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Non-canonical functions of autophagy proteins in immunity and infection

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ABSTRACT

The molecular machinery of macroautophagy, a catabolic pathway for cytoplasmic constituent degradation in lysosomes, remodels membranes by lipid phosphorylation and conjugation of LC3 and GABARAP proteins. In recent years it has become clear that these membrane modifications also regulate endo- and exocytosis. Here I will discuss recent evidence of how such non-canonical functions of the macroautophagy machinery with its autophagy related gene (atg) products influence infectious viral particle secretion, inflammation, and MHC restricted antigen presentation. Especially LC3-Associated Phagocytosis and ATG supported exocytosis will be high-lighted during immunity and infection.

1. Non-canonical functions of the macroautophagy machinery

Yoshinori Ohsumi started to define in the 1990s the molecular basis for the nitrogen starvation response in baker’s yeast (Tsukada and Ohsumi, 1993). The identified genes, originally called akg and now known as autophagy-related (atg) genes, form the machinery of macroautophagy, a process for cytoplasmic constituent degradation in lysosomes or the yeast vacuole (Mizushima et al., 2011). Yoshinori Ohsumi received the Nobel Prize for this discovery in 2016. Three ATG complexes are required to generate the double membrane surrounded autophagosome which then fuses with late endosomes or lysosomes for the degradation of its cargo and the inner autophagosomal membrane. These are the ULK1/ATG1 protein kinase complex that phosphorylates the phosphatidylinositol 3 (PI3) kinase complex containing VPS34 and Beclin-1/ATG6. The PI3 kinase complex generates PI3P marks that then recruit the third complex with ATG5, ATG12 and ATG16L1 that conjugates the mammalian ATG8 orthologues LC3A, LC3B, LC3C, GABARAP, GABARAPL1 and GABARAPL2 to phosphatidylethanolamine (PE) in the outer and inner membrane of the forming autophagosome. These ATG8 orthologues are involved in membrane elongation and closure of the autophagosome with the help of ATG2 channels and ATG9 vesicles, as well as recruitment of substrates either by binding to substrate proteins directly or to autophagy receptors like SQSTM1/p62, optineurin, NDP52, TAX1BP1 and NBR1 (Khaminets et al., 2016). These often contain LC3 interacting regions (LIRs) for their binding to LC3s (Birgisdottir and Johansen, 2020). The ATG8 orthologues are then removed by the ATG4 protease (ATG4A, ATG4B, ATG4C and ATG4D in higher eukaryotes) from the outer membrane of the completed autophagosome.

ATG4 C-terminally trims the LC3s and GABARAPs also prior to PE conjugation. The completed autophagosome then fuses with late endosomes or lysosomes in a syntaxin 17 and YKT6 dependent fashion (Itakura et al., 2012; Matsui et al., 2018). Transport along microtubules and recruitment of the fusion machinery depends on NDP52 or PLEKHM1 interaction with some of the ATG8 orthologues at the outer autophagosomal membrane (McEwan et al., 2015; Verlhac et al., 2015), presumably before ATG4 has entirely cleaved off LC3s and GABARAPs at its cytosolic side. The resulting degradation and nutrient recycling allow cells to survive starvation periods, but also clears damaged organelles, protein aggregates and even pathogens, processing some of them for presentation on major histocompatibility complex (MHC) class II molecules for helper CD4+ T cell stimulation (Fig. 1) (Münz, 2009; Schmid et al., 2007).

Beyond this canonical use of ATGs, Yoshinori Ohsumi’s group noticed ATG8 conjugation beyond autophagic membranes (Nakatogawa et al., 2012). If one provides C-terminally trimmed ATG8 to ATG4 deficient yeast, ATG8 gets conjugated to a wide variety of additional membranes, including vacuole, endosomes and the endoplasmic reticulum (ER). Indeed, a role for this non-canonical conjugation of LC3s and GABARAPs has now been suggested for endocytosis and unconventional secretion (Keller et al., 2018; Malhotra, 2013). The characteristic features of these non-canonical functions of the macroautophagy machinery are conjugation of LC3 to the lysosomal side of a single membrane surrounded phagosome (Sanjuan et al., 2007) or dependency on the LC3 conjugation machinery (ATG5, ATG12 and ATG16L1) for the secretion of proteins that do not contain an ER signal sequence for classical secretion, respectively (Duran et al., 2010; Manjithaya et al., 2010).

