently. Indeed, an hyperinflammatory phenotype, stimulating CD8⁺ T cell responses more efficiently seems to be the in vivo outcome of most Atg deficiencies in DCs or macrophages in vivo^{37; 38; 45; 46}.

Specifically for CD8⁺ T cell responses, MHC class I surface levels have recently been found to be regulated by Atgs. In both Atg5 and Atg7 deficient DCs and alveolar macrophages higher MHC class I surface levels were detected⁴⁶. In contrast to the above mentioned surface molecules, transcription and transport of newly synthesized and peptide loaded MHC class I molecules was unaffected by the absence of Atg5 and 7. Instead internalization was compromised. In particular, the recruitment of the adaptor associated kinase 1 (AAK1), involved in the internalization of MHC class I molecules, was not efficiently recruited to MHC class I in the absence of the Atg8 lipidation machinery. AAK1 was found to directly bind to LC3A and/or B, suggesting that recruitment of this kinase to MHC class I molecules is facilitated by the attachment of LC3 molecules to the cell membrane (Figure 1). The resulting MHC class I surface stabilization resulted in elevated influenza A virus and lymphocytic choriomeningitis virus specific CD8⁺ T cell responses in vitro and in vivo. These increased CD8⁺ T cell responses were protective in the case of influenza A virus infection, reducing viral titers and infection induced

weight loss. While AAK1 is usually involved in AP2 dependent clathrin mediated phagocytosis, no other components of this machinery were found to be enriched in MHC class I immunoprecipitates from Atg sufficient cells. However, Alzheimer precursor protein (APP) internalization and degradation was found to be LC3 lipidation dependent ⁴⁷. During this internalization AP2 complex components were found to contain LIR sequences for LC3 interaction. Mutating this LIR motif in the A1 subunit of the AP2 complex abolished its binding to LC3. In addition, a LIR motif was even found in clathrin by biochemical protein interaction studies ⁶. However, the biological relevance of LC3 binding by clathrin was never investigated. Nevertheless, these studies suggest that components of the macroautophagy machinery facilitate the internalization of surface receptors, possibly primarily facilitating clathrin mediated endocytosis.

5. Pathogen interference with antigen presentation via autophagy-related gene products

Mycobacterium tuberculosis (Mtb) has been suggested to be restricted by macroautophagy in macrophages⁴⁸. Both damaged endosomal Mtb replication compartments⁴⁹ and Mtb in the cytosol have been suggested to be targeted by macroautophagy⁵⁰. This antibacterial macroautophagy seems to also influence MHC class II presentation of Mtb antigens. In DCs it has been described that stimulation of autophagy leads to enhanced presentation of the Ag85B antigen to CD4⁺ T cells⁵¹. This also led to increased CD4⁺ T cell priming after adoptive transfer of the Mtb vaccine strain infected and macroautophagy stimulated DCs in vivo, resulting in improved protection against virulent Mtb challenge in mice. However, Mtb seems to counteract presumably both Mtb degrading and Mtb antigen processing macroautophagy. Mtb infection seems to repress macroautophagy by inducing miR33 expression⁵². MiR33 in turn lowers the expression of Atg5, Atg12, LC3B and lysosomal components, inhibiting macroautophagy of Mtb. In addition, a Mtb vaccine strain that carries listeriolysin and therefore damages Mtb containing endosomes was more efficiently targeted by LC3 positive membranes, when its anti-apoptotic molecules nuoG was deleted⁵³. This increased CD4⁺ T cell responses, particularly of certain memory helper subsets, after inoculation in mice. The respectively vaccinated animals were more resistant to Mtb challenge. Finally, the Mtb virulence factor PE-PGR47 was identified in a screen that aimed to characterize mycobacterial regulators of MHC class II restricted antigen presentation⁵⁴. PE-PGR47 was found to inhibit macroautophagy in Mtb infected mouse macrophages. PE-PGR47 deficient Mtb was attenuated during infection of mice, caused less pathology and was restricted by macroautophagy in murine macrophages and DCs. At the same time MHC class II restricted presentation of the Mtb antigens TB9.8 and Ag85B to specific T cells in vitro were enhanced in the absence of PE-PGR47 and sensitive to pharmacological macroautophagy regulation. Also in vivo PE-PGR47 deficient Mtb elicited increased CD4⁺ T cell responses to TB9.8 and Ag85B, but not to the ESAT6 antigen. These studies suggest that Mtb

has several pathways, by which it blocks antigen processing for MHC class II presentation via macroautophagy.

