Advances in the Regulation of Neutrophil Function by Autophagy: Postprint

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Abstract

Autophagy is an evolutionarily highly conserved intracellular degradation system designed to maintain cellular homeostasis in response to various cellular stresses. Under physiological conditions, autophagy levels are typically low; however, they are significantly upregulated under oxidative stress, nutrient starvation, and various pathogen stimuli. Numerous previous studies have demonstrated that autophagy plays a significant role in the regulation of various tissue cells and physiological functions. Early research identified a link between autophagy and neutrophil death, a necessary process closely associated with inflammation. Studies in human systems and mouse models have shown that autophagy plays a crucial role in neutrophil-driven inflammation and pathogen defense. Autophagy is essential for the execution of neutrophils' primary functions, including degranulation, reactive oxygen species production, and neutrophil extracellular trap release. In neutrophil production in the bone marrow, autophagy plays a key role in myelopoiesis and promotes the differentiation of myeloid progenitor cells into neutrophils. In summary, this review focuses on discussing the role of autophagy in neutrophils, from their generation in the bone marrow to inflammatory responses and NETotic cell death.

Full Text

Research Progress on the Regulation of Neutrophil Function by Autophagy

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Abstract

Autophagy is an evolutionarily highly conserved intracellular degradation system designed to maintain cellular homeostasis in response to various cellular stresses. Under physiological conditions, autophagy levels are typically low, but they become significantly upregulated under oxidative stress, nutritional starvation, and stimulation by various pathogens. Numerous past studies have demonstrated that autophagy plays a crucial role in regulating multiple tissue cells and physiological functions. Early research identified a link between autophagy and neutrophil death, a process closely associated with inflammation. Studies in both human systems and mouse models have shown that autophagy plays a vital role in neutrophil-driven inflammation and defense against pathogens. Autophagy is essential for the execution of neutrophils' primary functions, including degranulation, reactive oxygen species (ROS) production, and release of neutrophil extracellular traps (NETs). In neutrophil production within the bone marrow, autophagy plays a key role in myelopoiesis and promotes the differentiation of myeloid progenitor cells into neutrophils. In summary, this review focuses on the role of autophagy in neutrophils, from their generation in the bone marrow to inflammatory responses and NETosis cell death.

Keywords: autophagy; neutrophils; granulopoiesis; phagocytosis; degranulation; neutrophil extracellular traps

Introduction

In eukaryotic cells, macroautophagy (hereafter referred to as autophagy) represents a mechanism for regulating intracellular homeostasis that is essential for cells to cope with starvation and other types of stress, including hypoxia, oxidative burst, DNA damage, and infection. During autophagy, cytoplasmic components are sequestered within double-membrane vesicles called autophagosomes, which are subsequently delivered to lysosomes for degradation (forming autolysosomes). This dynamic and strictly regulated process protects cells by sensing and clearing damaged cellular components or intracellular pathogens. Degradation of intracellular macromolecules and organelles generates recyclable amino acids and other nutrients to sustain cellular metabolism [1]. In recent years, substantial evidence has demonstrated the involvement of autophagy in immune functions such as phagocytosis, elimination of intracellular pathogens, antigen presentation, thymic selection, maintenance of lymphocyte homeostasis, and cytokine production. Conversely, abnormal or excessive autophagy may lead to autophagy-dependent cell death [2]. Therefore, autophagy is intimately linked to both cell survival and death, depending on the cell type and stress conditions. Dysregulation of autophagy is associated with various diseases, including inflammatory disorders, neurodegenerative diseases, and cancer [3].

Neutrophils are the most abundant effector cells in the human immune system and the first to migrate to sites of tissue inflammation [4]. Neutrophils have a

very short lifespan, with a half-life ranging from 6-8 hours to a few days. Under homeostatic conditions, neutrophil turnover is ensured through continuous generation in the bone marrow [5]. However, during severe systemic inflammation, bone marrow progenitors are triggered to produce and mobilize large numbers of neutrophils, a process termed emergency granulopoiesis [6]. Neutrophils express diverse surface receptors that enable rapid responses to environmental cues and facilitate de novo cytokine synthesis [7]. This adaptability renders neutrophils a phenotypically and functionally heterogeneous cell population [8]. Upon activation, neutrophils exert their antimicrobial and pro-inflammatory effects through three distinct mechanisms: phagocytosis, degranulation, and the recently described formation and release of neutrophil extracellular traps (NETs) [9].