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LC3-Associated Phagocytosis (LAP) does not seem to require the ULK1/ATG1 complex. Both LAP and ATG supported secretion either utilize VPS34 generated PI3P marks on late endosomal membranes (Bilanges et al., 2019), including multivesicular bodies, for the recruitment of the LC3 conjugation complex or membrane binding by this complex in a PI3P independent fashion (Fletcher et al., 2018; Leidal et al., 2020). In the following I will discuss the contribution of these pathways that utilize ATG proteins in a non-canonical fashion in inflammation, pathogen replication and restriction, as well as MHC class I and II restricted antigen presentation to CD8+ and CD4+ T cells, respectively.

2. ATG mediated exocytosis for infectious viral particle release

A role for the LC3 conjugation machinery in unconventional protein secretion was first shown for Acrpy-CoA-binding protein (ACB1) (Duran et al., 2010; Manjithaya et al., 2010). In addition to ATG5, 12 and 16 it requires the Golgi-associated protein GRASP. More recently it has been shown that ACB1 is secreted together with antioxidants in a reactive oxygen species (ROS) dependent fashion (Cruz-Garcia et al., 2020). In higher eukaryotes this machinery seems to also be involved in the release of the pro-inflammatory cytokine IL-1β (Dupont et al., 2011; Zhang et al., 2015). In a recently published unbiased screen, proximity ligation was used to identify secreted proteins that had made contact with LC3B (Leidal et al., 2020). 200 targets were identified to be secreted in extracellular vesicles containing PE-conjugated LC3. Lipidated LC3 had been previously found in exosome like vesicles (Pallet et al., 2013). The ATG supported secretosome was enriched in RNA binding proteins, requiring also neutral sphingomyelinase 2 (nSMase 2) activity at multivesicular bodies for their release (Leidal et al., 2020). At least some of these RNA binding proteins directly interact with LC3 and are probably thereby sorted into LC3 containing extracellular vesicles.

This non-canonical use of the macroautophagy machinery seems to be also utilized for envelope acquisition by some viruses, including herpes and picornavirus (Nowag et al., 2014; Robinson et al., 2014). The γ-herpesvirus Epstein Barr virus (EBV) was found to stabilize LC3-conjugated membranes upon induction of infectious particle production (Nowag et al., 2014). RNA silencing of the LC3 conjugation complex compromised EBV production and viral DNA was enriched in the cytosol, the site for final envelope acquisition for herpesviruses. Furthermore, lipidated LC3 was found in purified virus particles. This suggests that EBV stabilizes LC3-conjugated membranes that are required for its envelope acquisition in the cytosol and then also found in mature virus particles. Similarly, the α-herpesvirus varicella zoster virus (VZV) incorporates LC3B conjugated membranes into its infectious particles (Buckingham et al., 2016). In addition, VZV seems to interact with Rab11 and mannose-6-phosphate receptor positive vesicles (Buckingham et al., 2016; Girsch et al., 2020) during envelope acquisition with the support of ATGs. Finally, the β-herpesvirus human cytomegalovirus (HCMV) also incorporates lipidated LC3 and GABARAPL1 into its viral particles (Taitse et al., 2019). LC3 accumulates at the viral assembly compartment, the site for HCMV envelope acquisition and RNA silencing of ATGs compromises virus replication. Thus, members of α-, β- and γ-herpesviruses, as well as other double-stranded DNA viruses, like coccolithovirus (Schatz et al., 2014), seem to use LC3-conjugated membranes to acquire their final envelope in the cytosol.

