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Autophagy in Pulmonary Innate Immunity

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Abstract
Autophagy is a major intracellular digestion system that delivers cytoplasmic components for degradation and recycling. In this capacity, autophagy plays an important role in maintaining cellular homeostasis by mediating the degradation of cellular macromolecules and dysfunctional organelles and regeneration of nutrients for cell growth. Autophagy is important in innate immunity, as it is responsible for the clearance of various pathogens. Deficiency of intracellular autophagy can result in exaggerated activation of the inflammasome. The latter is an innate immune complex that senses diverse pathogen-associated or danger-associated molecular patterns and activates the expression of inflammatory cytokines. In autophagy-deficient cells, accumulation of damaged organelles, misfolded proteins, and reactive oxygen species contribute to inflammasome activation. The lung is continuously exposed to pathogens from the environment, rendering it vulnerable to infection. The lung innate immune cells act as a crucial initial barrier against the continuous threat from pathogens. In this review, we will summarize recent findings on the regulation of autophagy and its interaction with innate immunity, focusing on the lung.

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Autophagy in Regulatory and Signaling Pathways

Autophagy is a conserved intracellular digestion and recycling pathway that is present in all eukaryotic cells [1]. It is a process that involves the engulfment of cytoplasmic components by double membrane-bound compartments, called autophagosomes, and fusion of these autophagosomes with lysosomes. It functions as a waste disposal system removing potential toxic cellular products by targeting aggregate proteins and malfunctioning organelles for degradation and the subsequent release of degraded products for cellular recycling purposes [2]. Autophagy provides substrates for biosynthesis and energy generation which promote cell survival during times of energy and nutrient deprivation [2, 3]. Thus, autophagy is a survival mechanism designed to maintain cellular homeostasis in response to metabolic stress

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[3]. In addition, autophagy is involved in various aspects of immunity, including the clearance of pathogens, cytokines, and immune signals, suggesting that autophagy also plays important roles in innate [3, 4] and adaptive immunity [5].

Cellular Regulation of Autophagy

The canonical autophagy pathway can be separated into three major steps: initiation, elongation, and maturation [6]. Each step is regulated by specific autophagy-related (Atg) proteins [6–9]. In the initiation step, with the activation of the ULK1 kinase complex, a small membranous sac called a phagophore is formed. A second kinase complex called the Vps34 complex is then recruited to the phagophore membrane [6]. Vps34 catalyzes the phosphorylation of cellular phosphatidylinositol to produce phosphatidylinositol 3-phosphate (PI3P), which serves as a platform to recruit more Atg proteins to promote the elongation of autophagic membranes (Fig. 1a) [8]. In the elongation step, two ubiquitin-like conjugation systems, Atg12–Atg5-Atg16L and LC3–PE, are involved. In the first ubiquitin-like system, Atg12 acts as a ubiquitin-like protein and, with the help of Atg7 and Atg10, becomes conjugated to Atg5. The resulting complex subsequently interacts with the coiled-coil protein Atg16L1 to form the Atg12–Atg5-Atg16L complex [10]. In the second ubiquitin-like system, pro-LC3 is cleaved by the cysteine protease Atg4, exposing a C-terminal glycine residue [11]. LC3 is then transiently linked to Atg7 and Atg3, and is eventually conjugated to phosphatidylethanolamine to form the mature form of lipid protein LC3II. The two systems work together to expand phagophore membrane formation that leads to mature autophagosomes (Fig. 1b) [6]. The mature autophagosomes then fuse with lysosomes to create autolysosomes and the acidic proteolytic environment of the autolysosome contributes to the degradation of the inner membrane as well as the luminal contents inside the autophagic vacuoles [6, 9].

Autophagosomes can be processed in the absence of some key autophagy components, in a process described as noncanonical autophagy [12, 13]. Noncanonical autophagy can also be processed without the ubiquitin conjugation proteins Atg5 and Atg7 [14, 15], and this type of autophagy has been found to play an important role in mitochondrial digestion during erythroid maturation in vivo [14–16]. LC3-associated phagocytosis (LAP) is a distinct form of noncanonical autophagy. It engages most of the canonical autophagy components, such as the Class III PI3K complex and ubiquitinylation-like protein-conjugation systems [17]. However, LAP proceeds without the ULK1 complex to form LC3-conjugated single-membraned phagosomes [17].

