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## Review

# Autophagy-mediated regulation of neutrophils and clinical applications

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## Abstract

Autophagy, an adaptive catabolic process, plays a cytoprotective role in enabling cellular homeostasis in the innate and adaptive immune systems. Neutrophils, the most abundant immune cells in circulation, are professional killers that orchestrate a series of events during acute inflammation. The recent literature indicates that autophagy has important roles in regulating neutrophil functions, including differentiation, degranulation, metabolism and neutrophil extracellular trap formation, that dictate neutrophil fate. It is also becoming increasingly clear that autophagy regulation is critical for neutrophils to exert their immunological activity. However, evidence regarding the systematic communication between neutrophils and autophagy is insufficient. Here, we provide an updated overview of the function of autophagy as a regulator of neutrophils and discuss its clinical relevance to provide novel insight into potentially relevant treatment strategies.

**Key words:** Autophagy, Neutrophil, Innate immunity, NETs, Clinical application, Neutrophil extracellular trap

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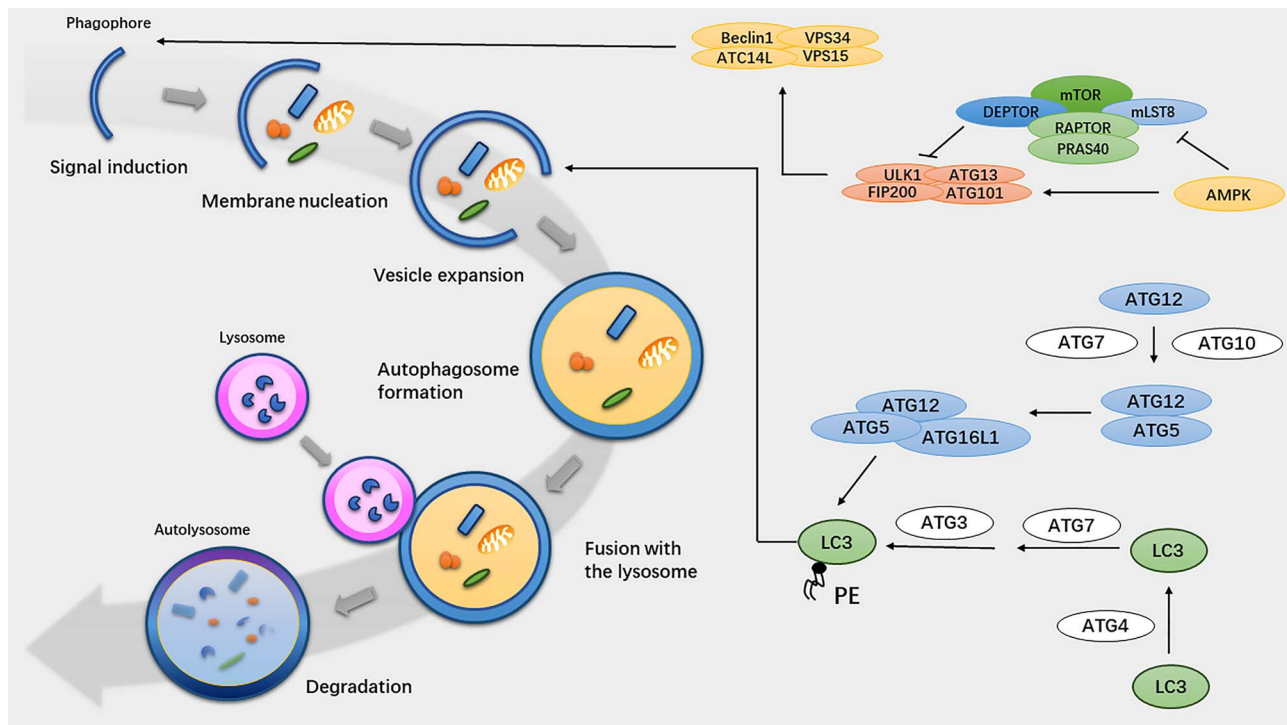
## Background

Neutrophils, the most abundant category of polymorphonuclear leukocytes in circulation, are professional killers during acute inflammation [1]. Neutrophils patrol the blood continuously and recruit cells to sites of inflammation at the onset of injury [2]. Activated neutrophils use multiple strategies, including phagocytosis, autophagy, degranulation, reactive oxygen species (ROS) release and neutrophil extracellular trap (NET) formation, to destroy infectious threats [3]. They modulate the innate immune response and have prolonged activity in ongoing inflammation in numerous diseases. Lately, neutrophils have come under increased scrutiny due to surprising findings regarding their heterogeneity and plasticity [4] and their abilities to be long-lived and regulate adaptive immune responses [5].

Autophagy, interpreted as self-eating from the Greek words *auto*, meaning “self”, and *phagein*, meaning “to eat”, is an evolutionarily conserved degradative process [6] that follows four major routes: macroautophagy, microautophagy, chaperone-mediated autophagy [7], and non-canonical autophagy (8). Macroautophagy (hereafter

referred to as autophagy) utilizes autophagosomes, which are double-membrane vesicles, to isolate portions of the cytoplasm, such as those containing harmful and unwanted material, and deliver them to lysosomes for degradation [9]. Autophagy is defined as non-selective or selective; selective autophagy targets various cargoes for degradation and includes organelle-specific autophagy (such as mitophagy, pexophagy, and reticulophagy) and xenophagy (the degradation of microorganisms) [10]. Macroautophagy, the best-characterized form of autophagy, is the major route of autophagy in neutrophils and will hereafter be referred to simply as autophagy. Autophagy is essential to maintain host health and helps the capture and clearance of invading pathogens by the immune system.

The cognition of the immunological functions of neutrophil autophagy has increased remarkably in recent years, and it is now appreciated that the primordial functions of autophagy have evolved and been incorporated into multiple innate and adaptive immune pathways [11]. Accumulating evidence indicates that



**Figure 1.** Overview of the autophagy pathway. Autophagy proceeds through several steps: signal induction, membrane nucleation, cargo targeting, vesicle expansion, autophagosome formation, fusion with the lysosome, cargo degradation, and nutrient recycling. Key proteins that regulate autophagy in mammals are shown on the right. Autophagosome activation is mediated by three initial signaling complexes: the ULK1, PI3KC3, and ATG16L1 complexes. Autophagosome elongation is regulated by two ubiquitin-like conjugate systems: the ATG12-ATG5-ATG16L1 and LC3-PE complexes. *AMPK* AMP-activated protein kinase, *mTOR* mammalian target of rapamycin, *LC3* microtubule-associated protein 1 light chain 3, *PRAS40*, 40 kDa Pro-rich AKT substrate, *RAPTOR* regulatory-associated protein of mTOR, *AMP* adenosine-triphosphate, *VPS* vacuolar protein sorting, *ULK1* unc-51 like autophagy activating kinase 1, *ATG* autophagy-related protein, *FIP200* family kinase-interacting protein of 200 kDa, *PE* phosphatidylethanolamine

autophagy has important roles in regulating neutrophil functions, including degranulation, metabolism, and NET formation. However, knowledge of the systematic relationship between neutrophils and autophagy is insufficient. In this review, we summarize the updated function of autophagy as a regulator of neutrophils and then focus on its clinical relevance to provide a novel therapeutic strategy.