In contrast picornaviruses, including coxsackie B and polio virus, are not thought to be enveloped. However, it was noticed that non-lytic release of coxsackie B virus results in shedding of multiple virus particles containing microvesicles (Robinson et al., 2014). These are surrounded by LC3-conjugated membranes. For their release coxsackie B virus might use macroautophagy of fragmented mitochondria and then direct the resulting autophagosomes for secretion (Sin et al., 2017). In addition, it has long been debated what contribution the macroautophagy machinery provides for the double-membrane vesicles (DMV) that form the picornavirus replication compartment, and also serve in a similar manner other RNA viruses (Dales et al., 1965; Fahmy and Labonte, 2017; Jackson et al., 2005; Lee et al., 2019; Reggiori et al., 2010). These DMVs seem to depend on the ATG5, 12 and 16L1 containing LC3 conjugation complex and the Vps34 and Beclin 1 containing PI3 kinase complex, the latter of which is recruited to DMVs via RACK1 (Fahmy and Labonte, 2017; Lee et al., 2019). However, curiously this does not lead to LC3 lipidation (Fahmy and Labonte, 2017; Reggiori et al., 2010) and it also remains unclear how these DMVs relate to non-lytic release of picornaviruses that are surrounded by LC3-conjugated membranes. Nevertheless, such release of picornavirus packages was also observed for polio virus and rhinovirus (Chen et al., 2015). An additional benefit of the surrounding autophagic membranes, apart from the protection against immune system recognition and neutralization, was found in the phosphatidylinerine exposure at the outer leaflet of the LC3-conjugated membranes. This allows these viral packages to be taken up by scavenger receptors on phagocytes which leads to more efficient picornavirus infection after envelope degradation in phagosomes. Thus, the sampling of autophagic membranes at ER and Golgi might allow enveloped picornavirus packages to more efficiently infect phagocytes after non-lytic release. The involved non-canonical function of the macroautophagy machinery in exocytosis might use...
LC3 binding to select cargo, including viral capsids, for release from cells.

3. ATG functions in endocytosis during inflammation and pathogen restriction

Apart from redirecting LC3-conjugated membranes for exocytosis, components of the molecular machinery of macroautophagy also modify endocytosis (Florey, 2018; Keller et al., 2018; Martinez, 2018). LC3-Associated Phagocytosis (LAP) was originally described for uptake of baker’s yeast or its cell wall components (zymosan) by mouse macrophages (Sanjuan et al., 2007). It is characterized by LC3 conjugation to the cytosolic membrane side of phagosomes that have taken up toll-like receptor 2 (TLR2) ligands (Fig. 1), dectin-1 ligands, integrin Mac-1 ligands, antibody opsonized material via Fc receptors and apoptotic material, possibly due to the TIM4 scavenger receptor (Gluschkko et al., 2018; Ma et al., 2012; Martinez et al., 2011; Romao et al., 2013). While the ULK1/ATG1 complex is not required for LAP, VPS34 is recruited to phagosomes and NADPH oxidase 2 (NOX2) is required for LAP (Bilanges et al., 2019; Gluschkko et al., 2018; Huang et al., 2009; Martinez et al., 2015; Romao et al., 2013). Furthermore, RUBICON, an inhibitor of the VPS34 complex that is involved in autophagosome fusion with lysosomes (Matsumaga et al., 2009; Tabata et al., 2010; Zhong et al., 2009), seems to be essential for LAP (Martinez et al., 2015). Indeed, VPS34 generated PI3P is required to assemble NOX2 from its transmembrane and cytosolic components (Ellson et al., 2006). ROS production by NOX2 is required for LAP (Romao et al., 2013). Both ROS production and PI3P are, however, not required for recruitment of the LC3 lipidation machinery to endosomes (Fletcher et al., 2018). Instead, the WD40 domain of ATG16L1 recruits the ATG5, ATG12 and ATG16L1 complex to phagosomes (Fletcher et al., 2018; Rai et al., 2019). This lipidation of ATG8 to endosomes has also been seen in ATG4 deficient yeast (Nakatogawa et al., 2012), even so its ATG16 does not contain a WD40 domain, suggesting additional recruitment mechanisms. The ROS production by NOX2 is however required to prevent removal of LC3 during LAP from yeast containing phagosomes (Ligeon et al., 2021). Indeed, ATG4 has been reported to be inhibited by ROS due to cysteine oxidation (Scherz-Shouval et al., 2007). Accordingly, phagosomes to which LC3 remains conjugated present with high and sustained ROS production (Ligeon et al., 2021). Upon mutation of the ROS sensitive cysteine in ATG4B its oxidation and oxidation-mediated aggregation during LAP is significantly reduced. Furthermore, oxidation insensitive ATG4B removes LC3 from phagosomes, even in the presence of oxidation sensitive endogenous ATG4A, ATG4B, ATG4C and ATG4D expression. These findings confirm that ATG4 is required to restrict ATG8/LC3 conjugation to certain membranes (Nakatogawa et al., 2012). Inhibition of ATG4 to sustain LC3 lipodation is also required at the forming autophagosome and is achieved by ATG1 mediated phosphorylation of ATG4 at this site (Sanchez-Wandelmer et al., 2017). While, therefore, the mechanisms leading to non-canonical LC3 conjugation to phagosomal membranes get more and more resolved, the consequences for late endosomal trafficking and the molecular machinery that is recruited by LC3 to phagosomes need to be better characterized in the future. LC3 conjugation might, however, influence trafficking and vesicular fusion events depending on the recruitment of LIR containing proteins that will guide LC3-conjugated phagosome fate. Along these lines, it was shown that NDP52 interacts with myosin VI for autophagosome transport along actin filaments (Verilac et al., 2015).

