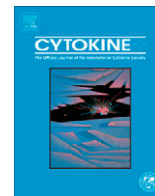




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Review Article

Autophagy and cytokines

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ABSTRACT

Autophagy is a highly conserved homeostatic mechanism for the lysosomal degradation of cytosolic constituents, including long-lived macromolecules, organelles and intracellular pathogens. Autophagosomes are formed in response to a number of environmental stimuli, including amino acid deprivation, but also by both host- and pathogen-derived molecules, including toll-like receptor ligands and cytokines. In particular, IFN- γ , TNF- α , IL-1, IL-2, IL-6 and TGF- β have been shown to induce autophagy, while IL-4, IL-10 and IL-13 are inhibitory. Moreover, autophagy can itself regulate the production and secretion of cytokines, including IL-1, IL-18, TNF- α , and Type I IFN. This review discusses the potentially pivotal roles of autophagy in the regulation of inflammation and the coordination of innate and adaptive immune responses.

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1. Introduction

Autophagy is a general term for the pathways through which cytoplasmic constituents, including macromolecules and organelles, are delivered to lysosomes for degradation. Three different types of autophagy have been identified so far; microautophagy, chaperone-mediated autophagy and macroautophagy. This review will focus on the latter (hereafter termed “autophagy”), which is characterised by the formation of an isolation membrane, or phagophore, which elongates around its target and eventually fuses with itself to form a double-membraned autophagosome. This then fuses with lysosomes to form an autolysosome, allowing degradation of the luminal contents. Autophagy acts as a major survival mechanism, orchestrating the sequestration and degradation of damaged/toxic cytosolic constituents, including organelles, and regulating energy and nutrient homeostases. Autophagy is induced in response to numerous different stimuli, including environmental and cellular stresses, such as nutrient deprivation/amino acid starvation, growth factor withdrawal, endoplasmic reticulum (ER) stress, mitochondrial damage and in response to various immune stimuli.

2. Autophagosome formation

In yeast, a number of autophagy-related (*ATG*) genes have been characterised and mammalian orthologs have been identified. The

processes involved in autophagosome formation and maturation are complex and not yet fully understood (for more comprehensive reviews, see [1,2]). Autophagy in response to environmental and cellular stress is typically dependent on the inhibition of mammalian target of rapamycin (mTOR) [2] (Fig. 1). This results in the translocation of a complex containing Atg1/unc-51-like kinase (ULK)1/2, Atg13, FIP200 and Atg101 complex from the cytosol to the ER [3]. This process is dependent on the interaction between ULK1 and AMP-activated protein kinase (AMPK) [4] and leads to the recruitment of the type III phosphatidylinositol-3-kinase (PI3K) VPS34, in complex with a number of proteins, including Beclin 1 (Atg6) which, in turn, generates phosphatidylinositol-3-phosphate (PtdIns(3)P or PI3P), leading to the recruitment of effectors such as double FYVE-containing protein 1 (DFCP1) and WD-repeat domain phosphoinositide-interacting (WIPI) family proteins (mammalian orthologs of Atg18) [5,6]. Elongation of the isolation membrane, derived from the ER, is dependent on at least two ubiquitin-like conjugates, the Atg12-Atg5 conjugate, which forms a complex with Atg16L1 [7] and the phosphatidylethanolamine (PE)-microtubule-associated protein 1 light chain 3 (LC3) conjugate. Unconjugated LC3 (LC3 I) is found in the cytosol, while PE lipids LC3 at the C-terminus, which then translocates to the autophagosomal membrane [7,8]. VPS34 is again involved in maturation of the autophagosome, this time in a complex with Beclin 1 and UV irradiation resistance-associated gene (UVRAG) [9]. Autophagy can be inhibited with the PI3K inhibitors wortmannin and 3-methyladenine (3-MA) and can be induced pharmacologically with rapamycin and rapamycin-related compounds, which bind to and inhibit mTOR. In addition, mTOR-independent