Numerous studies have demonstrated that autophagy is closely related to neutrophil biology and effector functions. This review summarizes the latest experimental and clinical findings on the regulation of autophagy and granulopoiesis, as well as the critical roles of autophagy in neutrophil and NET-mediated antimicrobial defense.

1. Autophagy Mechanisms and Regulatory Pathways

1.1 Concepts, Related Genes, and the Autophagic Process

Autophagy is classified into macroautophagy, microautophagy, and chaperone-mediated autophagy, with "autophagy" typically referring to macroautophagy. This process involves the formation of a double-membrane structure that buds from the ribosome-free region of the rough endoplasmic reticulum, engulfing portions of the cytoplasm and cellular components such as organelles and proteins that require degradation to form an autophagosome. The autophagosome then fuses with a lysosome to form an autolysosome, degrading its contents to fulfill cellular metabolic needs and enable organelle renewal. Moderate autophagy helps cells survive nutrient stress and maintain intracellular homeostasis, whereas excessive autophagy leads to autophagic cell death (ACD), also known as type II programmed cell death.

The autophagic process comprises autophagosome formation, subsequent maturation and fusion with lysosomes, and finally degradation. The roles of autophagy-related genes (ATGs) in controlling various steps of the autophagy pathway have been well established [10]. The first ATG gene, ATG1, encodes a protein kinase required for autophagy initiation, with its mammalian homolog ULK1 serving a similar function. ULK1 forms a complex with ATG13, ATG101, and FIP200. Following autophagy initiation, ULK1 phosphorylates and activates the class III PI3K complex I, which consists of VPS34, VPS15, ATG14L, and Beclin1. This complex generates phosphatidylinositol-3-phosphate (PtdIns3P) on nascent autophagosomal membranes to facilitate isolation membrane formation. ATG9 is the only transmembrane ATG protein. PtdIns3P recruits ATG16L1 and the ATG5-ATG12 conjugate to autophago-

somes through interaction with WIPI2. The ATG16L1-ATG5-ATG12 complex catalyzes the covalent conjugation of phosphatidylethanolamine molecules to the C-terminal glycine of LC3, which is activated by ATG4, ATG7, and ATG3 (forming LC3-I) to generate LC3-II [11]. LC3-II serves as an autophagy marker for quantifying autophagy in cells [12] and can also bind autophagy receptors such as SQSTM1, NDP52, NBR1, and OPTN [13]. Subsequently, autophagosomes mature and fuse with lysosomes, a process mediated by class III PI3K complex II composed of Vps34, VPS15, Beclin1, and UVRAG, ultimately leading to degradation by lysosomal enzymes.

1.2 Regulatory Mechanisms of Autophagy

1.2.1 Regulation by AMPK and mTOR Autophagy is primarily induced by nutrient starvation, oxidative stress, endoplasmic reticulum stress, and energy depletion. During starvation, cells utilize specialized mechanisms to degrade their own components, delivering large amounts of cytoplasmic content (proteins, amino acids, organelles, and other macromolecules) to lysosomes for degradation and recycling to provide nutrients for essential cellular activities. In addition to general autophagy, evidence indicates the existence of mitophagy, a selective degradation process that removes damaged mitochondria. The autophagy machinery involves multiple protein complexes, among which AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) regulate various aspects of this mechanism.

AMPK has been shown to regulate autophagy in both yeast and mammalian cells. One regulatory mechanism involves direct phosphorylation of mTOR upstream regulator TSC2 at Ser122 and Ser1345; another involves direct phosphorylation of mTORC1 subunit RAPTOR at Ser722 and Ser792. Under energy stress conditions, these phosphorylation events reduce mTOR activity, alleviating its inhibition of ULK1 to activate autophagy. In summary, induction of the autophagy machinery is regulated by the AMPK/mTOR complex 1 (mTORC1) energy-sensing system. During nutrient deficiency or starvation, ATP levels decrease and AMPK becomes activated, leading to mTORC1 inhibition. Suppression of mTORC1 activation serves as the primary trigger for autophagy.

