AUTOPHAGY AS A DEFENSE MECHANISM AGAINST PATHOGENS

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Autophagy has evolved as a conserving process for the bulk degradation and the recycling of cytoplasmic components, such as long-lived proteins and organelles. Various types of autophagy, including micro-, macro- and chaperone-mediated autophagy (CMA), differ in their mechanisms and functions. Both micro- and macro-autophagy can engulf large structures through both selective and non-selective mechanisms, whereas CMA degrades, in a selective manner, mostly soluble proteins. The autophagic machinery participates in the cellular defense against pathogens as cells use it to selectively capture and deliver microorganisms to lysosomes.

HOW DOES AUTOPHAGY WORK?

Autophagy involves the sequestration of certain cytosolic constituents, like proteins and organelles, in two layer membrane vesicles called autophagosomes (1, 2). The autophagosomes then fuse with lysosomes to form autolysosomes (Figure 1). The whole process is inducible and tightly regulated by the mammalian target of rapamycin (mTOR) pathway, which acts as an energy and amino acid sensor inside the cell. The autophagic process is controlled by "autophagy related genes" (Atgs), many of which are involved in the formation of the autophagosome. Beclin1 (Atg6) and class III PI3K are needed for the first step of autophagy, the vesicle nucleation (the isolation membrane). The subsequent vesicle elongation process features two ubiquitin-like conjugation systems which are well conserved among all eukaryotes. One pathway involves the conjugation of Atg12 to Atg5 with the help of Atg7 and Atg10. The second pathway involves the conjugation of phosphatidylethanolamine to Atg8 (microtubule-associated protein 1 light chain 3, or LC3) by the sequential action of Atg4, Atg7 and Atg3. This conjugation leads to the conversion of LC3-I (the soluble form of LC3) to LC3-II (the autophagic vesicle-associated form), which alone or fused to fluorescent proteins is the most used molecular marker for autophagy (Figure 2)(3). After the establishment of animal models carrying targeted deletions of specific Atg genes, significant insights into the autophagy regulation have been made. Interestingly, while some gene deletions are lethal, others allow a normal embryonic development. Homozygous Beclin1 (Atg6)-deficient mice are embryonic lethal at 7.5–8.5 days of embryogenesis, while mice deficient in either Atg5 or Atg7, express a nearly normal phenotype at birth, but die within 1 day of the delivery. A possible explanation would be that neonates face severe nutrient deprivation until lactation starts, leading to increased mortality(4, 5).

The capacity for large-scale degradation is important in autophagy, but it carries a certain risk, as unregulated degradation of the cytoplasm is likely to be lethal. On the other hand, the basal level of autophagy is important for maintaining normal cellular homeostasis. Thus, it is crucial for this autophagic process to be tightly regulated, so that it is induced when needed, but otherwise maintained at basal levels. An interesting example of the double-edged sword nature of autophagy is found in the heart, where cardiomyocytes require the basal level of autophagy to adjust to the high energy requirements. However, in response to various stresses such as ischemia/reperfusion or as in cardiac hypertrophy and heart failure, this