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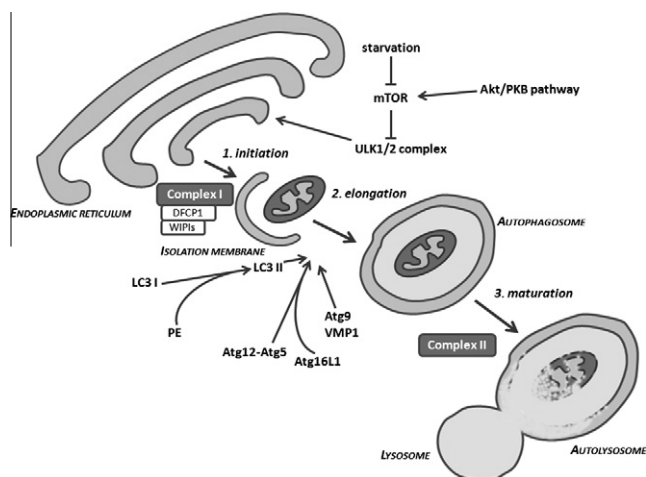


Fig. 1. mTOR-dependent autophagosome biogenesis. Autophagosome biogenesis occurs in three stages. 1. *Initiation*: Mammalian target of rapamycin (mTOR) is inhibited by amino acid starvation which, in turn, leads to translocation of a protein complex containing ULK1/2, Atg13, Atg101 and FIP-200 from the cytosol to the endoplasmic reticulum (ER). This allows recruitment of a complex consisting of the type III PI3K VPS34, Beclin 1 (Atg6), VPS15 and Atg14 (complex I). This allows the recruitment of DFCP1 and WIPI family proteins which translocate to the autophagosome formation site and generate ER-associated Ω -like structures, called omegasomes, from which the isolation membrane is thought to emerge. 2. *Elongation*: The isolation membrane elongates around its cytosolic target until it fuses with itself to form an autophagosome with a double membrane. This process is dependent on the lipidation of cytosolic LC3 I by phosphatidylethanolamine (PE) to form LC3 II, which is recruited to the autophagosomal membrane and on the Atg5-Atg12-Atg16L1 complex. Elongation is also dependent on the transmembrane proteins Atg9 and VMP1, although their precise role is not yet clear. 3. *Maturation*: The outer membrane of the autophagosome is able to fuse with lysosomes, allowing the degradation of the luminal contents. This process is dependent on the VPS34-Beclin 1-UVRAG complex (complex II). Activation of mTOR (for example, through activation of the Akt signalling pathway) leads to the inhibition of ULK1/2 translocation and inhibition of autophagy.

autophagy induction has been reported, involving the cAMP-Epac-PKC ϵ pathway [10].

3. Regulation of autophagy by cytokines

Autophagy plays important roles in innate and adaptive immunity, both in the direct elimination of intracellular bacteria (although many microorganisms target or modulate autophagy for their own survival) and in the processing and presentation of endogenously expressed antigens via major histocompatibility complex class I and II molecules [2,11,12]. The fact that many cytokines, as well as TLR and NOD-like receptor (NLR) ligands are potent inducers of autophagy further suggests that it has evolved as an important mechanism in the immune response against pathogenic microorganisms [13–16].

Studies on the role of autophagy in the response of macrophages to *Mycobacterium tuberculosis* have helped to elucidate the role of cytokines in regulating autophagy. It is well established that a predominantly Th1-biased response, directed by IFN- γ , IL-12 and TNF- α is essential for host protective responses against *M. tuberculosis* [17]. Similarly, it has been known for some years that activation of macrophages with IFN- γ , in the presence or absence of LPS, leads to increased maturation of mycobacteria-containing phagosomes [18,19] and that the IFN- γ -inducible GTPase Irgm1 (formerly LRG-47, IRGM in humans) is important for the protective response to *M. tuberculosis* [20]. More recently, activation of human and murine macrophages with IFN- γ has been shown to induce autophagy in an Irgm1/IRGM-dependent manner [21,22]. In