Nevertheless, the differences in molecular requirements for macroautophagy and LAP have allowed to distinguish the importance of these two pathways during autoimmunity, infections, neurodegenerative diseases and cancer biology (Cunha et al., 2018; Beretci, 2021; Gluschkko et al., 2018; Heckmann et al., 2019; Henault et al., 2012; Is et al., 2016; Martinez et al., 2016). While macroautophagy was previously shown to restrict inflammation induced by activation of inflamasomes, the cytosolic DNA sensing cGAS-STING pathway or the cytosolic RNA sensing RIG-I-MAVS pathway (Matsuzawa-Ishimoto et al., 2018; Saitoh et al., 2008), LAP has been shown to deliver DNA complexes with antibodies, as they occur during the systemic autoimmune disease lupus erythematous, to vesicular compartments that harbor the DNA sensor TLR9 in plasmacytoid cells (Henault et al., 2012). This elicited potent type I interferon (IFN) responses that are a hallmark of lupus (Blanco et al., 2001). Similarly, B cell receptor (BCR) mediated antigen uptake delivers BCR ligands to TLR9 containing vesicles in an ATG dependent fashion for improved B cell activation (Chaturvedi et al., 2008). A similar delivery of RNA to its cognate TLR7 receptor in endosomes could also explain the enhanced ATG5 dependent lupus-like disease upon TLR7 overexpression in mice (Weindel et al., 2015). The autophagosomes in lupus, including DNA recognizing immunoglobulins, can result from deficient clearance of apoptotic cell debris and some of these autophagosomes develop in mice that cannot efficiently clear dead cells due to deficiency in LAP (Martinez et al., 2016). Deficient degradation of apoptotic cell bodies by LAP deficient mice resulted in increased inflammatory cytokine production and autoantibody generation. In addition to apoptotic bodies, pathogen clearance is also compromised in LAP deficient macrophages (Gluschkko et al., 2018). Furthermore, neurodegeneration in a mouse model of Alzheimer’s disease was accelerated in mice with deficiency in LAP in microglia, the CNS resident macrophages (Heckmann et al., 2019). Deficient clearance of β-amyloid led to increased plaque formation and inflammation. Such a shift toward inflammation was also seen upon LAP deficiency in tumor associated macrophages (Cunha et al., 2018). This improved tumor immune control by T cells in a STING and type I IFN dependent fashion. Therefore, one of the main phenotypes of ATG deficiency in myeloid cells is hyperinflammation. This can result from lacking restriction of cytosolic inflammation initiators by macroautophagy, including damaged mitochondria driven inflammasome activation (Zhong et al., 2016; Zhou et al., 2011), or defective clearance and redirection of inflammation stimulators by LAP, like DNA to TLR9 containing endosomes (Henault et al., 2012).