## Review

### Mechanisms of autophagy

Autophagy, an adaptive catabolic process, plays a cytoprotective role to enable cellular homeostasis [12]. Neutrophil autophagy occurs at a basal level in nutrient-rich conditions, but can be markedly induced in response to many cellular stresses, including starvation, endoplasmic reticulum (ER) stress, oxidative stress, and exposure to certain chemicals, radiation, and hypoxia [10, 13, 14]. In the innate immune response, neutrophils utilize autophagy as an antimicrobial effector function, which can be regulated in the process of a memory response [15].

**Steps in the autophagic pathway and autophagy-related proteins**  
Neutrophil autophagy involves the following pivotal steps: signal induction, membrane nucleation, cargo targeting, vesicle expansion, autophagosome formation, fusion with the lysosome, cargo degradation, and nutrient recycling [16] (Fig. 1).

Autophagosome formation is the key event in the autophagy pathway. The autophagosome is activated by three initial signaling complexes: the serine/threonine protein kinase unc-51-like autophagy-

activating kinase 1 (ULK1) complex (comprising ULK1, a focal adhesion kinase (FAK) family kinase-interacting protein of 200 kDa (FIP200, also known as RB1CC1), autophagy-related protein (ATG) 13 and ATG101, [17–19]); the phosphoinositide 3-kinase catalytic subunit type III (PI3KC3) complex (comprising Beclin-1, vacuolar protein sorting (VPS) 34, VPS15 and ATG14L [20]); and the ATG16L1 complex (comprising ATG16L1, ATG5, and ATG12, [21]). The serine/threonine kinase mammalian target of rapamycin complex 1 (mTORC1) is phosphorylated under conditions of nutrient excess, whereas nutrient deprivation and immune signaling activation lead to the inhibition of mTORC1 phosphorylation and the activation of the Beclin-1-VPS34 complex by ULK1 complex formation [22, 23].

During the phagophore membrane nucleation step, phosphatidylinositol-3-phosphate (PtdIns3P)-rich regions are produced by the lipid kinase VPS34 and recruited to the surface of donor membranes, including the ER, Golgi apparatus, ER-mitochondria contact sites, endosomes, and plasma membrane [24–28]. The ULK1 complex recruits Beclin-1, ATG14L, and phosphoinositide 3-kinase regulatory subunit 4 (PIK3R4), along with the phagophore, to the phagophore assembly site (PAS). PtdIns3P is recognized by PtdIns3P-binding factor WD repeat (WDR) domain phosphoinositide-interacting protein 1 (WIPI1)-WIPI4 [29]. ATG9 plays a role in PAS formation and expansion by momentarily interacting with omegasomes [30, 31].

Autophagosome elongation is regulated by two ubiquitin-like conjugate systems: the ATG12-ATG5-ATG16L1 and microtubule-associated protein 1 light chain 3-phosphatidylethanolamine (LC3-PE) complexes. ATG12-ATG5 conjugation is activated by ATG7 and

ATG10 [32]. The ATG16L complex is generated subsequently and non-covalently binds to the ATG5–ATG12 conjugate [21]. In parallel, LC3 is cleaved at its C-terminal arginine by the ATG4 protease to expose a glycine residue [33]. There are seven mammalian orthologs of ATG8 (LC3A, microtubule-associated protein 1 light chain 3 beta (LC3B), LC3C, GABA Type A Receptor Associated Protein (GABARAP), GABA Type A Receptor Associated Protein Like 1 (GABARAPL1), GABARAPL2, and GABARAPL3; these are referred to collectively as LC3 in this review) [34]. The ATG16L1 complex conjugates LC3 to phosphatidylethanolamine (PE) to form the second ubiquitin-like conjugate system [35], which involves ATG7 and ATG3 [36]. LC3 regulates membrane tethering and fusion and recruits membranes to extend the isolation membrane and form autophagosomes; thus, LC3 is widely used as a microscopic detection marker [34, 37].

During the final maturation step, autophagosomes become degradative autolysosomes. The small guanosine triphosphatase (GTPase) Ras-related protein 7 (RAB7, [38]), the autophagosomal SNARE protein syntaxin 17 [39] and the lysosomal SNARE vesicle-associated membrane protein 8 (VAMP8), as well as lysosomal membrane proteins such as lysosomal-associated membrane glycoprotein 2 (LAMP2) [40, 41], are required for autophagosome and lysosome fusion. Lysosomes break the inner autophagosomal membrane, and lysosomal hydrolases regulate the degradation of autophagosome cargo.

**Regulation of the autophagy pathway** Autophagy is an intricate physiological mechanism that can be regulated by numerous molecules and pathways. Nutrient signaling mediates autophagy progression by targeting mTOR [42]. The Class I phosphatidylinositol-3-kinase (PI3K)–also known as Protein Kinase B (AKT) pathway negatively regulates autophagy by activating mTOR [43]. Adenosine-triphosphate (AMP)-activated protein kinase (AMPK) inhibits mTOR and phosphorylates ULK1 to promote autophagy [6]. mTOR regulates the activation of autophagy. The Beclin-1-interacting complex also mediates autophagy progression, and upregulating PI3P promotes autophagosomal membrane nucleation [44]. Other interacting factors, such as ATG9, may also regulate this process [34]. Autophagosome elongation is mainly mediated by the ATG12–ATG5–ATG16L1 and LC3–PE complexes [45].

Therefore, neutrophil autophagy is an intricate catabolic process that involves the following pivotal steps: signal induction, membrane nucleation, cargo targeting, vesicle expansion, autophagosome formation, fusion with the lysosome, cargo degradation, and nutrient recycling. Several molecules and pathways are involved in regulating autophagy progression.

### Autophagy-mediated regulation of neutrophil differentiation

Neutrophil differentiation is dependent on extensive cytoplasmic and nuclear remodeling. Every second, approximately  $1 \times 10^6$  neutrophils are generated in the bone marrow of humans. Exquisite regulation is required for granulopoiesis. Multipotent progenitors (MPPs) give rise to granulocyte–monocyte progenitors (GMPs) and myeloblasts (MBs), which are considered the initial granulocyte precursors [46]. Through the promyelocyte (MC), metamyelocyte (MM), and band cell (BC) stages, MBs differentiate into mature polymorphonuclear neutrophils (PMNs) [4, 47]. Autophagy was shown to be involved in neutrophil differentiation and proliferation in bone marrow and lymphoid organs in a mouse model and to have different effects on various stages of differentiation [48, 49].