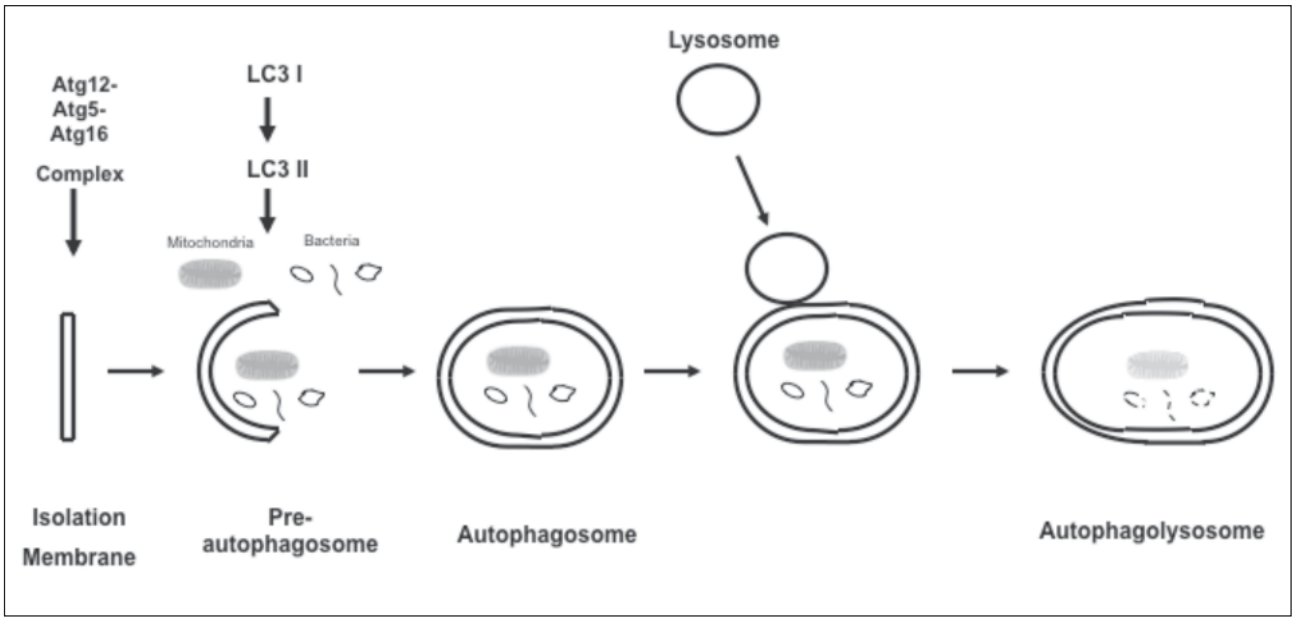

Figure 1. *The Molecular Machinery of Macroautophagy*

Atg6 (Beclin1) is part of the type III PI3K complex that initiates autophagosome formation. Two ubiquitin-like systems are required for the formation of the isolation membrane and coupling of Atg8 (LC3) to phosphatidylethanolamine (PE) and Atg12 to Atg5. The five C-terminal amino acids of Atg8 (LC3) are cleaved by Atg4 to reveal glycine 120 and produce the cytosolic LC3-I form. When autophagy is activated, LC3-I is linked by Atg7 and Atg3 to PE in the autophagosomal membrane (red circles in Figure 2) producing the LC3-II form. Similarly, glycine 140 is used by Atg7 and Atg10 to couple Atg12 to Atg5. This complex localizes to the outer membrane of the forming isolation membrane of the pre-autophagosome. Upon autophagosome completion, the Atg12-Atg5 complex recycles from the outer membrane, and only Atg8 (LC3) remains associated with the completed autophagosome. Autophagosomes then fuse with lysosomes for degradation of their cargo and their intravesicular membranes (autophagolysosome).

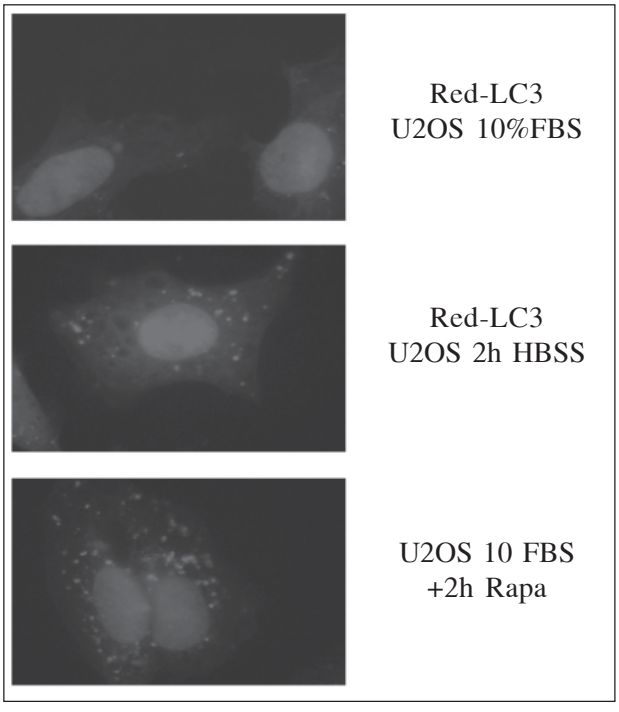

Figure 2. Analysis of Autophagy Using a Red-LC3 Reporter.