Even so best investigated for Mtb, also other pathogens might compromise macroautophagy to affect antigen presentation on MHC class II molecules. It was suggested that the HIV envelope glycoprotein inhibits macroautophagy in DCs⁵⁵, even so it seems to stimulate it in CD4⁺ T cells⁵⁶. LC3 silencing prevented HIV gag antigen presentation to CD4⁺ T cells, suggesting that the HIV mediated down-regulation of macroautophagy also affected intracellular HIV antigen presentation to CD4⁺ T cells. Moreover, HSV encodes the ICP34.5 protein which inhibits macroautophagy⁵⁷. In the absence of ICP34.5 an HSV encoded model antigen was more efficiently presented on MHC class II molecules of HSV infected mouse DCs. Finally, nuclear localization of some viral antigens also limits their processing by macroautophagy for MHC class II presentation. EBNA1 derived CD4⁺ T cell epitopes were more efficiently presented on MHC class II molecules via macroautophagy, when the nuclear localization sequence of EBNA1 was removed¹⁴. While only a subset of MHC class II restricted epitopes was macroautophagy dependent in the presence of nuclear localization of EBNA1, all investigated epitopes became macroautophagy dependent in the absence of the nuclear localization sequence. Thus, also viruses seem to compromise MHC class II antigen processing via macroautophagy either by actively inhibiting this pathway or by hiding their antigens in cellular compartments that are less accessible to engulfment by autophagosomes.

6. Macroautophagy inhibition by DNA viruses

The above pathogens, for which an inhibition of macroautophagy has been directly linked to their antigen presentation on MHC class II molecules, is just the tip of the iceberg with respect to macroautophagy regulation by viruses⁵⁸. A comprehensive discussion of viral regulation of macroautophagy would also go beyond the scope of the current review. I just want to highlight one DNA virus family, the herpes viruses, which are a rich source of modulators of macroautophagy and contain members, which hijack Atg supported exocytosis for their release from infected cells.

Viruses mainly modulate macroautophagy at two check-points. While DNA viruses have been found to often interfere with autophagosome formation, RNA viruses mainly regulate autophagosome fusion with lysosomes⁵⁸. This was first demonstrated for the α -herpesvirus HSV⁵⁷. Its ICP34.5 protein was found to bind to Atg6/Beclin-1 and thereby seems to inhibit autophagosome formation. Similarly, the Bcl-2 homologues of the two γ_2 -herpesviruses Kaposi sarcoma associated herpesvirus (KSHV) and murine γ -herpesvirus 68 (MHV68) also interact with Atg6/Beclin-1 to prevent autophagosome formation⁵⁹. Interestingly, most of these herpesviruses have more than one macroautophagy regulator. HSV encodes a second protein US11 that blocks macroautophagy by targeting the dsRNA-dependent protein kinase PKR⁶⁰. KSHV even encodes two additional gene products that affect macroautophagy. K7 interacts with Rubicon, a negative regulator of autophagosome maturation⁶¹. Furthermore, the viral FLICE-like inhibitory protein (vFLIP) inhibits Atg3 mediated processing of the Atg8 orthologues to prevent autophagosome formation⁶². The γ -herpesvirus saimiri encodes a vFLIP protein that fulfills the same function. Also the β -herpesvirus HCMV encodes two proteins, TRS1 and IRS1, which both inhibit autophagosome formation via Atg6/Beclin-1 binding^{63; 64}. It has been suggested that this inhibition of macroautophagy is required for efficient lytic replication of HSV and KSHV^{65; 66}, while macroautophagy stimulation even enhances HCMV production⁶⁴.