macrophages infected with mycobacteria, pre-treatment with IFN- γ leads to increased intracellular killing of the bacilli and engulfment of mycobacteria-containing phagosomes by autophagosomes, which in turn fuse with lysosomes [21]. The full extent to which autophagy contributes to IFN- γ -induced phagosome maturation in *M. tuberculosis*-infected macrophages is not clear. We have found that while infection of macrophages with the avirulent H37Ra strain of *M. tuberculosis* does induce autophagy in murine macrophages, the mycobacteria-containing phagosomes are typically LC3-negative (Ni Cheallaigh and Harris, unpublished observations). However, activation of macrophages with IFN- γ prior to infection increases co-localisation of BCG with LC3 [21]. Moreover, silencing of Beclin 1 in murine macrophages with siRNA completely abrogates the effect of IFN- γ on the maturation of BCG-containing phagosomes [23], suggesting that this is an entirely autophagy-dependent process.

In BCG- or *M. tuberculosis*-infected human macrophages, IFN- γ -induced phagosome maturation is abrogated by the TNF blockers adalimumab and infliximab (monoclonal antibodies against TNF- α) and etanercept (soluble TNF receptor/Fc fusion protein) [24], suggesting that IFN- γ -induced phagosome maturation – which may be dependent on autophagy – might in turn be dependent on TNF- α . A number of studies have demonstrated a role for TNF- α in stimulating autophagy in cells, including human and murine macrophages, human T lymphoblastic leukaemic cells, human vascular smooth cells, human skeletal muscle cells and rat epithelial cells [25–31]. How TNF- α stimulates autophagy is not well understood and may differ between different cell types. In murine macrophages CD40 ligation, coupled with TNF- α signalling, aids autophagic elimination of *Toxoplasma gondii* [25,31]. In human atherosclerotic vascular smooth cells TNF- α up-regulates expression of the autophagy genes LC3 and Beclin 1 and this is dependent on signalling via the Jun kinase (JNK) pathway, as well as the inhibition of Akt activation [28]. In MCF-7 human breast cancer cells, induction of autophagy by TNF- α is dependent on the ERK1/2 pathway [32], while in Ewing sarcoma cells, activation of NF- κ B inhibits TNF- α -induced autophagy, which is dependent on the generation of reactive oxygen species (ROS) [33]. In rat intestinal epithelial cells, TNF- α induces mitochondrial dysfunction, characterised by increased mitochondrial ROS, decreased oxygen consumption and a drop in mitochondrial membrane potential, which in turn leads to increased autophagy of mitochondria (mitophagy) [26].

While IFN- γ and TNF- α , both archetypal Th1 cytokines, induce autophagy, the classical Th2 cytokines IL-4 and IL-13 inhibit it. Again, in the context of infection with *M. tuberculosis*, both IL-4 and IL-13 inhibit starvation- or IFN- γ -induced autophagosome formation and lead to decreased phagosome maturation and increased intracellular survival of the bacilli [23]. This is dependent on two distinct signalling pathways; inhibition of starvation-induced autophagy is dependent on the Akt pathway, while inhibition of IFN- γ -induced autophagy is dependent on signalling via STAT6 [23]. Similarly, in HT-29 human epithelial cells IL-13 is a potent inhibitor of starvation-induced autophagy and this is dependent on activation of the Akt pathway [34,35]. These data would suggest that, in the context of infection with *M. tuberculosis*, autophagy is an effector of Th1/Th2 polarisation. In murine macrophages, IL-10 also inhibits starvation-induced autophagy via the Akt pathway [36] and rapamycin-induced autophagy via both the Akt and STAT3 pathways [37]. In addition, IL-10 has been shown to inhibit LPS-induced autophagy, although the signalling pathway responsible for this has not been determined [38]. Thus, IL-10 may have an important role in regulating autophagy, particularly in the context of infection. Moreover, given that IL-4, IL-13 and IL-10 are largely secreted by monocytes/macrophages and lymphocyte subsets, T cell-mediated and autocrine regulation of autophagy in