Autophagy Regulation

While the autophagy cascade could be affected in all three steps of the pathway, most regulation of autophagy occurs at the initiation step [9, 18]. The initiation complex ULK1 kinase is mainly regulated by mammalian target of rapamycin serine/threonine kinase complex 1 (mTOR) and adenosine monophosphate-activated protein kinase (AMPK). mTOR acts as a cellular nutritional sensor, phosphorylating ULK1, thus inhibiting autophagy initiation in nutrient-sufficient conditions [9, 18]. Under starvation or treatment with mTOR inhibitors such as rapamycin, mTOR activity is inhibited and ULK1 is rapidly dephosphorylated, resulting in activation of the ULK1 kinase (Fig. 1a). AMPK acts as an energy sensor that is activated by a decrease in the ATP/AMP ratio. AMPK activates autophagy initiation through inhibiting mTORC1 and activating the ULK1 complex (Fig. 1a) [18]. Vps34 kinase activity depends on Vps34-Beclin1 interaction as the direct downstream target of ULK, and it is inhibited by kinase inhibitor 3-methyladenine (3-MA) and wortmannin [18]. A recent study showed that the activity of Vps34 is also inhibited by acetyltransferase p300 [19]. Deacetylation of LC3 by Sirt1 affects LC3's distribution between the nuclear and cytoplasmic compartments and modulates the LC3-Atg7 conjugation process [20]. NAD+-dependent deacetylase Sirt1 directly deacetylates other Atg proteins, including Atg5, Atg7 [21], and Beclin1 [22], thus affecting autophagy processing (Fig. 1c).

Autophagy can be regulated at the transcriptional level. Forkhead transcription factors (FoxOs) upregulate autophagy by promoting the transcription of Atg genes, including Atg4, LC3B, and ULK1 [23, 24]. The activity of FoxOs depends on its nuclear localization. It has been shown that AKT/protein kinase B mediates the phosphorylation of FoxOs, leading to nuclear exclusion and inactivation [23, 24] (Fig. 1d). Transcription factor EB (TFEB) is another newly identified master transcription regulator of autophagy [25, 26]. Like FoxOs, the activation of TFEB is also dependent on nuclear translocation of the non-phosphorylated form