Upper panel: U2OS cells stable transfected with Red-LC3 in complete medium containing 10% FBS (as negative control). Middle and lower panels: To induce autophagy, the culture medium was changed to HBSS (an amino acid- and glucose-free solution which induces massive autophagy) or 50 nM rapamycin (a mTOR inhibitor) was added in medium containing 10% FBS for 2 h. Autophagy is detected as the formation of the autophagosomes aggregates (dots) in the cytosol, which could be then quantified as a measure of the autophagy activation (e.g. expressed as number of dots per cell).
(Red-LC3: Red fluorescent protein (Clontech) fused to LC3, courtesy of Alessia Di Nardo, Harvard Medical School, Boston; FBS fetal bovine serum; HBSS: Hank's Balanced Saline Solution)

basal level of autophagy is altered, possibly contributing to the development of the cardio-vascular disease(6). Many aspects of the autophagy regulation and its role in the onset of heart diseases, neurodegenerative diseases and cancer have been covered in recent reviews(2, 7). Here we are addressing the role of autophagy in regulating cellular fate and the response to infectious agents.

AUTOPHAGY: A PRO-SURVIVAL OR PRO-APOPTOTIC MECHANISM?

The pro-survival function of autophagy has been demonstrated at both cellular and organism level in different contexts, including during nutrient or growth factor deprivation, endoplasmic reticulum stress, development, microbial infection, and diseases characterized by the accumulation

of protein aggregates. An apparent conundrum is that autophagy acts both in cytoprotection and in cell death. In response to most forms of cellular stress, autophagy plays a cytoprotective role, as Atg knockdown or knockout accelerates rather than delays cell death. However, in certain settings of uncontrolled upregulation of autophagy (as in overexpression of the autophagy protein Beclin1 in mammalian cells), autophagy can lead to cell death, possibly through activation of apoptosis or as a result of the inability of cells to survive the non-specific degradation of large amounts of their cytoplasmic contents(5). Many examples of Atg-gene-dependent cell death occur in cells deficient in apoptosis, suggesting that autophagy, as a route to cell death, maybe a choice of last resort. Autophagic programmed cell death was originally described in tissues undergoing active development. Mice lacking Beclin1 or Atg5 display increased numbers of apoptotic cells in embryonic tissues, arguing against a requirement for the autophagic machinery in developmental programmed cell death. In adult animals with tissue-specific Atg knockout, there is perhaps clearer evidence of an anti-apoptotic function of autophagy *in vivo*(4, 5). Many signals, originally studied in the context of apoptosis activation, induce autophagy, whereas signals that inhibit apoptosis also inhibit autophagy. Anti-apoptotic proteins, such as Bcl-2 family members, inhibit Beclin, while pro-apoptotic factors, such as members of BH3-only proteins, disrupt this inhibitory interaction and thereby activate autophagy. Another link between the autophagic machinery and apoptosis is the observation that calpain-mediated cleavage of Atg5 generates a pro-apoptotic fragment functioning in the intrinsic mitochondrial death pathway.

Thus, it is likely that coordinated regulation of 'self-digestion' by autophagy and 'self-killing' by apoptosis might underlie diverse aspects of development, tissue homeostasis and disease pathogenesis(2).