Similar to HCMV, the formation of autophagic membranes seems to benefit the replication of the γ_1 -herpesvirus Epstein Barr virus (EBV) and the α -herpesvirus Varicella Zoster Virus (VZV)^{67; 68; 69}. Both viruses seem to prevent LC3 coupled membranes to be degraded and benefit from these membranes for their exocytosis, as discussed in more detail below. In addition, EBV also induces macroautophagy via its two latent membrane proteins (LMPs)^{70; 71}. These macroautophagy induction mechanisms seem to prevent cell death due to either LMP1 overexpression or p53 dependent apoptosis during B cell transformation by EBV^{70; 72; 73}. Thus, most herpesviruses regulate macroautophagy by multiple mechanisms, either to prevent its anti-viral functions or to utilize autophagic membranes as well as its pro-survival capacities.

7. RNA virus release with the help of autophagic membranes

In contrast to the inhibition of macroautophagy by most DNA viruses, RNA viruses often allow autophagic membranes to form, but then block their maturation and fusion with lysosomes⁵⁸.

Among these are members of the picorna-, alpha-, retro-, flavi- and orthomyxoviruses. I will only high-light in this chapter the viruses that stabilize autophagic membranes and will become later important with respect to the discussion of Atg dependent exocytosis, namely primarily picorna- and orthomyxoviruses.

The first virus for which accumulation of double-membrane vesicles upon infection was shown is the polio picornavirus⁷⁴. Poliovirus causes the accumulation of these vesicles with its 2BC and 3A proteins⁷⁵. The accumulating double-membrane vesicles do not fuse with each other and contain LC3B as well as the lysosome associated membrane protein 1 (LAMP1). Silencing of LC3 and Atg12 prevents the formation of these vesicles and inhibits infectious poliovirus release. The respective membranes are not thought to be required for viral genome replication, but rather for the exocytosis of viral particles^{76; 77}. Similar membrane accumulations can be also found upon infection with other members of this virus family. Namely, the rhinoviruses 2 and 14 accumulate LC3 positive vesicles upon infection⁷⁵. Finally, the foot-and-mouth disease virus also contains with 2B, 2C and 3A proteins that associate with LC3 positive vesicular structures⁷⁸. Similar to poliovirus inhibition of macroautophagy compromises replication of this virus, while stimulation leads to enhanced infectious viral particle release. Finally, also the coxsackievirus B of this virus family seems to use autophagic membranes for its exocytosis from infected cells⁷⁹. Interestingly, Atg13 and FIP200, two proteins that are early involved in autophagosome formation, were recently found to rather restrict coxsackievirus B replication and the infectious particle production of yet another picornavirus, encephalomyocarditis virus (ECMV)⁸⁰. Thus, picornaviruses stabilize autophagic membranes for their release from cells, while other Atgs might inhibit picornavirus replication in a rather macroautophagy-independent fashion.

A similar block in the degradation of LC3 coupled membranes can be observed in orthomyxovirus infections. Influenza A virus accumulates autophagic membranes in infected cells⁸¹. In contrast to poliovirus infection these membranes fuse with each other and form large perinuclear vesicular structures. The viral gene product that is responsible for these accumulations is matrix protein 2 (MP2) of influenza A virus. Since the proton channel activity of MP2 was suggested to be responsible for autophagic membrane accumulation⁸², MP2 might compromise acidification of autophagosomes to prevent their maturation towards fusion with lysosomes. At the same time MP2, however, also contains a LIR motif and thereby directly interacts with the LC3 coupled membranes⁸³. This interaction is required to divert LC3 and with it most likely autophagic membranes to the cell membrane (Figure 2). This membrane supply allows filamentous budding of influenza A viruses and seems to alter viral envelope composition, allowing for an increased stability of virus particles. Similarly, parainfluenza virus also interferes with autophagosome maturation⁸⁴. Its phosphoprotein blocks syntaxin 17 mediated fusion of autophagosomes with lysosomes. This activity is required for efficient virus replication. Thus, both influenza and parainfluenza viruses arrest autophagic membrane degradation for increased virus stability and virus replication, respectively.