During early granulopoiesis, autophagy is essential for normal and induced neutrophil differentiation by governing these energy-consuming changes. Neutrophil differentiation depends on free fatty acids generated by autophagy, and these free fatty acids support mitochondrial respiration. Autophagy-deficient (ATG7 knockout at the GMP–MB stage) neutrophil precursors show excessive glycolysis, lipid droplet accumulation, and adenosine-triphosphate (ATP) depletion but no change in glycolytic activity [47]. However, autophagy controls the earliest precursor stage, and defects at the hematopoietic stem cell (HSC) level (Lyz2-Cre-mediated ATG5 deletion) cause the rapid production of granulocyte precursors, which do not show morphological changes or functional deficiencies [49].

Autophagy is important for differentiation in response to specific stimulants, including granulocyte colony-stimulating factor (G-CSF) and all-*trans* retinoic acid (ATRA). G-CSF induces autophagy in both neutrophils and HSCs in human and mouse models. Autophagy protects neutrophils from cytokine-induced stress to prolong their life span and counteract neutropenia after cytotoxic chemotherapy. Autophagy ensures HSC survival for transplantation [50]. Beclin-1-independent autophagy is positively correlated with ATRA-induced neutrophil differentiation [51]. The expression of ATG3, ATG4D, ATG5, and WIPI1 is increased by the Ets family hematopoietic transcription factor PU.1, which is important for neutrophil differentiation [52, 53]. PU.1 regulates autophagy via microtubule-associated protein 1S (MAP1S, also known as C19ORF5), which plays a role in microtubule dynamics [54]. Damage-regulated autophagy modulator-1 (DRAM-1) is involved in ATRA-induced neutrophil differentiation [55].

In general, autophagy has different effects on the different stages of normal neutrophil differentiation. During early granulopoiesis, autophagy is essential for neutrophil differentiation because it governs energy-consuming changes. At the HSC level, autophagy controls the earliest precursor stage. Autophagy is important for differentiation in response to specific stimulants, including G-CSF and ATRA.

### Autophagy-mediated regulation of neutrophil lifespan

Neutrophils, which are terminally differentiated cells in the innate immune system, have a short life span. In the absence of activation, neutrophils are constitutively committed to apoptosis. Autophagy is considered a “double-edged sword” that controls the fate of neutrophils. Autophagy can help cells survive by detecting oxidative stress and eliminating damaged cellular components [56]; however, it also jeopardizes cell survival and accelerates cell death. Here, we elaborate on the effect of autophagy on apoptotic and neutrophil-derived giant phagocytes (*G $\phi$* ) to explore the mystery between autophagy and neutrophil lifespan.

**Autophagy and apoptosis** Autophagy and apoptosis, two classical pathways of programmed cell death, exhibit complex interactions. Apoptosis can suppress autophagy for digesting crucial autophagy-related proteins [57, 58], and autophagy can both promote and suppress apoptosis. On the one hand, autophagy can prevent neutrophils from undergoing intrinsic apoptotic by multiple mechanisms, such as mitophagy and the degradation of pro-apoptotic proteins. Autophagy can suppress ER stress and help neutrophils survive apoptotic stimuli [59]. A reduced autophagy capacity results in a compensatory increase in apoptosis [60]. 3-Methyladenine (MA) and chloroquine (CQ), two autophagy inhibitors, markedly promote spontaneous neutrophil apoptosis by downregulating Mcl-1 without affecting B-cell lymphoma-XL (BCL-XL) [61]. On the other

hand, autophagy can facilitate apoptosis. Caspase 8 can activate autophagosomes by serving as a platform [60]. Caspase 3 and poly ADP-ribose polymerase (PARP) can be activated by autophagy via a Fas-associated exogenous pathway. Autophagy also degrades endogenous inhibitors of apoptosis. Although autophagy has pro-apoptotic activity, this activity is relatively weaker than its anti-apoptotic activity. Ultimately, autophagy has an inhibitory effect on apoptosis.

Many molecules, such as ATG5, BCL-2, and p53, simultaneously modulate autophagy and apoptosis [62]. ATG5 and BCL-2 might be the key regulators. ATG5, an important regulator of autophagy, can translocate from the cytoplasm to mitochondria and activate caspases. The proteolytic cleavage of ATG5 weakens autophagy and induces apoptosis. In mice, BCL-2, an anti-apoptotic protein, inhibits pro-apoptotic members of the BCL-2 family and inhibits Beclin-1 to interfere with autophagy. High BCL-2 levels can abolish the pro-apoptotic function of truncated ATG5 [63].

**Autophagy and neutrophil-derived G $\phi$**  Autophagy is involved in the development of the long-living subset of neutrophils, neutrophil-derived G $\phi$ , which are positive for CD15, CD66b, CD63, CD11b, myeloperoxidase (MPO), and neutrophil elastase (NE) [64]. Autophagy is a basic characteristic of these cells and largely controls their formation. G $\phi$  act as cellular “cannibals” to eliminate dead neutrophils and debris. The phagocytic centers of G $\phi$  include LC3B-coated vacuoles and microtubule-associated protein 1 light chain beta (LCB) aggregates. LC3B accumulation increases during oxidized LDL (oxLDL) internalization. Treatment with 3-methyladenine (3MA) and bafilomycin A1 (BafA1), two specific autophagy inhibitors, arrests the development of G $\phi$  [65].

In short, autophagy should be considered an important mechanism that prolongs the lifespan of cells. Autophagy exhibits bidirectional regulation of apoptosis, but in general, it inhibits apoptosis. Autophagy facilitates the development of long-living neutrophil subsets, such as neutrophil-derived G $\phi$ .

### Autophagy-mediated regulation of neutrophil degranulation

Autophagy regulates neutrophil-mediated inflammation and affects neutrophil degranulation and ROS production by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase nitrogen oxide (NOX) [66]. There are more than four types of granules in neutrophils [67]. Primary and tertiary granules originate from the ER–Golgi network. Primary, or azurophilic, granules store MPO,  $\beta$ -glucuronidase, elastase, and other antimicrobial factors. Tertiary (gelatinase) granules contain matrix metalloprotease-9 (MMP-9). Secondary (specific) granules originate from both the ER–Golgi and endocytosis and contain lactoferrin and MMP-9. These granules express significant molecules, including CD11b/CD18 and complement receptors, that are key for neutrophil migration and activation on the membrane; another such important protein in secondary granules is flavocytochrome b558, a component of the NOX machinery [68]. The fusion of secondary granules can produce ROS by activating the NOX complex. Autophagy deficiency disrupts neutrophil degranulation, especially that of secondary and tertiary granules. Knockout of ATG5 or ATG7 inhibits degranulation, indicating that this event likely occurs through autophagy, not an ATG-independent mechanism. Granule fusion helps induce NOX-mediated ROS generation, and ROS are vital for neutrophil apoptosis. Under inflammatory conditions, ER stress can induce neutrophil autophagy, and autophagy can inhibit ER stress [59].