AUTOPHAGIC DEFENSE AGAINST VIRUSES, INTRACELLULAR BACTERIA AND PARASITES: INVOLVEMENT OF BOTH INNATE AND ADAPTIVE IMMUNITY

The disposal of intracellular organisms, similar to the one of cellular organelles, represents a challenge for cellular degradative pathways which can only be achieved through autophagy. The same autophagic machinery used to selectively capture cellular organelles is also used for the selective delivery of microorganisms to the lysosomes, in a process called xenophagy (or degradation of foreign microbes by autophagy, Figure 1) (8, 9). The cell biology of xenophagy is less studied than that of the classical autophagy, and it is still unclear whether the membranes engulfing microorganisms have a biogenesis similar to or different than that of classical autophagosomes. Nevertheless, the same molecular components (Atgs) are involved in the two processes. In addition, while xenophagy appears to be a selective form of autophagy, almost nothing is known about how bacteria, viruses or the membranous compartments containing microbes are recognized by the autophagic machinery(8).

The interaction between viruses and the autophagic machinery is just beginning to be elucidated, and so far three major outcomes of this interaction have been found. First, the induction of autophagy can successfully limit the viral replication. Second, it has been reported that viruses inhibit autophagy to avoid restriction of their replication, and third, viruses can use accumulated autophagosomes for their replication. For example, in the case of certain RNA viral infections, autophagy is required for the delivery of viral nucleic acids to the endosomal toll-like receptor TLR7, and subsequent activation of type I interferon signaling(9).

In contrast to viruses, a number of microbial pathogens have been described as being successfully targeted by autophagy during the innate immune responses. Bacteria and parasites have been found to be susceptible to macroautophagy in two cellular compartments after entering their host cells. Free bacteria in the cytosol can fall prey to autophagy, while pathogen-conditioned phagosomes can either fuse with or get engulfed by autophagosomes. Since most of the studies have been performed *in vitro*, it is still an open question whether autophagy can restrict bacteria and parasites invasion *in vivo*(2, 9).

In addition to limiting pathogen replication in host cells, xenophagy can also deliver viral, parasitic and bacterial antigens to late endosomal compartments where autophagy substrates are then degraded by lysosomal hydrolases. The fusion vesicles between autophagosomes and late endosomes, called amphisomes, have been isolated; the amphisomes display multivesicular and multilamellar morphology, the same characteristics that

have been reported for major histocompatibility complex (MHC) class II loading compartments. The latter are equipped with the molecular machinery to load antigenic fragments onto MHC class II molecules for presentation to T cells (CD4+), thus allowing the establishment of an adaptive immune response(8, 9).

Given the diverse roles of autophagy in innate and adaptive immunity, it is not surprising that many pathogens have devised strategies to outsmart autophagy. Some intracellular bacteria and viruses co-opt the autophagic machinery to use Atg protein-dependent dynamic membrane rearrangements for their own replicative advantage. More commonly, successful intracellular pathogens modulate the signalling pathways that regulate autophagy or block the membrane trafficking events required for autophagy-mediated pathogen delivery to the lysosome. Notably, microbial evasion of autophagy may be essential for microbial pathogenesis under certain conditions. For example, fatal herpes simplex virus encephalitis requires inhibition of the autophagy protein Beclin1 by a specific viral protein(10). Thus, selective disruption of interactions between microbial virulence factors and their targeted host autophagy proteins may help reduce infection-induced pathology. Other postulated roles of autophagy in immunity that warrant further scientific attention include T-cell homeostasis, central and peripheral tolerance induction, and prevention of unwanted inflammation and autoimmunity(8, 10).

CONCLUSIONS AND FUTURE DIRECTIONS

To better understand the immunity it is important to identify the mechanisms by which autophagy is activated in response to microbial invasion, the targets that allow specific recognition of intracellular pathogens, and the roles of autophagy in immune cell function. The recent introduction of conditional knockout mice will help in part overcome this problem as it makes possible to compare the consequences of impaired autophagy from birth. Thus, although tremendous advances have been made in our understanding of autophagy, many unanswered questions about its role in immunity remain. A better understanding of all types of autophagy is necessary before we can start manipulating these pathways to treat human disease.

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