1.2.2 Regulation by AMPK and ULK1 A direct link exists between AMPK and the autophagy pathway. Activation of ULK1 upon glucose starvation depends on AMPK-mediated phosphorylation. When AMPK is knocked down, ULK1 cannot be activated under glucose deprivation, demonstrating AMPK' s essential role in ULK1 activation. Lee et al. [14] reported that AMPK and ULK1 play important roles in autophagy induction. AMPK directly phosphorylates ULK1 at a minimum of four residues: Ser467, Ser555, Thr574, and Ser637. Furthermore, AMPK-mediated ULK1 activation is required for mitophagy to remove damaged mitochondria. Cells expressing ULK1 mutants that cannot be phosphorylated by AMPK accumulate defective mitochondria, indicating that AMPK-ULK1 signaling is important not only for general autophagy

but also for selective removal of damaged mitochondria. Subsequent studies have confirmed ULK1' s essential role in mitophagy under various conditions. Besides phosphorylating ULK1, AMPK also phosphorylates residues on other core components of the autophagy pathway, such as Ser761 on ATG9, Thr133 and Ser135 on VPS34, Ser91 and Ser94 on Beclin1, Thr50 on VPS34-associated protein RACK1, and Thr32 on PAQR3 [15]. However, many of these AMPK phosphorylation sites have not been independently verified, leaving numerous details of AMPK and ULK1 regulation of autophagy to be elucidated.

2. Regulation of Autophagy in Granulopoiesis

Granulopoiesis, the production of granulocytes under both steady-state and stress conditions including systemic inflammation, involves hematopoietic stem cells and progenitor cells [9]. Evidence indicates that autophagy serves as a regulator of hematopoietic stem cell (HSC) metabolism and plays an important role in controlling their differentiation [16]. Clearance of damaged mitochondria through mitophagy prevents ROS accumulation and subsequent damage that would otherwise lead to apoptosis in HSCs. Deletion of Atg7 in HSCs impairs their function, likely due to accumulation of damaged mitochondria and ROS production. This group also confirmed that Atg7 deletion causes myeloproliferation resembling acute myeloid leukemia. Another study by Warr et al. [17] demonstrated that FOXO3A-mediated autophagy protects HSCs, enabling their survival under metabolic stress. The homeostatic role of autophagy in hematopoietic progenitor cell function was further confirmed by a recent study showing that excessive mitophagy due to deletion of the gene encoding AAA+-ATPase Atad3a adversely affects HSC homeostasis [18]. Ho et al. [19] used several mouse models to further demonstrate that interactions between autophagy and cellular metabolism in HSCs lead to epigenetic changes and stem cell loss. Autophagy activity in HSC subsets during aging is associated with declining hematopoietic progenitor cell function, consistent with autophagy' s beneficial role in maintaining cellular health. In summary, autophagy primarily participates in the regulation of early hematopoietic progenitor cells.

Although autophagy' s role in HSCs is well established, its involvement in later stages of myeloid lineage progenitor progression has been less studied. Myeloid cell-specific deletion of Atg5 has been shown to positively regulate neutrophil proliferation rates, leading to accumulation of neutrophils in the bone marrow, blood, and spleen without affecting neutrophil effector functions such as apoptosis and migration.

A study by Riffelmacher et al. [20] further confirmed the importance of autophagy in granulopoiesis. This research demonstrated that autophagic degradation of fatty acids is necessary for the metabolic switch from glycolysis to oxidative phosphorylation (OXPHOS), a process essential during late-stage neutrophil differentiation. Sidaway [21] first demonstrated dynamic regulation of autophagy at different stages of neutrophil differentiation in vivo using a transgenic mouse model, with the highest autophagy levels observed during early

stages of myeloblast (myelocytes) and promyelocyte differentiation, and lower levels during final stages. Finally, a recent study by Huang et al. [22] reported differential expression of 22 autophagy-related genes between monocyte and granulocyte differentiation, suggesting that autophagy may play distinct roles in myeloid differentiation toward granulocytic and monocytic lineages.

3. Interaction Between Autophagy and Neutrophil Phagocytosis

Neutrophil phagocytosis and ROS production are key mechanisms for microbial killing. Studies have shown interactions between autophagy and phagocytosis in macrophage host defense [23]. Autophagy can detect and eliminate intracellular pathogens. Pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain proteins (NOD)1/2, and ubiquitin-binding protein p62/SQSTM1 are activated by sensing various pathogen-associated molecular patterns (PAMPs) on cell membranes or in the cytoplasm, inducing a selective form of autophagy termed "xenophagy." Additionally, autophagy can be activated through extracellular PRR signaling during pathogen phagocytosis. A novel selective autophagy form called LC3-associated phagocytosis (LAP) has been described in murine macrophages. In LAP, the autophagy protein LC3 associates with conventional phagosomes, promoting phagolysosome formation and maturation while enhancing phagocytosis. Besides engulfing pathogens, LAP also mediates uptake and clearance of apoptotic and necrotic cells or immune complexes via phosphatidylserine or Fc receptors. Thus, LAP may protect the organism from aberrant inflammation.