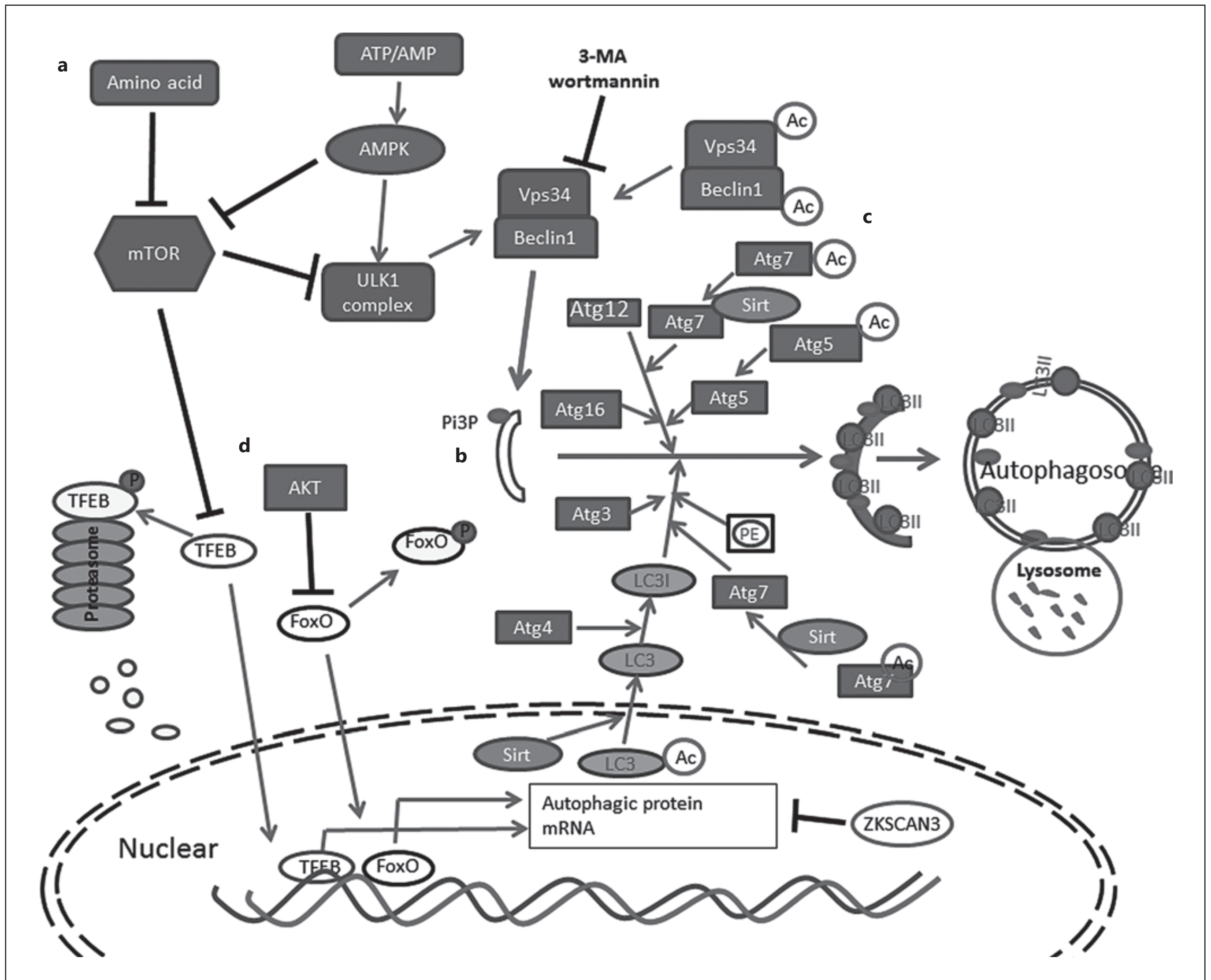

Fig. 1. Simplified autophagy signaling pathway and regulation. **a** Nutrition deprivation inhibits mTOR, which in turn activates ULK1 complex. Energy deprivation is sensed by AMPK to suppress mTOR and activates ULK1 complex. Activated ULK1 complex recruits Vps34-Beclin-1 complex to produce PI3P and initiates phagosome formation. **b** Two conjugation systems, Atg12–Atg5-Atg16L and LC3–PE, facilitate phagophore membrane elongation and autophagosome maturation. **c** NAD-dependent deacetylase Sirt1 catalyzes deacetylation of Vps34, Beclin1, Atg5, Atg7, and LC3 to promote autophagy. **d** Transcription factors TFEB and FoxO translocate to the nuclear to induce autophagy-related gene expression. Their transcription activity can be inhibited by phosphorylation caused by mTOR or AKT, respectively. Phosphorylated TFEB is digested by proteasome; ZKSCAN3 repress autophagy-related gene transcription.

of TFEB. mTOR and MAPK are extracellular signaling kinases that catalyze TFEB phosphorylation and restrain its nuclear translocation, inhibiting its activity (Fig. 1d) [25, 27, 28]. Our studies show that TFEB activity could also be regulated by proteasome-mediated degradation of phosphorylated TFEB [29, 30]. In contrast to FoxOs and TFEB, the transcriptional factor ZKSCAN3, which controls cell proliferation [31], has recently been identified to act as a repressor to a wide range of Atg genes. Knockdown of ZKSCAN3 has been shown to induce autophagy and lysosome biogenesis (Fig. 1d) [32].