Autophagy downregulates ROS and increases neutrophil circulation to control inflammation.

### Autophagy-mediated regulation of NETs

NETs are an important neutrophil antimicrobial effector in response to various stimuli [69]. Classical NET release occurs in a complex series of orchestrated events regulated in five stages, including ROS generation, peptidyl arginine deiminase-4 activation, distinct vesicle formation, chromatin decondensation and active extrusion of the DNA/histone/cathelicidin antimicrobial peptide cocktail into the extracellular space [70]. Classical autophagy plays a multilayer regulatory role in NET formation. Autophagy participates in pathogen elimination as an alternative degradative platform to phagosomes with lysosomes [71]. Autophagy is involved in the externalization of membrane-bound and cytosolic proteins, thereby influencing stage 3 of NET vacuolization [60, 72]. Autophagy may also play a role in inhibiting the respiratory burst, blocking cytoskeletal dynamics and chromatin decondensation and inducing histone citrullination in stages 1, 4 and 5 of NET formation [73–75]. However, chaperone-mediated autophagy (CMA) has no effect on NET formation [76].

**Is autophagy the answer?** According to the classical theory, autophagy is indisputably involved in neutrophil NET formation. However, a controversial point of view was recently proposed: a key molecule in autophagy, rather than autophagy itself, is involved in the regulation of neutrophil NET formation. Is autophagy the answer? To answer this question, we analyzed all autophagy-related molecules involved in NET generation and outlined the potential mechanism in Table 1. We found no evidence contradicting the notion that PI3K–AKT–mTOR signaling affects NET formation in the early stage of autophagy. The focus of the debate is whether ATG5 is essential for NET formation and whether the later stage of autophagy has the same effect as the early stage. ATG5 levels increase when autophagy induces NET formation [43, 77]. ATG5-dependent autophagy is required for NET formation [60], which is inhibited by 3MA and wortmannin. However, neutrophils from ATG5-knockout mice show normal NET release. The late autophagy inhibitors BafA1 and CQ had no effect on normal NET formation [78]. From the perspective of signaling pathways, NET formation was not regulated by autophagy but rather by the PI3K–AKT–mTOR pathway, which interacts with signaling cascades initiated by triggers of NET formation. However, considering the metabolism of neutrophils, autophagy has a positive effect on NET formation. Thus, we deduced that autophagy affects NET formation, but this hypothesis has not been proven.

**mTOR, a bridge between autophagy and NETs** The PI3K–AKT–mTOR axis connects autophagy and NET induction and has a considerably influence on both processes. mTOR is a serine/threonine kinase that regulates cellular stress, growth, and autophagy. Inactivation of mTOR by dephosphorylation of Ser-2448 downregulates autophagy [42]. Inorganic polyphosphate induces NET formation, and interferon lambda-1 (IFN- $\lambda$ 1)/interleukin-29 (IL-29) treatment exerts the opposite effect by targeting mTOR. Rapamycin and WYE-354, autophagy inducers that inhibit mTOR, promote NET formation by autophagy downstream of formyl peptide receptor (FPR) signaling in human neutrophils [79, 80]. The increase in NET release is sensitive to the inhibition of respiratory burst and the blockade of cytoskeletal dynamics [74].

**Autophagy and ROS, dependent or independent factors?** Autophagy is dependent on ROS production by NOX. NOX2-generated



**Table 1.** Autophagy-related molecules involved in NET formation

Key molecule	Species	Model	Observed effect of autophagy on NET	Associated mechanism	Reference
mTOR	Human	Patients with ST segment elevation acute myocardial infarction (STEMI) and controls	NET formation was induced by the inhibition of mTOR and the induction of autophagy	The effect of polyP on autophagy and NET formation is dose-dependent	[42]
mTOR	Human	Neutrophils stimulated with ANCA-positive IgG	Autophagy promoted NET formation	ANCA-positive IgG-induced NET formation is enhanced by rapamycin and suppressed by 3MA	[79]
mTOR	Human	Patients with lupus nephritis	Autophagy affected NET formation and release	The mTOR inhibitors rapamycin and WYE-354 (which can induce autophagy) increase the number of SYTOX-positive neutrophils and the expression of NE	[80]
mTOR, Beclin-1	Human	Patients with systemic lupus erythematosus	NET formation activated neutrophils through autophagy	Pharmacologic inhibition of autophagy prevents intracellular chromatin decondensation, which is necessary for NETosis and NET formation	[81]
mTOR	Human	Neutrophils stimulated with fMLP	The mTOR pathway plays a pivotal role in NET formation by regulating autophagy downstream of FPR signaling	NET inducer PMA activates autophagy	[74]
ATG5, mTOR	Mouse	Aged mice, TLR2 KO mice and MyD88 KO mice	TLR2 ligand-induced NETosis in aged mice was compromised by an ATG5 defect, leading to a subsequent impairment of autophagy	Pharmacologic inhibition of mTOR accelerates NET release	[60]
mTOR	Mouse	Mincle <sup>-/-</sup> mice	Mincle regulated autophagy to control NET formation	Rapamycin initiates autophagy by inhibiting mTOR and increases PMA-induced NET formation	[82]
PI3K, ATG5	Human	Human neutrophils with the D39 strain of <i>Pneumococcus</i>	Extensive NET formation in neutrophils induced by pneumococci was critically dependent on autophagy	Pneumococci induce autophagy in neutrophils in a PI3K-dependent manner requiring ATG5	[43]
ATG5, Beclin-1	Human	Septic patients	Autophagy primed neutrophils for increased NET formation	In neutrophils, the expression of autophagy-related proteins, such as ATG5 and Beclin-1, increases during sepsis	[77]
ATG5	Mouse	ATG5-deficient mice	ATG5-dependent autophagy was not required for extracellular DNA trap formation. The late autophagy inhibitors BafA1 and CQ had no effect on NETs	3MA and wortmannin inhibit ET formation by blocking class I PI3Ks	[78]
ATG5, Beclin-1	Mouse	Mouse model of sepsis	Neutrophil autophagy enhanced survival in a mouse model of sepsis via increased NET formation	Neutrophil autophagy is not normally induced in septic mice	[77]
PI3K	Human	Neutrophils stimulated with CAH, CALY, or CAIY	Autophagy was involved in NET release induced by <i>C. albicans</i> morphotypes	Pre-treatment with WT, which suppresses autophagy through the persistent inhibition of class III PI3Ks, significantly reduces NET formation	[83]
PI3K	Human	Patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV)	Anti-LAMP-2 antibody-induced NET formation involved autophagy	LAMP-2 is a major constituent of the lysosomal membrane and plays a role in autophagy	[84]
PI3K	Human	Patients with gout	NET formation was associated with autophagy-related signaling in gout	PI3K inhibition prevents NET formation	[85]