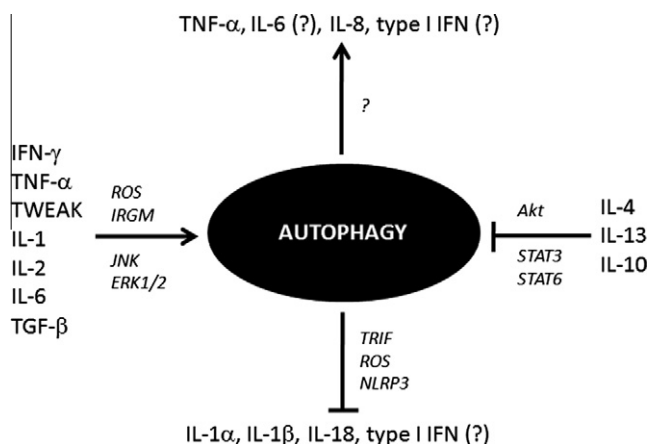


Fig. 2. Interactions between autophagy and cytokines. Autophagy is induced by a number of cytokines, including IFN- γ in macrophages, TNF- α in macrophages, TWEAK in C2C12 myotubes, IL-1 α and IL-1 β in macrophages, IL-2 in CD4 $^+$ T cells, IL-6 in CD11b $^+$ peripheral blood mononuclear cells and TGF- β in hepatocarcinoma cell lines. Induction of autophagy by TNF- α has been shown to be dependent on the generation of reactive oxygen species (ROS) and on the JNK and ERK signalling pathways. IFN- γ -induced autophagy is dependent on IRGM (Irgm1/LRG-47 in mice). IL-4 and IL-13 inhibit autophagy through the STAT6 and Akt pathways, while IL-10 inhibits autophagy through the STAT3 and Akt pathways. Autophagy negatively regulates the processing and secretion of IL-1 β and IL-18, as well as the secretion of IL-1 α , by macrophages and dendritic cells. Conversely, autophagy positively regulates the transcription and secretion of TNF- α , IL-8 and possibly IL-6. There is conflicting data on the role of autophagy in the production of type I IFN in the response to viral infection of cells; it may depend on the specific virus, as well as the cell type.

macrophages may represent an important factor in specific immune responses to *M. tuberculosis* and other infectious microorganisms.

Other cytokines, chemokines and growth factors have been implicated in the control of autophagy (Fig. 2). In some carcinomas, TGF- β has growth inhibitory effects and has been shown to induce autophagosome formation and increased expression of Beclin 1, Atg5 and Atg7 mRNA in human hepatoma cells, and increased autophagy in MDA-MB-231 mammary carcinoma cells [39,40]. However, the CC chemokine CCL2 (monocyte chemoattractant protein-1) and IL-6 both stimulate autophagy and upregulate antiapoptotic proteins in human CD11b $^+$ peripheral blood mononuclear cells [41]. Moreover, CCL2 and IL-6 drove CD11b $^+$ cells toward a CD14 $^+$ /CD205 $^+$ tumour-promoting M2-type phenotype. Thus, autophagy has conflicting roles in the immune regulation of tumorigenesis. IL-1 α and IL-1 β have been demonstrated to stimulate autophagy in human macrophages [42], which, given the role of autophagy in regulating IL-1 secretion (discussed later), could suggest a negative feedback loop in certain inflammatory responses. A recent study has also demonstrated that the proinflammatory cytokine TNF-like weak inducer of apoptosis (TWEAK) increases transcription of Beclin 1, LC3B, Atg5 and Atg12 in the mouse myoblastic cell line, C2C12 [43]. Moreover, TWEAK-induced atrophy in C2C12 cell cultures is prevented by treatment of the cells with 3-MA or transfection with Beclin 1 siRNA [43]. IL-2 induces autophagy in human CD4 $^+$ T lymphocytes, although in these cells it appears to contribute to growth factor withdrawal-induced cell death, rather than act as a cell protective mechanism [44]. This may represent a difference in the roles of autophagy between lymphocytes and macrophages (and other antigen-presenting cells), an important consideration when designing therapeutics that target autophagy. Conversely, insulin-like growth factor 1 (IGF1), fibroblast growth factor 2 (FGF2) and Leukaemia inhibitory factor (LIF) all inhibit autophagy in HEK293, HeLa, MCF7 and H4 cell lines [45]. Further studies are required to determine the importance of

these findings in the context of immunity and disease and to better understand the cytokine networks involved in autophagy regulation.