Autophagy and the Elimination of Pulmonary Pathogens

In addition to its role in maintaining cellular homeostasis, autophagy is actively engaged in cellular host defense by reducing the pathogen burden [33]. *Mycobacterium tuberculosis* bacterium is a notorious pulmonary pathogen. *M. tuberculosis* primarily infects alveolar phagocytic cells, in which it resides and multiplies within the host-derived phagosomes [34]. *M. tuberculosis* developed strategies to escape phagocytotic clearance by preventing phagosome-lysosome fusion [35], disrupting vacuolar H-ATPase recruitment and phagosome acidification [36, 37], and inhibiting PI3P-dependent membrane trafficking [38, 39]. *M. tuberculosis* suppress the apoptosis of infected macrophages and trigger necrosis that results in the spreading of more bacteria to infect adjacent cells [40, 41]. Multiple studies have shown that autophagy plays an important role in innate defense against *M. tuberculosis* infection [42–45]. Stimulation of autophagy by starvation, rapamycin, IFN-γ [4], ATP [46], or lipopolysaccharides (LPS) [44, 47] promoted the transfer of intracellular mycobacteria to lysosomes to be killed. Furthermore, vitamin D, which was used to treat tuberculosis in the preantibiotic era, has been shown to exert anti-*M. tuberculosis* effects by stimulating autophagy through induction of the antibacterial peptide, cathelicidin [48–50]. Antituberculosis drugs such as isoniazid and pyrazinamide have been shown to be partly dependent on autophagy activation, because in autophagy-defective cells antibiotic treatment was less effective against mycobacteria [51]. Additionally, autophagy deficiency also indirectly affects *M. tuberculosis* infection by enhancing macrophage uptake of mycobacteria through upregulation of scavenger receptor expression [52] and inhibiting antigen presentation [5, 47]. Some *M. tuberculosis* virulence factors facilitate intracellular bacterial survival by autophagy inhibition. *M. tuberculosis* secrete phosphatase SapM [39] and mycobacterial cell wall glycolipid mannose-capped lipoarabinomannan ManLAM [38], which are major virulence factors that can cause mycobacterial phagosome maturation arrest by interfering with the PI3K/PI3P pathway and suppressing PI3P production. Recent evidence indicates that SapM blocks autophagosome-lysosome fusing by binding with GAPase RAB7 [53]. ManLAM was also found to suppress autophagosome formation [54]. Another mycobacterial secreted protein is Eis, which is an N-acetyltransferase that enhances the survival of mycobacteria in human monocytic cells. It suppresses autophagy by acetylating JNK-specific phosphatase MKP-7 to inhibit JNK-dependent autophagy efflux initiation [55]. The *M. tuberculosis* ESX-1 secretion system [56] was found to inhibit autophagic flux by blocking autophagosome-lysosome fusion in human dendritic cells [57]. A recent study of *M. tuberculosis* virulence-related *PE_PGR* genes revealed that the PE_PGRS47 protein actively suppresses autophagy by blocking mycobacterial phagosome acidification and phagolysosomal fusion. By such inhibition, PE_PGRS47 restricts MHC class II antigen presentation in dendritic cells (Fig. 2a) [58].

Legionella pneumophila is a common pulmonary pathogen infecting human lung alveolar macrophages and causing pneumonia [59]. It evades the immune response by residing in a special vacuole formed from the endoplasmic reticulum membrane and by inhibiting lysosome fusion [60, 61]. Autophagy was shown to be critical for *L. pneumophila* elimination in an in vitro study showing that knockdown of Atg5 in mouse macrophages enhanced bacterial replication [62]. Furthermore, in vivo studies using the Atg9 mutant *Dictyostelium discoideum* showed a critical defect in the clearance of *L. pneumophila* [63]. Legionella developed strategies to counter cellular autophagy elimination. The Legionella I Dot/Icm type IV secretion system secretes RavZ, a cystine protease, and delipidates the LC3, blocking its membrane conjugation [64]. Another effector protein, LpSpl, acts as sphingosine-1 phosphate lyase, decreasing host cell sphingolipid levels to inhibit autophagosome formation (Fig. 2b) [65]. A common pulmonary virus pathogen, Influenza virus A, induces autophagy but blocks the autophagosome-lysosome fusion by the viral Matrix 2 (M2) ion-channel protein [66]; thus, the virus adapts the multifunctional autophagosomes to reproduce the virus components and replicate (Fig. 2c) [66, 67]. Consistent with the role of autophagy in host defense, recent studies have addressed the augmentation of autophagy as a method to enhance the clearance of pathogens including *Pseudomonas aeruginosa* [68] and *Burkholderia cenocepacia* [69].