Continued

Table 1. Continued

Key molecule	Species	Model	Observed effect of autophagy on NET	Associated mechanism	Reference
P13K	Human	Patients with CDG	NET-mediated cell death required both autophagy and superoxide generation	Autophagy prevents intracellular chromatin decondensation, which is essential for NETosis and NET formation	[86]
P13K	Caprine	Healthy caprine	The inhibition of PMN autophagy by blocking the P13K-mediated signaling pathway failed to influence tachyzoite-induced NETosis	Parasite-triggered NETosis is independent of NOX, SOCE, ERK1/2 and p38 MAPK activity	[87]
DDIT4/REDD1	Human	Active ulcerative colitis patient and control	NETs were positively regulated by autophagy, and the autophagy inhibitors BafA1 and HCQ abolished NET release	REDD1 is a key inducer of autophagy-mediated NETosis	[88]
V-ATPase	Human	Patients with <i>H. pylori</i> -positive gastritis	Clarithromycin upregulated autophagy to promote NET formation	PMNs pretreated with BafA1 prior to stimulation with clarithromycin show reduced NET formation	[89]
HMGB1/RAGE	Human	Patient with acute myocardial infarction	High-mobility group protein B1 (HMGB1) promoted autophagy and primed neutrophils for NET generation	Wortmannin and 3MA abrogate NET formation elicited by activated platelets or HMGB1	[90]
G6PD	Human	Taiwanese Hakka	Autophagy was involved in PMA-stimulated NET formation	ROS can elicit NETosis independent of autophagy	[91]
HMGB1	Human	Neutrophils in thrombosis	Tissue factor (TF) localized in autophagosomes prior to extracellular delivery via NETs	Autophagy functions as a secretory mechanism for the externalization of membrane-bound or cytosolic proteins in NETs	[72]
HMGB1	Human	Patients with Gram-negative sepsis	Autophagy mediated the delivery of TF to NETs in sepsis patients	Autophagy regulates the translocation of certain neutrophil proteins, including TF and HMGB1, to NETs	[92]
HMGB1, RAGE	Mouse	RAGE <sup>-/-</sup> mouse model	NETs were upregulated in pancreatic cancer through RAGE-dependent autophagy pathways	Circulating HMGB1 induces autophagy intrinsically and extrinsically by binding to RAGE	[93]
P140	Mouse	MRL/lpr mouse model	Neutrophil NET formation was not directly influenced by the CMA-targeting peptide P140	P140 does not influence NET formation, cytokine/chemokine production, or CMA in neutrophils	[76]
NFκB	Mouse	Gulo <sup>-/-</sup> mice	Autophagy was necessary for the induction of intracellular chromatin decondensation during PMA-induced NETosis	ATG3, ATG5, ATG6, ATG7, and ATG8 mRNA levels were significantly upregulated in PMNs from VItC-deficient mice	[73]

AAV associated vasculitis, ANCA antineutrophil cytoplasmic antibodies, CMA chaperone-mediated autophagy, HMGB1 high-mobility group protein B1, LAMP2 lysosomal-associated membrane glycoprotein 2, mTOR mammalian target of rapamycin, NETs, neutrophil extracellular traps, NFκB nuclear factor kappa-light-chain-enhancer of activated B cells, PMN polymorphonuclear neutrophil, REDD1 regulated in development and DNA damage response-1, STEMI ST-Elevation myocardial infarction, TF tissue factor, ATG autophagy-related protein, P13K phosphoinositide 3-kinase, RAGE receptor for advanced glycation end products, FPR formyl peptide receptor, CQ chloroquine, 3MA 3-methyladenine, NOX nitrogen oxide, ERK1/2, extracellular regulated protein kinases 1/2, ROS reactive oxygen species, PMN polymorphonuclear neutrophil, FADD Fas-associated death domain

ROS promote antibacterial autophagy, which recruits microtubule-associated proteins 1A/1B light chain 3 (LC3) to the phagosome via inhibiting the PI3K-I-Akt-mTOR signaling pathway [94]. ROS, and more specifically  $O_2^{2-}$ , stimulate cellular autophagy and accelerate intracellular pathogen clearance [81]. NETosis, a distinct form of cell death, requires autophagy and superoxide generation [95, 96]. Interestingly, both ROS and autophagy have independent effects on NET formation, and a lack of either axis results in apoptosis [74]. On the one hand, NET release depends on NOX2 activation by the Raf-MEK-ERK pathway [97]. 3MA cannot inhibit ROS-induced NET formation. On the other hand, as reported, Mincle regulates NET formation by mediating autophagy without affecting ROS [82]. *Candida albicans* hyphal (CAH) cells upregulate NET formation through autophagy in a ROS-independent manner in the early phase [83]. Inhibiting either autophagy or ROS results in impaired NET formation due to impeded intracellular chromatin decondensation [81, 86]; thus, we can target autophagy to regulate NET formation but preserve ROS production to maintain innate immune defense.

In summary, classical autophagy has a multilayer regulatory function in NET formation and a positive effect on NET formation, considering the metabolism of neutrophils and the kinetics of NET formation. The PI3K-AKT-mTOR axis connects autophagy and NET induction, and mTOR inhibition promotes NET formation via autophagy. Autophagy is dependent on ROS production by NOX but has an independent effect on NET formation.

### Autophagy-mediated regulation of neutrophils in bacterial infection

Neutrophils are considered the most effective and abundant front-line defenders, especially at the early phase of infection. Neutrophils play a significant role in pathogen clearance through multiple antimicrobial strategies, including the exocytosis-mediated release of antimicrobial molecules, phagocytosis, neutrophil NET formation, and autophagy. Autophagy is a significant pathway for the degradation of content trapped by phagocytosis. When pathogens escape phagosomes, autophagy becomes the only weapon available to neutrophils to capture the intracellular “escaped prisoner.” Autophagy plays a role in the detection and elimination of invasive pathogens such as intracellular bacteria, viruses, and parasites [98]. During autophagy, pathogens are engulfed by the autophagosome membrane and then degraded by lysosomes. Autophagic death can reduce intracellular pathogen survival and cytokine production. Autophagy of infected neutrophils regulates cytokine production to control inflammation [99]. Autophagy and NET formation have decisive effects on the fate of neutrophils that are invaded by pathogens. Neutrophils can selectively release NETs by discriminating different bacteria, which is dependent on autophagy [100].