While research on autophagy as a defense mechanism has focused primarily on macrophages, studies in mice with knockout of the autophagy factor Atg5 in monocytes or macrophages have confirmed autophagy's critical role in pathogen defense. Subsequently, an autophagy-independent role for Atg5 in preventing neutrophil-mediated lung immunopathology was proposed in vivo [24]. In 2010, evidence demonstrated that human neutrophils possess autophagy machinery. Subsequent transmission electron microscopy analysis of autophagosomes containing bacteria, along with autophagy inhibition by 3-methyladenine (3-MA) or bafilomycin A1, showed that xenophagy exerts antimicrobial effects in human neutrophils [25].

Similar to macrophages, interactions between phagocytosis and autophagy pathways have been described in neutrophils. *Escherichia coli* triggers the autophagy machinery in neutrophils. Studies have also shown that LC3B presence in phagosomes of mouse and human neutrophils requires NADPH oxidase activation and ROS generation. Human neutrophils have been shown to undergo autophagy upon *Streptococcus pneumoniae* infection in vitro, which depends on type III PI3K and ATG5 and enhances bacterial phagocytosis. ATG5 dependence was demonstrated using siRNA-transfected neutrophils incubated with granulocyte-macrophage colony-stimulating factor (GM-CSF) [26].

However, many pathogens have evolved strategies to evade or exploit autophagy in macrophages to establish long-term intracellular survival and replication. Microbial subversion of autophagy in neutrophils has been less studied. Previously, adherent-invasive *E. coli* (AIEC) strains isolated from Crohn' s disease patients were shown to invade human neutrophils and trigger autophagy. However, AIEC can evade killing in neutrophil-like PLB cells by disrupting autophagic flux at the autolysosomal step, allowing bacterial survival within cells. In summary, neutrophils may utilize both autophagy and phagocytosis to kill pathogenic microorganisms and clear cellular debris, and these two defense mechanisms may be functionally interrelated.

4. Autophagy and Neutrophil Degranulation

Upon activation, neutrophils release antimicrobial and inflammatory proteins stored in cytoplasmic granules into phagosomes or through secretion, a process known as degranulation. Neutrophils contain four distinct granule types: primary (azurophilic) granules, secondary (specific) granules, tertiary granules, and secretory vesicles. Primary granules store elastase, myeloperoxidase (MPO), cathepsins, and defensins. Secondary granules primarily contain NADPH oxidase, lactoferrin, and matrix metalloproteinase 9 (gelatinase). Tertiary granules are rich in gelatinase but lack lactoferrin, while secretory vesicles are enriched in alkaline phosphatase and various cell membrane and plasma proteins derived from endocytosis [27]. Granule-derived proteins are essential for neutrophil functions including chemotaxis, antimicrobial activity, and NET release. Elastase and MPO are also required for NET formation [28]. Therefore, degranulation and NET release are interconnected and exhibit complementary roles during neutrophil activation.

The importance of autophagy in regulating neutrophil degranulation has been demonstrated in myeloid-specific autophagy-deficient mouse models. Autophagy deficiency in neutrophils significantly reduces degranulation both in vitro and in vivo [29]. In the same study, ROS production was also diminished in autophagy-deficient neutrophils, and inhibition of NADPH oxidase reduced neutrophil degranulation, suggesting that NADPH oxidase mediates autophagy' s effects on degranulation [29].

5. Autophagy and NET Formation

In 2004, Brinkmann et al. [30] identified a novel neutrophil microbicidal mechanism through NET release. NETs are fibrous meshworks that trap and kill extracellular microorganisms, composed of various highly active neutrophil-derived granule proteins, cytoplasmic proteins, and chromatin [9,31]. In contrast to apoptosis and necrosis, NET-mediated cell death (NETosis) involves chromatin decondensation, nuclear envelope disintegration, and plasma membrane rupture to release NETs [2].