4. LC3-Associated Phagocytosis in antigen processing of MHC class II presentation

In addition to on the one hand redirecting DNA and possibly RNA as inflammation stimuli to TLR containing endosomes and on the other hand degrading inflammation stimuli contained in apoptotic cell bodies, LAP has also been shown to deliver endocytosed material for antigen presentation by MHC class II molecules (Fig. 1) (Keller et al., 2018). Already in an early study on ATG5 deficiency in dendritic cells it was noted that also extracellular antigen was less efficiently presented to CD4+ T cells (Lee et al., 2010). In addition to the ovalbumin coated splenocytes that were used in this study, deficient MHC class II presentation in the absence of ATG5 was also extended to apoptotic oligodendrocytes and the autoantigen myelin oligodendrocyte glycoprotein (MOG) (Keller et al., 2017). This ATG5 dependency of especially sustained MHC class II restricted antigen presentation was also found for Candida albicans derived antigens (Ma et al., 2012; Romao et al., 2013). Both Dectin-1 and TLR2 were implicated in the respective Candida antigen uptake stimulating LAP. Moreover, ATG5 is also required for MHC class II presentation of surface bound antigen after its BCR mediated uptake by B cells (Arbogast et al., 2018). In addition to ATG5, ATG16L1 was also found to be essential for this LAP mediated antigen processing for efficient MHC class II presentation in both human and mouse dendritic cells and macrophages (Fletcher et al., 2018; Romao et al., 2013). More specifically the WD40 domain of ATG16L1 that is essential for LAP but dispensable for macroautophagy was required for the presentation of the mouse MHC class II H2-E alpha chain on the mouse MHC class II molecule H2-K^d as measured with an antibody that is specific for the respective MHC class II plus peptide complex (Fletcher et al., 2018). The LAP dependency of extracellular antigen processing for MHC class II presentation was also documented by its
dependency on NOX2 (Keller et al., 2020). Both ATG5 and NOX2 deficiency in dendritic cells and/or CD11c positive macrophages diminished central nervous system (CNS) autoimmunity induced by adoptive transfer of MOG specific CD4+ T cells (Keller et al., 2017, 2020). The NOX2 dependency of extracellular antigen processing for MHC class II presentation depends also at least partially on ROS regulation of ATG4 (Ligeon et al., 2021). A ROS insensitive ATG4B mutant that compromises LAP also inhibits MHC class II presentation of phagocytosed Candida antigens to CD4+ Th17 cells. Thus, all professional antigen presenting cell populations, including dendritic cells, macrophages and B cells, seem to use LAP to process endocytosed antigens for efficient MHC class II presentation to CD4+ T cells. This includes infectious disease antigens (Candida) and autoantigens (MOG). However, how LC3 positive phagosomes facilitate MHC class II presentation of endocytosed antigens remains unclear. In human phagocytes, maintenance of endocytosed antigen in long-lived LC3 positive phagosomes (Ligeon et al., 2021; Romao et al., 2013) might sustain MHC class II presentation (Delamarre et al., 2005). Indeed, such maintenance and protection from rapid lysosomal degradation of phagocytosed antigens has previously been shown to increase their immunogenicity (Delamarre et al., 2006). However, different strategies might be used by different antigen presenting cells to efficiently deliver endocytosed material to MHC class II compartments (MIICs) and fusion of LC3 positive phagosomes with MIICs needs to be better characterized in the future.