Autophagy and Lung Inflammation

Recent studies have found that autophagy is a negative regulator of inflammation in general, and of NLRP3 inflammasome in particular. The inflammasome is a multiprotein complex responsible for caspase-1 activation. Activation of caspase-1 leads to the release of the active form of potent inflammatory cytokines, including IL-1β

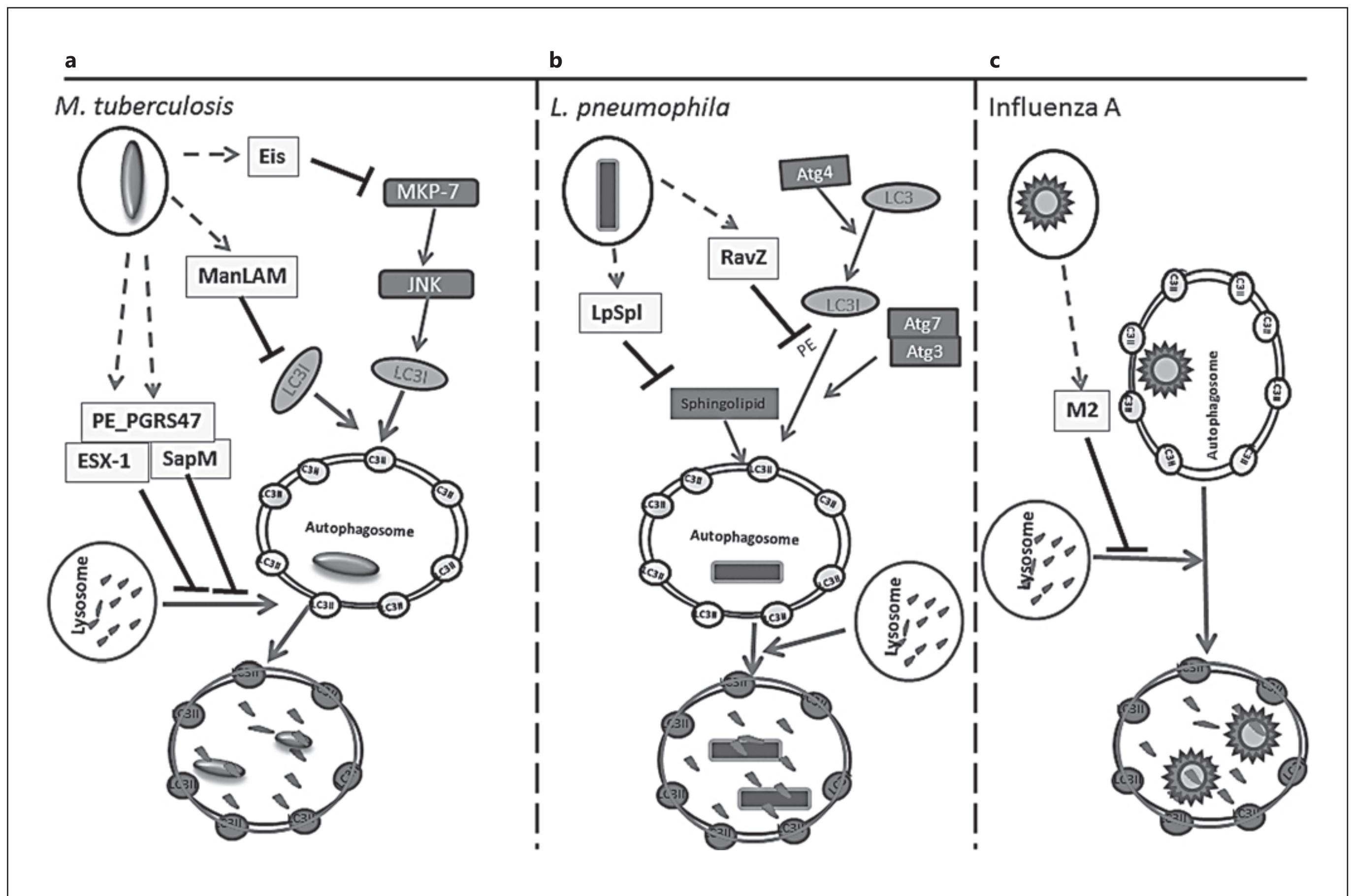

Fig. 2. Strategies used by pulmonary pathogens to avoid host autophagy. **a** *M. tuberculosis* has five identified anti-autophagy factors. *M. tuberculosis* secretion system Esx-1, the secreted phosphatase SapM, and the virulence protein PE_PGRS47 inhibit autophagy by blocking autophagosome lysosome fusing. Eis is an N-acetyltransferase. It acetylates JNK-specific phosphatase MKP-7 to initiate the inhibition of JNK-dependent autophagy. Mannose-capped lipoarabinomannan ManLAM interferes with trafficking proteins in autophagy and also affects LC3 protein expression levels and inhibits accumulation of autophagic vacuoles. **b** RavZ, the bacterial effector protein of *L. pneumophila*, inhibits autophagy by hydrolyzing the release of LC3II phosphatidylethanolamine at the carboxyl-terminal glycine residue. The hydrolyzed LC3 cannot be reconjugated by Atg7 and Atg3 to form mature LC3II for autophagosome localization. LpSpl with sphingosine-1 phosphate lyase activity reduces sphingolipid levels, reducing autophagosome formation. **c** Influenza A's transmembrane protein M2, which arrests autophagosome degradation by blocking autophagosome and lysosome fusion.

and IL-18, by proteolytic cleavage. Macrophages from Atg16L1-deficient mice produced exaggerated quantities of IL-1β and IL-18 in response to LPS [70]. Depletion of other autophagic proteins such as Atg7, LC3B, or Beclin 1, or treatment with autophagy inhibitors wortmannin or 3-methyladenine, enhanced the production of IL-1β and IL-18 by macrophages [70, 71]. These studies indicated that autophagy deficiency is associated with increased inflammasome activity. Furthermore, autophagy deficiency in myeloid-derived cells was shown to cause spontaneous pulmonary inflammation in two independent studies [72, 73]. In both of these studies, mice lacking either Atg5 or Atg7 in myeloid cells spontaneously developed lung inflammation characterized by enhanced recruitment of inflammatory cells into the lung, increased levels of pro-inflammatory cytokines, submucosal thickening, goblet cell metaplasia, and increased collagen content [72, 73]. Following LPS challenge, these autophagy-deficient mice had higher levels of pro-inflammatory cytokines in serum and in bronchoalveolar lavage, severe pulmonary inflammation, as well as increased mortality compared to wild-type mice [72, 73]. In addition, mice lacking Atg5 or Atg7