**Xenophagy** Xenophagy is a specialized form of autophagy in which invading bacteria are recognized, captured, and killed, and it plays a significant role in the bacteriostatic activity of neutrophils. Downregulating xenophagy increases the susceptibility to bacterial infection and decreases the resistance to pathogens. As reported previously, xenophagy is vital for the  $\alpha$ -defensin-mediated killing of *Bacillus anthracis* bacilli by neutrophils [101]. *ATG5*<sup>-/-</sup> mice are more susceptible than wild-type mice to *Listeria monocytogenes* and *Toxoplasma gondii* and show increased susceptibility to viral encephalitis [102]. Autophagy-deficient myeloid cells are more susceptible to *C. albicans* infection [103]. However, bacteria subvert neutrophils by

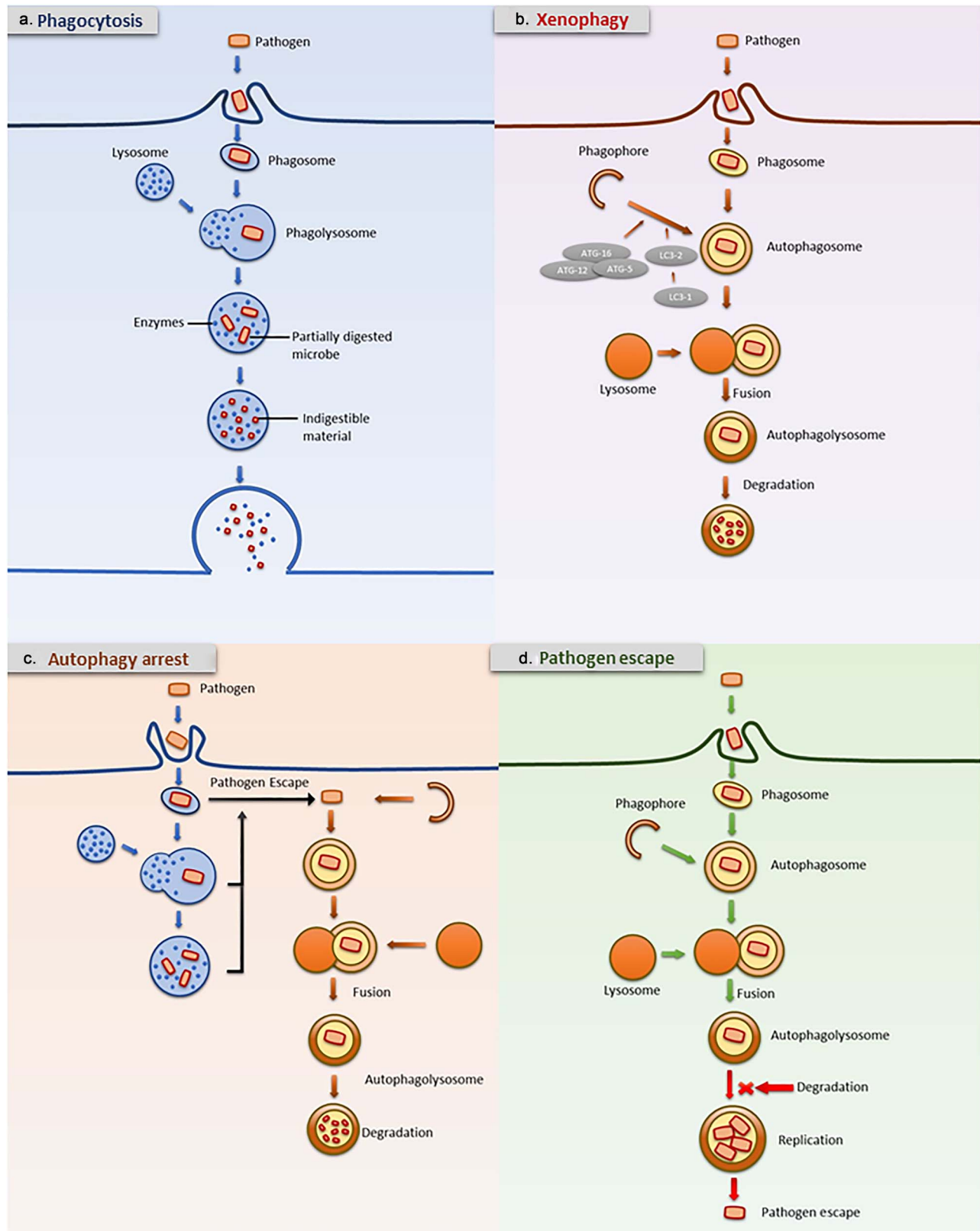
manipulating xenophagy to help bacterial spread via the bloodstream [104, 105]. Pathogens counter host attacks by sabotaging xenophagy at the degradation step to prolong their lifespan. Pathogens also utilize autophagy as a mechanism to acquire nutrients [43]. In adherent-invasive *Escherichia coli* (AIEC)-infected neutrophils, autophagy is downregulated, which limits the antibacterial inflammatory response [106]. *Burkholderia pseudomallei* induces xenophagy in a T3SS-dependent manner and show significantly increased survival in neutrophils in which autophagy is inhibited [107]. Both live and dead bacteria can induce autophagy [83]. Interestingly, specific differences between live and dead bacteria are key to determining whether bacteria escape autophagy. As reported previously, autophagosomes are considered a niche for intracellular *S. aureus* replication, which results in pathogen dissemination. *S. aureus* can survive autophagy and be transported by neutrophils in the bloodstream [108]. In the study of the scavenger receptor-induced autophagy elimination of *Listeria* and *Yersinia*, autophagy often failed to eliminate live bacteria in vivo [109] (Fig. 2).

**Macroautophagy-independent effect of ATG5** There is increasing focus on the macroautophagy-independent effect of ATG5 in *M. tuberculosis* (Mtb) infection. ATG5 plays a special role in Mtb infection and limits intracellular bacterial growth [110]. However, the conventional autophagy pathway does not have the same effect, suggesting that autophagy is not the essential reason for restricting Mtb. Our analysis indicated two possible explanations for this observation. One explanation is that ATG5 regulates this autophagy process in a manner independent of other ATG genes. Another possible explanation is that ATG5 limits Mtb infection in an autophagy-independent manner. ATG5 is involved in other vesicle-trafficking processes, including endocytosis, protein secretion, and LC3-associated phagocytosis [111, 112]. Proteolytic cleavage of ATG5 induces apoptosis [108]. ATG5 associates with BCL-XL and Fas-associated death domain (FADD) to regulate cell death, which is linked to inflammation and infection [113, 114]. ATG5 also interacts with many proteins involved in pathogenesis [115]. Further research is needed to reach the final conclusion.