8. Viral envelope acquisition from autophagic membranes

Why do different DNA and RNA viruses, including Epstein Barr virus (EBV), Varicella Zoster virus (VZV), poliovirus and influenza A virus, stabilize autophagic membranes? A possible benefit for viral replication was initially unveiled for EBV. In the absence of Atg12 or Atg16L1, viral genomes accumulated in the cytosol⁶⁷, the location of envelope acquisition for this herpesvirus⁸⁵. Indeed, lipidated LC3B was then found enriched in purified virus particles and could be localized to the viral envelope by immune electron microscopy. Similarly, VZV particles were also found to contain LC3B⁶⁹. Thus, herpesviruses that acquire their second envelope after initial budding through the inner nuclear membrane and deenveloping through the outer nuclear membrane in the cytosol from ER and Golgi, seem to recruit stabilized autophagic membranes for efficient release from infected cells (Figure 2).

Why would viruses use this membrane source and not simply bud through the cell membrane? The RNA viruses that affect autophagic membrane accumulation might provide some clues to answer this question. Even so picornaviruses are not enveloped, it has been observed that coxsackievirus B gets released from infected cells in LC3B positive membranes⁷⁹. These contain multiple virus particles and might be the reason why also poliovirus spreads more efficiently from Atg expressing cells without lysing them⁸⁶. These autophagic membrane surrounded packages of picornaviruses might protect them during extracellular transfer from proteases and antibodies. Furthermore, these autophagic membranes have been found to be enriched in phosphatidylserine (PS) due to their cell organelle origin from PS rich ER and Golgi⁷⁷. PS is usually detected on apoptotic cells by scavenger receptors for efficient clearance by macrophages⁸⁷. Similarly, poliovirus containing autophagic membranes (Figure 2) are more efficiently taken up by scavenger receptor positive cells, which therefore assist in poliovirus infection⁷⁷. Therefore, it could also be beneficial for other viruses to incorporate autophagic membranes into their envelope in order to influence its lipid composition and more efficient infection of bystander cells.

It is tempting to speculate that, even so influenza A virus does not incorporate lipidated LC3B in its envelope directly, it changes the lipid composition of the cell membrane via recruiting autophagic membranes to it⁸³. Since the cell membrane is the source of the influenza A virus envelope this could lead to altered lipid composition in the virus surrounding membrane and result in the increase virion stability. Such an alternative use of autophagic membranes for the regulation of virus envelope composition is obviously quite distinct from the use of Atg proteins for lysosomal degradation of cytoplasmic constituents. It rather points towards viruses hijacking an Atg mediated exocytosis mechanism that leads to non-canonical secretion of cellular components.

9. Non-canonical secretion with autophagy-related gene products

Indeed a variety of eukaryotic gene products exit cells without signal peptide sequence for incorporation into the ER^{88; 89}. These include IL-1 β , IL-33, HMGB1, acyl CoA binding protein (Acb1), tissue transglutaminase, galectin-3, macrophage migration inhibitory factor and insulin degrading enzyme. While some of them, like IL-1 β , are probably also released during cell death^{90; 91; 92}, an involvement of Atgs has been claimed for some of them. The underlying cellular mechanism could constitute the basis of autophagic membrane acquisition by viruses, which might have hijacked this cellular pathway. Acb1 secretion from yeast and amoeba has been first described to rely on Atgs^{93; 94}. Both of these studies found Acb1 secretion to be dependent on the Golgi-associated protein GRASP. Similarly, IL-1 β secretion by mammalian cells was found to depend on GRASP55⁹⁵. Interestingly, a wide variety of Atg proteins has been implicated in this unconventional protein secretion, including Atg1, 5, 6, 7, 8, 9, 11, 12 and 17^{93; 94; 95}. Four different cellular compartments have been put forward that might execute this unconventional protein secretion. They were named compartment for unconventional protein secretion (CUPS)^{96; 97}, defective ribosome products-containing autophagosome-rich blebs (DRibbles)^{98; 99; 100}, secretory lysosomes¹⁰¹ or require HSP90 dependent insertion of IL-1 β into the intermembrane space of forming autophagosomes¹⁰². CUPS, which have mainly been investigated for Acb1 secretion, form from Golgi membranes under starvation conditions. Atg8 is thought to get attached from the cytosolic side to these membrane compartments, as does the cargo Acb1. Small vesicles from the elongated CUPS membrane compartment then fuse with endosomes and the cargo gets engulfed into intravesicular compartments with the assistance of the multivesicular body (MVB) machinery. The resulting MVBs then fuse with the cell membrane and release vesicles containing Acb1 that burst open to release their cargo. DRibbles secretion also results in extracellular vesicles containing Atg8/LC3 recruited cargo. It had been noted that antigen transfer for cross-presentation on MHC class I molecules benefits from intact Atg proteins in the antigen donor cells (Figure 1)^{103; 104}. Inhibition of lysosomal and proteasomal