in myeloid cells were more susceptible to bleomycin and silica challenge [74]. Spontaneous lung inflammation was also found in mice with Atg5 deletion in dendritic cells [75]. Knockout of other autophagy-related genes such as Atg14, Fip200, or Epg5 in myeloid cells led to sterile lung inflammation, thus confirming the essential role of autophagy in lung homeostasis, which is not specific to a particular autophagy-related gene [76].

During active infection, autophagy also functions to prevent extensive inflammation [56, 76, 77]. In vivo studies of *M. tuberculosis* infection showed that, compared to wild-type, mice with myeloid cell-specific Atg5 knockout had a higher bacterial burden, severe necrotic lung lesions, elevated levels of IL-17 and IL-1α, and higher mortality. These studies suggest that in addition to suppressing *M. tuberculosis* growth, autophagy in myeloid-derived cells is responsible for controlling damaging inflammation [56, 77]. A recent study showed that the loss of Atg5 in polymorphonuclear cells causes excessive inflammation and predisposes to *M. tuberculosis* infection. This study suggested that the role of Atg5 in *M. tuberculosis* inhibition could be at least partially independent of autophagy [78].

Autophagy Regulates Inflammasome Activity

The innate immune system is an important component that acts as an initial barrier to protect against microbial pathogens or damaging agents. Cross-talk between autophagy and the innate immune system balances protection of the host against an exaggerated immune response, while enabling the neutralization of infectious and damaging threats. This is crucial at sites such as the lung, skin, and colon, where the host is continuously exposed to potential hazardous elements, such as inhaled toxins, toxic food products, as well as chemicals and commensal and pathogenic bacteria. The innate immune system is able to recognize and orchestrate a protective inflammatory response against harmful insults. Such responses should be tightly controlled to prevent exaggerated damage to the host.