4. Regulation of cytokines by autophagy

Recent studies have shown that autophagy, as well as being regulated by cytokines, can itself directly influence the transcription, processing and secretion of a number of cytokines. In particular, disruption of normal autophagic pathways has been linked to increased secretion of the pro-inflammatory cytokines IL-1 α , IL-1 β and IL-18 [46–50]. The processing and secretion of IL-1 β is tightly regulated and largely dependent on the specific activation of caspase 1, following the formation of an inflammasome [51]. Secretion of IL-1 β is typically a two stage process; firstly transcription of pro-IL-1 β is induced (by, for example, LPS), then inflammasome assembly and caspase 1 activation is stimulated by endogenous signals, such as ROS, uric acid crystals or ATP. Autophagy regulates IL-1 β secretion through at least two separate mechanisms. Loss of autophagy in macrophages or dendritic cells, either through knock down of Atg7, Atg16L1 or Beclin 1, or by treatment with the autophagy inhibitor 3-MA, stimulates the processing and secretion of IL-1 β in response to TLR agonists [46,47,49]. In murine macrophages and dendritic cells this effect is dependent on TIR-domain-containing adaptor-inducing interferon- β (TRIF) and mitochondrial ROS and/or mitochondrial DNA and at least partially dependent on NLRP3 [47–50], while in human peripheral blood mononuclear cells it may be independent of TRIF, but dependent on p38 MAPK signalling [46]. The dependence on mitochondrial ROS could, in turn, suggest a role for TNF- α in mediating these effects, as the cytokine has been shown to induce oxidative stress via mitochondrial ROS [26,52,53] and is highly up-regulated in macrophages and dendritic cells treated with TLR agonists. In mice with a conditional deletion of Atg7 in the intestinal epithelium, LPS induces higher levels of IL-1 β mRNA, compared to wild type controls [54], while LC3B $^{-/-}$ mice produce higher levels of IL-1 β and IL-18 in response to LPS- or caecal ligation and puncture (CLP)-induced sepsis [48]. In humans, polymorphisms in Atg16L1 have been associated with an increased risk of Crohn's disease [55,56] and mice lacking Atg16L1 in haematopoietic cells have been shown to be more susceptible to dextran sulphate sodium (DSS)-induced colitis [49], suggesting that autophagy may be an important mechanism for the control of inflammation *in vivo*.

These data would indicate that autophagy is required to control endogenous inducers of inflammasome activation/IL-1 β processing, such as mitochondrial ROS. Conversely, induction of autophagy with rapamycin inhibits the secretion of IL-1 β in murine dendritic cells in response to LPS with ATP or alum [47]. Moreover, following treatment with rapamycin and LPS or PAM3Cys, levels of pro-IL-1 β in macrophages were reduced. Coupled with the observation that, in macrophages treated with TLR agonists, IL-1 β could be seen in autophagosomes, these data suggest that autophagy specifically targets pro-IL-1 β for lysosomal degradation, thus acting as an internal regulatory mechanism controlling the availability of pro-IL-1 β for subsequent processing [47]. Treatment with rapamycin also reduces the amount of IL-1b in the serum of mice concurrently treated with LPS [43]. Given that IL-1 α and IL-1 β have both been shown to induce autophagy [42], this may act as a negative feedback loop to control IL-1-induced inflammation. Moreover, as IL-1 (along with IL-23) drives IL-17 secretion by lymphocytes [57–59], autophagy may play an important role in the modulation of Th17 responses, which in turn could have implications for specific responses to pathogens and vaccination, as well as in the regulation of certain autoimmune disorders, such as multiple sclerosis.