Autophagy plays a role in the detection and elimination of invasive pathogens. Intracellular bacteria, viruses, and parasites are involved. Autophagy induces autophagic death to reduce intracellular pathogen survival and cytokine production. Bacteria subvert neutrophils by manipulating xenophagy to increase their spread via the bloodstream. Autophagy often fails to eliminate live bacteria in vivo. ATG5 plays a unique role in Mtb infection and limits intracellular bacterial growth. Neutrophil autophagy is now considered a novel therapeutic target in infection.

### Neutrophil autophagy and aseptic inflammation

Neutrophils are the first line of innate immunity and migrate to sites of inflammation upon activation. Neutrophil autophagy is involved in this biological process. As reported, autophagy can be activated under aseptic inflammatory conditions, as evidenced by strong LC3 immunoreactivity [99, 116]. The relationship between neutrophil autophagy and inflammation is intricate [117]. Autophagy is a protective mechanism in neutrophil-mediated inflammation, and blocking autophagy may cause uncontrolled inflammation [118]. Autophagy decreases cytokine production and downregulates neutrophil influx to control inflammation [119, 120]. Autophagy limits degranulation and affects ROS generation, resulting in the downregulation of apoptosis and influencing neutrophil tissue infiltration



**Figure 2.** Autophagy arrest and pathogen escape. Neutrophils utilize multiple antimicrobial strategies to eliminate bacteria, including phagocytosis (a) and autophagy (b). Autophagy plays a role in detecting and eliminating invasive pathogens. In neutrophil autophagy, pathogens are engulfed by autophagosome membranes and degraded by lysosomes. Autophagy is an important pathway for the degradation of phagocytosed content. When pathogens escape phagosomes, they can be captured by autophagy (c). However, autophagy often fails to kill live bacteria. Autophagosomes are considered a niche for pathogen replication, resulting in dissemination (d). *LC3* microtubule-associated protein 1 light chain 3, *ATG* autophagy-related protein



**Table 2.** Pharmacologic regulation of neutrophil autophagy and potential applications

Pharmaceutical	Associated mechanism	Potential application	Reference
IVIG	Upregulates autophagy	Infectious diseases	[123]
Vitamin D	Upregulates autophagy	Tuberculosis	[124]
Carbamazepine	Upregulates autophagy	Infectious diseases	[124]
Valproic acid	Upregulates autophagy	Infectious diseases	[125]
Celastrrol	Upregulates autophagy	Colitis	[126]
Rapamycin	Inhibits mTOR and upregulates autophagy	Cigarette smoke exposure	[119]
PolyP	Inhibits mTOR and upregulates autophagy	Arterial STEMI thrombi	[42]
SLC37A4	Inhibits mTOR and upregulates autophagy	Glycogen storage disease	[127]
Clarithromycin	Upregulates autophagy and increases NET formation	Infectious diseases	[89]
NLRP3/2 and NLRP3 inhibitor	Downregulates autophagy and upregulates phagocytosis	Sepsis	[128]
Ethyl pyruvate	Downregulates autophagy and granule release	Lipopolysaccharide-induced acute lung injury	[129]
Low-molecular-weight heparin (LMWH)	Inhibits autophagy and NET formation	Inflammation	[130]
Bafilomycin A1 and HCQ	Inhibits autophagy and NET formation	Active ulcerative colitis	[88]
Chloroquine (CQ)	Inhibits autophagy and NET formation	Pancreatic cancer	[93]
3-Methyladenine (3MA)	Downregulates autophagy and apoptosis	ANCA-associated vasculitis	[84]
2-Morpholin-4-yl-8-phenyl-chromen-4-one	Downregulates autophagy and apoptosis	ANCA-associated vasculitis	[84]

3MA 3-methyladenine, CQ chloroquine, IVIG intravenous immunoglobulin, LMWH low-molecular-weight heparin, mTOR mammalian target of rapamycin, NLRP3/2 NLR family pyrin domain containing 3/2, NLRP3 NLR family pyrin domain containing 3, SLC37A4 solute carrier family 37 member 4, NET neutrophil extracellular trap, STEMI ST-Elevation myocardial infarction, ANCA antineutrophil cytoplasmic antibodies

[109]. Many signaling molecules are involved in both autophagy and apoptosis and regulate the balance between these two processes, as described previously in detail [119].

### Neutrophil autophagy and cancer

Neutrophils are significantly involved in the pathophysiology of cancer and differentiate into anti-tumor neutrophils (N1) and pro-tumor neutrophils (N2) neutrophils, two subsets with pro-tumorigenic and tumor suppressor effects during oncogenesis [121]. Similar to neutrophil subsets, neutrophil autophagy also has pro-tumorigenic and anti-tumorigenic functions depending on the tumor environment. Neutrophil autophagy influences the progression of solid tumors. For example, in human hepatocellular carcinoma (HCC), neutrophil autophagy is activated by extracellular regulated protein kinases 1/2 (ERK1/2), p38, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) signaling. The upregulation of autophagy maintains mitochondrial stabilization and sustains neutrophil survival in the tumor microenvironment. Neutrophil autophagy promotes tumor growth by increasing the levels of the pro-metastatic proteins oncostatin M (OSM) and MMP-9 and promoting cancer cell migration [122]. However, in hematological malignancies, neutrophil autophagy is essential for blocking disease progression. In acute myelocytic leukemia (AML), autophagy is essential for neutrophil differentiation. Impairing autophagy limits neutrophil degranulation and decreases the release of inflammatory molecules [52, 54]. Neutrophil autophagy may be a novel target for oncotherapy, but the goal of upregulating or down-regulating neutrophil autophagy as a therapeutic depends on the type of tumor.

### Clinical applications

Neutrophil autophagy has clinical relevance in many pathologies, including inflammation, cancer and, infectious diseases. So neutrophil autophagy is placed under the spotlight for pharmacologists and clinicians [11]. The pharmacological regulation of neutrophil autophagy may represent a novel strategy for the treatment of certain diseases [6] (Table 2).

**Infectious diseases** Neutrophil autophagy is now considered a novel therapeutic target in infection. The majority of pharmacological agents stimulate autophagy, including intravenous immunoglobulin (IVIG), clarithromycin, vitamin D, carbamazepine, and valproic acid. IVIG boosts the ability of neutrophils to kill multidrug-resistant bacteria and eliminate drug-sensitive strains by stimulating autophagy. Inhibiting autophagy weakens the antibacterial activity of neutrophils [123, 131]. Clarithromycin plays an immunomodulatory role as an inducer of neutrophil autophagy to amplify NET formation [89]. Vitamin D can prevent exacerbated inflammation by upregulating autophagy to suppress the release of pro-inflammatory cytokines during Mtb infection [132]. Carbamazepine and valproic acid are autophagy inducers that show similar effects [133]. NLR family pyrin domain containing 3/2 (NLRP3/2) and NLR family pyrin domain containing 3 (NLRP3) inhibitors downregulate autophagy and upregulate phagocytosis to improve bacterial clearance in polymicrobial sepsis [128].