The innate immune system relies on a group of pattern-recognition receptors that include toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), and absent in melanoma (AIM2)-like receptors (ALRs). Both NLRs and ALRs can form a cytoplasmic multiprotein complex called the inflammasome upon sensing a wide variety of ligands. Inflammasome assembly involves the adapter protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) which recruits caspase-1. Activation of caspase-1 leads to the release of the active form of IL-1β and IL-18 by proteolytic cleavage and can also lead to a form of cell death called pyroptosis [79]. The most widely studied inflammasome is NLRP3. Its activation depends on two steps. In the first step, pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are recognized by TLRs to activate NF-κB signaling-dependent expression of the inflammasome components and pro-cytokines (Fig. 3a) [47, 80]. In the second step, specific stimuli trigger inflammasome complex assembly and the inflammasome processes the pro-cytokine to generate mature cytokines by active caspases (Fig. 3b) [81, 82]. Many forms of such stimuli have been discovered, such as DAMP/PAMP-induced mitochondria damage [81, 82]. A recent study revealed that newly synthesized mitochondrial DNA may act as an NLRP3 ligand and directly associate with the NLRP3 inflammasome complex, thereby promoting its activity [80]. There have been several mechanisms proposed for how autophagy deficiency can lead to inflammasome activation. Accumulation of damaged mitochondria, leading to the release of reactive oxygen species (ROS) and/or mitochondrial DNA, and failure to degrade misfolded proteins have been proposed as mechanisms for inflammasome activation in autophagy-deficient cells [71, 83]. Autophagy has also been suggested to suppress inflammasome activation by directly digesting inflammasome components such as ubiquitinated ASC (Fig. 3c) [84]. Furthermore, autophagy was found to directly sequester pro-cytokines such as pro-IL-1β for digestion to reduce mature cytokine production (Fig. 3c) [85]. Mice with Atg7 deficiency in myeloid cells developed spontaneous lung inflammation that was mostly mediated by IL-18. Neutralization of IL-18, but not IL-1β or IL-17, attenuated lung inflammation in these mice. In contrast, increased mortality in response to endotoxin was caused by increased IL-1β [62]. In addition to the effect of autophagy on inflammasome-associated cytokines, several studies have suggested an effect of autophagy or autophagic proteins on cytokines that are not associated with inflammasome activation. In mice, Atg5-deficient macrophages produced more pro-inflammatory cytokine IL-1α in an inflammasome-independent way [77]. In Influenza A virus infection, excessive immune responses, including increased neutrophil and macrophage infiltration, contribute to lung injury and pathology more than the effects of viral replication [86].