5. The influence of the macroautophagy machinery on MHC class I restricted antigen presentation

In contrast to antigen presentation on MHC class II molecules which benefits from both LAP and canonical macroautophagy for extra- and intracellular antigen processing, respectively, the role of ATG proteins in antigen presentation on MHC class I molecules to CD8+ T cells is much less clear. There are at least three aspects to it, namely regulation of MHC class I and co-stimulatory molecule trafficking by ATGs, their role in intra- and extracellular antigen processing for MHC class I presentation, and ATG support of antigen packaging by antigen donor cells for efficient MHC class I restricted cross-presentation by neighboring antigen presenting cells. In studies that compromised the LC3 conjugation machinery in dendritic cells and pancreatic tumor cells, elevated MHC class I and co-stimulatory molecule expression on the cell surface was reported (Hubbard-Lucey et al., 2014; Loi et al., 2016; Yamamoto et al., 2020). Different mechanisms by which ATGs contribute to this regulation of surface receptors were identified, ranging from enhanced AP2 associated kinase 1 (AAK1) mediated MHC class I internalization and degradation, diminished A20 mediated CD80 transcription and redirection of MHC class I molecules for lysosomal degradation by the autophagy receptor NBR1 (Fig. 2). Upon ATG16L1, ATG5, ATG7 or NBR1 deficiency, as well as dominant negative ATG4B overexpression graft-versus-host disease (GVHD) mediated by CD8+ T cells (Hubbard-Lucey et al., 2014), antiviral CD8+ T cell responses (Loi et al., 2016) and pancreatic tumor cell restriction by CD8+ T cells were increased (Yamamoto et al., 2020). Thus, ATGs seem to enhance MHC class I degradation in lysosomes and thereby restrict CD8+ T cell stimulation. Intracellular antigen processing for MHC class I presentation to CD8+ T cells is not significantly enhanced by antigen targeting to autophagosomes (Schmid et al., 2007). However, aggregation prone antigens that get efficiently removed by macroautophagy get better presented on MHC class I molecules in macroautophagy deficient cells (Wenger et al., 2012). The antigen format also influences ATG dependence of extracellular antigen processing for MHC class I cross-presentation. Cross-presentation of soluble but not cell-associated or DEC205 entry receptor targeted antigen was sensitive to deficiency in ATG5, ATG7 or ATG12 in dendritic or B cells (Dasari et al., 2016; Mintern et al., 2015). Consistent with a LAP like process being involved in this cross-presentation, antigen was found to be maintained longer in NOX2 sufficient dendritic cells facilitating antigen processing for MHC class I presentation (Savina et al., 2006). However, if antigens escape endosomes to the cytosol and aggregate, similar to intracellular antigen, their degradation by macroautophagy limits cross-presentation on MHC class I molecules (Ho et al., 2020). Thus, the influence of ATGs on antigen processing for MHC class I presentation seems to strongly depend on the antigen’s endosomal trafficking and cytosolic aggregation behavior.

A contribution of the macroautophagy machinery to MHC class I restricted antigen cross-presentation was also found in antigen donor cells (Fig. 2). This was initially described for influenza virus infected and tumor cells (Li et al., 2008; Uhi et al., 2009). It most likely requires an ATG supported exocytosis pathway similar to the above-described extracellular vesicle release from multivesicular bodies that is supported by the LC3 conjugation machinery and high-jacked by some viruses. It was found that this immunogenic vesicle release can be increased by proteasomal and lysosomal inhibition (Fan et al., 2019; Li et al., 2011, 2012; Twitty et al., 2011; Ye et al., 2014; Yi et al., 2012). Proteasomal inhibition presumably reroutes more antigens to autophagosomes that are in part released if they cannot be degraded due to lysosomal inhibition.

6. Conclusions and outlook

The various mechanisms by which the macroautophagy machinery influences pathogen clearance, inflammation and MHC restricted antigen presentation make it difficult to design strategies to regulate ATG proteins even only with respect to immune responses in disease settings. However, it becomes clear that inhibition of the autophagy machinery might be beneficial to increase inflammation and CD8+ T cell responses for better immune control of viral infections and tumors and to compromise CD4+ T cell responses in autoimmunity. During bacterial...
and fungal infections, however, improved pathogen clearance and CD4+ T cell responses might benefit from the stimulation of the macroautophagy machinery. With the recent realization that ATGs are also involved in the regulation of endo- and exocytosis, and the increasing molecular understanding how the macroautophagy machinery influences these pathways, specific inhibitors and activators of these different non-canonical roles of ATGs can be developed to regulate the respective pathways more specifically in the various associated disease settings. Along these lines genetic ATG deficiencies have been identified in various human diseases, including destabilizing ag161 mutations in inflammatory bowel disease (Crohn’s disease), beclin-1/atg6 haploinsufficiency in breast carcinoma and ATG5 deficiency in autoimmune systemic lupus erythematosus (SLE) (Mizushima and Levine, 2020). These diseases would particularly benefit from specific therapeutic interventions that target the macroautophagy machinery.

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