**Respiratory diseases** In respiratory diseases, neutrophil autophagy plays a crucial role in smoking, acute lung injury, emphysematous, asthma, and fibrosis. Increased neutrophil autophagy is a marker of smoke exposure and a protective mechanism of the lung. The mTOR

inhibitor rapamycin increases autophagy in neutrophils and reduces alveolar inflammation during cigarette smoke exposure [119]. Ethyl pyruvate is a novel potential treatment for lipopolysaccharide-induced acute lung injury that inhibits neutrophil autophagy and downregulates granule release to alleviate disease progression [129]. IFN regulatory factor 3 (IRF3) activates autophagy, phagocytosis and apoptosis in neutrophils, and the IRF3 pathway is considered to ameliorate the lipopolysaccharide-triggered exacerbation of emphysematous [48]. Autophagy prevents NF- $\kappa$ B activation, and the inhibition of autophagy reduces lung injury after prolonged low-pressure mechanical ventilation [134]. In asthma patients, autophagy augments the effects of immune cells, including neutrophils, and increases asthma severity [135]. Inhibiting autophagy can reduce neutrophil migration, and autophagy is becoming a new therapeutic target in corticosteroid-resistant asthma patients [136]. In pulmonary fibrosis patients, neutrophil autophagy is a target for controlling fibrosis by regulating NET formation [137].

**Cardiovascular diseases** Neutrophil autophagy is a novel therapeutic target in cardiovascular disease, including arterial ST-Elevation myocardial infarction (STEMI) thrombi and antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis. In arterial STEMI thrombi, autophagy regulates NETs, which contribute to disease development. mTOR inhibition by polyP counteracts the effects of IFN- $\lambda$ 1/IL-29 treatment, resulting in the inhibition of NET formation. IL-29 and polyP are important regulators of thromboinflammation that control neutrophil autophagy [42]. Low-molecular-weight heparin (LMWH) inhibits autophagy and NET formation in activated neutrophils to mobilize granule content. LMWH is the preferred treatment for inflammation, and autophagy provides the theoretical basis for the use of this agent [130]. In ANCA-associated vasculitis, the autophagy inhibitors 3MA and 2-morpholin-4-yl-8-phenyl-chromen-4-one can attenuate the decreased apoptosis rate. NET formation and autophagy are associated during autoimmune attacks [84]. In thrombosis, autophagosomes engulf tissue factor (TF) before it is delivered extracellularly in NETs. HMGB1, a component of the autophagy pathway, is involved in the externalization of membrane-bound and cytosolic proteins in NETs [72].

**Endocrine diseases** The regulation of neutrophil autophagy has shown a considerable curative effect in endocrine diseases. In diabetes, the increases in apoptosis and ROS production in neutrophils may be related to the suppression of autophagy by mTOR. In a diabetic rat model, neutrophils expressed low levels of autophagy-related molecules such as LC3B and had few punctate structures labelled as autophagosomal membranes. Autophagy is considered to regulate neutrophil-induced inflammation by reducing MPO activity and ROS production [138]. Glycogen storage disease type Ib (GSD-Ib), an autosomal recessive glycogen storage disorder with a phenotype of metabolic and myeloid defects, is caused by glucose-6-phosphate translocase (G6PT) deficiency and results in severe congenital neutropenia. Solute carrier family 37 member 4 (SLC37A4) and G6PT are key activators of neutrophil autophagy that negatively regulate mTORC1. SLC37A4 increases the interaction between N-terminal Venus-tagged ULK1 (ULK1-VN) and C-terminal Venus-tagged ATG9 (ATG9-VC) to affect mTORC1 function through calcium mobilization. Neutrophil autophagy may be a potential target for the treatment of neutropenia in GSD-Ib [127]. In fasted volunteers, neutrophils activated autophagy to counteract the nutrient deprivation, whereas leukocyte subpopulations showed only a significant increase in the number of LC3B+ puncta. Therefore,

neutrophil autophagy is an indicator of autophagy at the whole-body level under starved pathological conditions [139].

**Digestive diseases** Neutrophil autophagy is an effective therapeutic target in digestive inflammation and cancer. In active ulcerative colitis, autophagy is thought to control neutrophil-driven inflammation. Regulated in development and DNA damage response-1 (REDD1), a component of the autophagy pathway, was reported to be a vital inducer of autophagy-mediated NETosis and NET-associated IL-1 $\beta$  release. The autophagy inhibitors BafA1 and HCQ are used to negatively regulate NET release [88]. In pancreatic cancer, inhibiting autophagy with CQ or genetic ablation of RAGE decreased the propensity for NET formation, serum DNA levels, and citrullinated histone H3 expression. Patients treated with an autophagy inhibitor show decreased NET formation in the pancreatic tumor microenvironment and in peripheral blood, leading to lesion control [93]. Autophagy defects are an important factor in the pathogenesis of Crohn's disease (CD), and autophagy plays a crucial role in host defense against infection. As reported previously, the ATG16L1 variant T300 AD decreases phagocytic and bactericidal activities, resulting in CD [140]. Celastrol ameliorates inflammation via the PI3K-Akt-mTOR pathway, which is implicated in autophagy [126].

## Conclusion

Autophagy has received considerable attention in the field of cellular immunology over the past five years. Autophagy evolved as a metabolic and quality control system in neutrophils; it is a first line of defense against microbial infection and affects many aspects of neutrophil biology, including differentiation, lifespan, degranulation, and NET formation. Neutrophil autophagy is an attractive target for the development of new treatments of multiple diseases, including inflammation, cancer and infectious diseases. However, the existing understanding of neutrophil autophagy is still in its infancy, and the explicit functions of ATG proteins and the particular regulatory mechanisms underlying autophagy on neutrophils are still being explored. Whether every component of autophagy is involved in NET formation and whether all the components of canonical autophagy participate in xenophagy remain unknown. It is difficult to determine whether the induction or suppression of autophagy is better for pathogen clearance from neutrophils. The mechanism by which bacteria inhibit autophagosome-lysosome fusion and the involved bacterial effectors still need to be fully elucidated. Further research is needed to clarify the hypotheses regarding the detailed function of autophagy in neutrophils.

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## Conflicts of interest

The authors declare that they have no competing financial interests.

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