degradation was described to allow the accumulation of ubiquitinated defective ribosomal products (DRiPs) in vesicular fractions of tumor cell supernatants ¹⁰⁵. This cargo might be recruited into forming autophagosomes in a p62 dependent manner, but never get degraded under conditions of lysosome inhibition ¹⁰⁶. Instead it seems to exit cells from an MVB like compartment. Related to this secretion after frustrated macroautophagy, might also be the contribution of the Atg machinery to the release of secretory lysosomes. It has been described that osteoclasts, the bone macrophages, release hydrolases for bone degradation via a pathway, in which LC3 facilitates lysosome fusion with the cell membrane ¹⁰¹. Finally, Zhang and coworkers have shed some additional light on the unconventional secretion of IL-1 β by expressing mature IL-1 β in cells other than macrophages ¹⁰². In these studies the authors describe that IL-1 β gets inserted between the inner and outer autophagosomal membrane by a mechanism that is dependent on KFERQ sequences in IL-1 β and their interaction with HSP90. Upon completion of the respective autophagosomes these then fuse with MVBs for release into the supernatant. Thus two unconventional secretion mechanisms utilizing Atgs have been described that release cargo from cells in vesicles (CUPS and DRibbles), while two mechanisms release cargo directly into the supernatant (secretory lysosomes and intermembrane autophagosomes space). In future studies it needs to be addressed which contributions these unconventional secretion mechanisms make to overall protein secretion. Nevertheless, from the so far available experimental information it is already clear that the topological inversion from cytosolic to extracellular/vesicular space that is achieved for lysosomal degradation during macroautophagy can also be used for signal peptide independent non-canonical protein secretion and uses parts of the autophagic machinery.

10. Perspectives

The multitude of non-canonical uses of the Atg machinery, as described above, clearly indicate a modular usage of Atgs for a variety of cellular processes, including endocytosis and exocytosis in addition to macroautophagy, by eukaryotic cells. Primarily, attachment of Atg8 and its higher eukaryotic orthologues (LC3A-C, GABARAP, GABARAPL1 and 2) seems to act as membrane tags, which allow for the recruitment of LIR containing proteins to the sites. Depending on the respective activities of this LIR containing proteins different biological functions might be executed. This notion is strengthened by earlier findings from the Ohsumi lab that indicated Atg8 attachment to various membranes in yeast cells deficient for Atg4 and transgenic for C-terminally processed Atg8¹⁰⁷. Future studies now need to address how this Atg4 mediated Atg8 deconjugation is selectively regulated in the above discussed endocytic and exocytic pathways and how the different LIR containing proteins and not only macroautophagic effector molecules are selectively recruited to Atg8/LC3 conjugated membranes to fulfill diverse biological functions in both antigen presentation and virus infection.

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Figure 1: Atgs contribute to intracellular and extracellular antigen processing for MHC presentation. Autophagosomes engulf intracellular antigen and deliver them to MHC class II loading compartments. Extracellular antigen is delivered by LC3 associated phagocytosis (LAP) to MHC class II loading compartments. Antigen is exported in autophagic membranes for efficient cross-presentation on MHC class I molecules. MHC class I molecules are internalized upon adaptor associated kinase 1 (AAK1) recruitment via LC3 to early endosomal compartments. Green dots represent LC3.

Figure 2: Autophagic membranes support viral exocytosis. Influenza A virus redirects LC3 coupled membranes to the cell membrane to support filamentous budding of this virus. Picornaviruses like polio get secreted in LC3 coupled membranes as packages of multiple virus particles. The herpesviruses Epstein Barr virus (EBV) and Varicella Zoster virus (VZV) incorporate autophagic membranes into their envelopes. Green dots represent LC3.

Figure 1

Figure 2