Similar to IL-1 β , IL-18 is processed by caspase 1 and requires the formation of an inflammasome and its secretion is similarly enhanced by inhibition of autophagy [47,48]. LPS-induced secretion of IL-1 α is also increased in the presence of 3-MA, although this is independent of NLRP3 [47]. It is, however, unclear whether these cytokines, like IL-1 β , are targeted for degradation by autophagosomes. However, they do further suggest that autophagy has a specific role to play in the regulation of inflammatory cytokines.

There is also evidence that autophagy is involved in the regulation of TNF- α secretion by macrophages and dendritic cells. In particular, treatment of both human and murine cells with 3-MA strongly inhibits TLR-dependent TNF- α (and IL-6) secretion [46,47]. Moreover, in human PBMC, treatment with 3-MA decreased transcription of TNF- α (and increased transcription of IL-1 β) in response to LPS or PAM3Cys [46]. We have observed similar inhibition of LPS-induced IL-6 transcription in response to 3-MA in murine macrophages (Oleszycka, Lavelle and Harris, *unpublished observations*). It is not yet clear whether this is a specific effect of autophagy inhibition, or some other off-target or toxic effect of 3-MA; experiments using autophagy-deficient cells or cells subjected to siRNA silencing of autophagy genes would help to clarify this. Silencing of Atg7 in intestinal epithelial cells has been shown to down-regulate TLR-dependent IL-8 production [60], suggesting that autophagy may play a role in regulating the recruitment of granulocytes to sites of tissue damage and/or inflammation.

There is conflicting data on the role of autophagy in the regulation of type I IFN secretion by virally-infected cells. The production of Type I IFN by plasmacytoid dendritic cells (pDC) is dependent on TLR7 and TLR9 [61,62]. In addition, pDC deficient in Atg5, or treated with 3-MA or wortmannin, fail to produce IFN- α in response to infection with vesicular stomatitis virus (VSV) [63]. Similarly, IFN- α production is abrogated in autophagy-deficient pDC stimulated with herpes simplex virus (HSV) or CpG, suggesting that autophagy may have a role to play in TLR7/9 signalling or endosomal maturation [63]. In contrast, other studies have demonstrated that embryonic fibroblasts from Atg5^{-/-} mice are more resistant to VSV infection and produce higher levels of IFN- α and IFN- β mRNA in response to VSV or stimulation with dsRNA (poly(I:C)), compared with WT controls [64,65]. This response is dependent on mitochondrial dysfunction and ROS release, which enhances signalling by RIG-I-like receptors (RLR) [65], suggesting that the removal of damaged mitochondria by autophagy may on the one hand hamper antiviral responses, but on the other control excessive inflammatory responses. A more recent study has demonstrated that the knockdown of either Beclin 1 or Atg7 by siRNA increases the expression of IFN- α and IFN- β in immortalised human hepatocytes infected with hepatitis C virus (HCV) [66]. Interestingly, HCV has been shown to induce autophagy [67], suggesting that the virus may use the host's autophagic machinery to its own advantage. These studies suggest that the role of autophagy in virus-induced production of type I IFN may depend on a number of factors, including the cell type and the specific receptors involved.

5. Conclusions

The many bi-directional interactions with cytokines suggest important roles for autophagy in the immune response, particularly in response to infection with bacteria and viruses, as well as in the control of inflammation. In certain bacterial infections, particularly with *M. tuberculosis*, autophagy may be an important effector of Th1/Th2 polarisation, aiding in the killing of bacilli. Conversely, through the negative regulation of IL-1 and type I IFN, autophagy may be essential in the control of inflammation and fine-tuning of the immune response. Research into this cross-talk between autophagy and cytokines is still very much in its infancy,

but represents a potentially crucial target for the specific regulation of immune responses and inflammation. Pharmacological modulation of autophagy could have considerable potential in the treatment of inflammatory and autoimmune disorders and perhaps as an adjunctive therapy in the treatment of specific infectious diseases, such as tuberculosis. Only through further research will we be able to uncover the full repertoire of interactions between autophagy and cytokines, the mechanisms and pathways involved and the potential for therapeutic benefit.

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