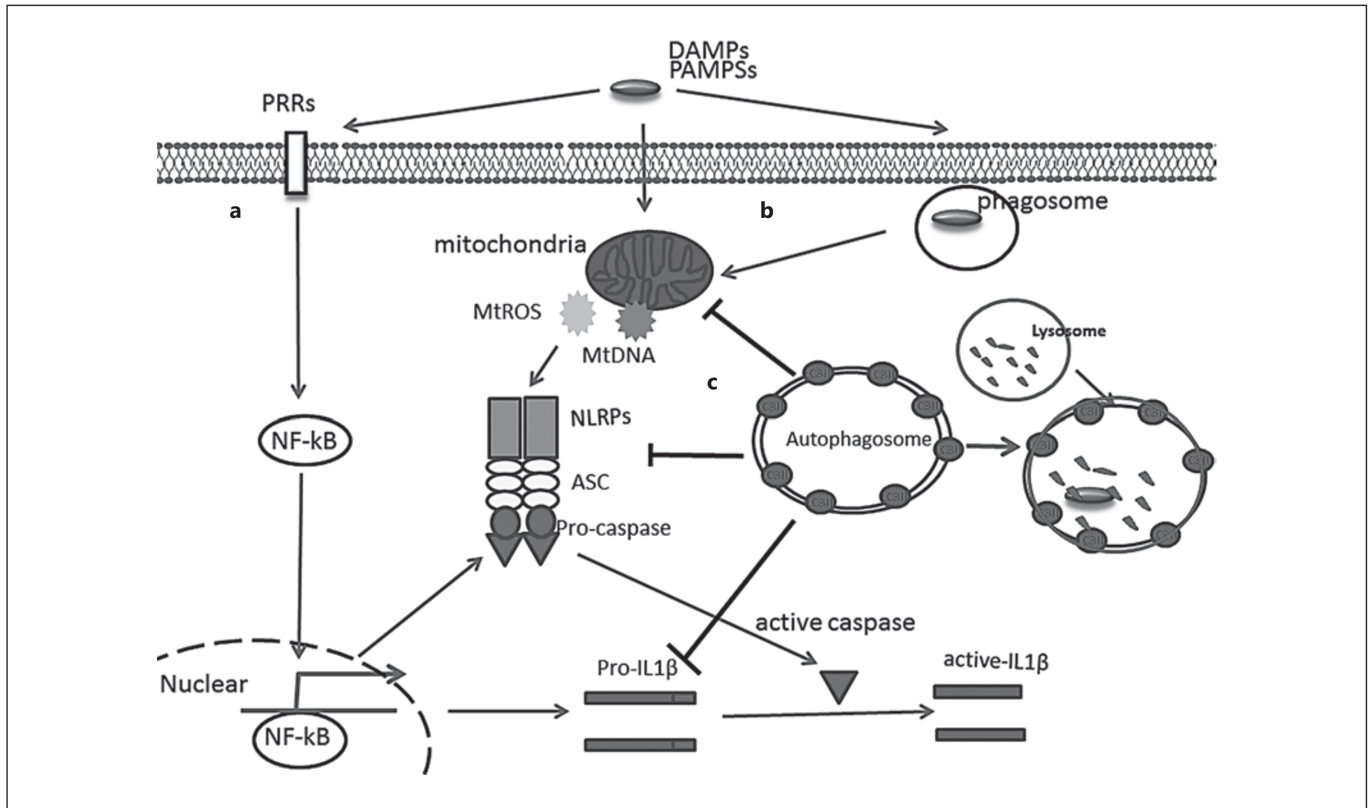

Fig. 3. Autophagy in host defense and inflammasome regulation. **a** PAMPs or DAMPs, recognized by pattern-recognition receptors, result in NF-κB activation. Active NK-κB promotes inflammasome components and cytokine expression. **b** PAMPs or DAMPs cause mitochondrial damage and the release of mitochondrial ROS and DNA, triggering the assembling of NLRP3, ASC, and Pro-caspase into active inflammasome. Caspase-1 is activated by autocleavage and then cleaves the pro-inflammatory cytokines IL-1β into active cytokines. Bacteria-containing phagosome membrane disruption leads to the release of PAMPs. **c** Activated autophagosomes can engulf damaged mitochondria, NLRP3, ASC, and Pro-caspase, and target them to lysosomal degradation, reducing the production and secretion of active cytokines.

Conclusion and Future Prospects

Autophagy is an important intracellular recycling system with diverse functions implicated in multiple cellular signaling pathways. Autophagy is regulated at the transcriptional, translational, and posttranslational levels. Phosphorylation and de-phosphorylation on some key proteins in the initiation complexes has been found to be a major mechanism of autophagy regulation [18]. Recent studies revealed that acetylation could modify autophagy proteins and influence the autophagy cascade [21]. Further elucidation of these regulatory mechanisms could provide potential therapeutic targets in diseases in which autophagy modulation is desired.

Since the discovery of autophagy's involvement in the innate immune response, extensive in vitro and in vivo studies have shown that autophagy plays an important role in lung homeostasis and tissue protection [68, 72, 73, 77]. During host infection, autophagy eliminates pathogens by mediating pathogen autolysosomal killing and facilitating antimicrobial antigen presentation [5, 77, 87]. In addition to pathogen elimination, autophagy tames the host inflammatory response by negative regulation of inflammasome activity. Multiple studies have shown that the induction of autophagy can have beneficial effects in combating infections, suggesting that promoting autophagy may be a beneficial strategy to control lung infection [43, 44]. However, some pathogens have evolved adaptive strategies to resist autophagy elimination, potentially limiting the impact of autophagy in immune defense. Some of these anti-autophagy strategies employed by pathogens include anti-autophagy factors

such as SapM, ManLAM [54, 88], Eis [55], PE_PGRS47 [58], and ESX-1 from *M. tuberculosis* [57], RavZ and LpSpl from *L. pneumophila* [64, 65], and M2 ion-channel protein from Influenza A [67] (Fig. 2). These virulence factors contribute to the drug resistance of those pathogens by enhancing pathogen survival in spite of host autophagy. Thus, developing drugs that inactivate pathogen virulence factors involved in autophagy avoidance may represent the next generation of anti-microbial agents.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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