Beyond their antimicrobial function, accumulating evidence implicates NETs

in the pathogenesis of many non-infectious inflammatory diseases [32]. Furthermore, clinical and experimental studies have shown that neutrophils release qualitatively distinct NETs in different disease contexts, with expression of bioactive proteins determined by the inflammatory environment. For example, NETs in classic autoinflammatory diseases such as familial Mediterranean fever (FMF) and Still' s disease carry IL-1 [33-35]. NET autoantigens are associated with autoimmune diseases including lupus, rheumatoid arthritis, and ANCA-associated vasculitis [36,37].

Neutrophil extracellular trap formation is triggered by numerous pathogenic factors and various pro-inflammatory stimuli such as cytokines (IL-8, TNF) and interferons (IFN), while granule enzymes (MPO, elastase) and ROS regulate NET release [38]. A close relationship exists between ROS production and autophagy, which represent two major regulators of NETosis. ROS burst induces autophagy, which in turn maintains ROS production. Remijsen et al. [39] showed that both autophagy and ROS synthesis are required for PMA-induced NET formation in human neutrophils. Inhibition of either autophagy or NADPH oxidase prevents chromatin decondensation, which is essential for NETosis and leads to apoptosis instead. Additionally, neutrophils isolated from chronic granulomatous disease patients lacking NADPH oxidase activity cannot produce NETs [39]. Meanwhile, studies have confirmed that neutrophils from patients with acute gouty arthritis exhibit autophagy that mediates NET release, first linking autophagy-associated NETosis to sterile inflammation. Subsequently, mTOR and cytoskeletal machinery have been shown to play key roles in regulating autophagy-mediated NET formation in human neutrophils. Pharmacological inhibition of the mTOR pathway significantly promotes autophagosome formation and histone citrullination, facilitating NET release in response to N-formylmethionyl-leucyl-phenylalanine (fMLP), whereas blocking cytoskeletal dynamics abolishes mTOR/autophagy-mediated NETosis [40]. Furthermore, silencing ATG5 in AIEC-infected neutrophil-like PLB human cell lines blocked NET formation. Recently, reduced Atg5 expression levels were shown to impair NET formation capacity in neutrophils from aged mice, suggesting an important role for autophagy in maintaining the NET machinery [41]. In vitro NET production in response to LPS and IL-8 is impaired [42], with reduced ATG5 gene expression [41,43]. Consistently, autophagy inhibition via pharmacological inhibitors or small interfering RNA against ATG7 attenuates LC3 autophagosome formation and significantly reduces NET generation in promyelocytes [44]. Moreover, inhibiting the PI3K/AKT/mTOR pathway or PTEN reduces NET production in PMA-stimulated, HL-60-differentiated neutrophils [45].

Conversely, conflicting data have been reported regarding autophagy' s contribution to NET release. Specifically, Atg5-knockout mouse neutrophils with reduced autophagy activity retain their ability to release cellular DNA. Furthermore, although PI3K inhibition prevents NET formation in human neutrophils, autophagy is not inhibited by bafilomycin A1 or chloroquine [46]. This suggests the existence of autophagy-independent NETosis pathways [47]. Recently, Bendorius et al. [48] found that P140, which mediates autophagy and chaperone-

mediated autophagy, inhibits NET release from neutrophils. Pre-incubation of neutrophils with P140 dose-dependently inhibited NIC-induced NET release, but the same P140 concentration was ineffective at inhibiting NET release induced by fMLP, LPS, or phorbol myristate acetate, while having minimal effect on mitophagy.

Angelidou et al. [49] used immunofluorescence confocal microscopy, ELISA, immunoblotting, flow cytometry, and quantitative PCR to analyze NET-associated markers in colon biopsy specimens and peripheral blood neutrophils. Their study revealed that intestinal inflammation in ulcerative colitis (UC) is characterized by REDD1 expression in neutrophils and formation of NETs modified by autophagy-dependent bioactive IL-1 and tissue factor (TF). REDD1 emerges as a key factor linking stress to autophagy-mediated NET formation and IL-1 expression in autoinflammation. These findings provide new hope for clinically relevant diagnostic and therapeutic targets.

Summary and Outlook

Autophagy is a degradation mechanism involved in intracellular homeostasis. During starvation, cells utilize specialized mechanisms to degrade their own components, delivering large amounts of cytoplasmic content to lysosomes for degradation and recycling to provide nutritional resources for essential cellular activities. Autophagy regulates neutrophil function, and induction of the autophagy process in neutrophils in response to invading pathogens represents an important mechanism of innate immunity. Neutrophils kill pathogens through degranulation, phagocytosis, and NET release, all of which depend on timely activation of the autophagy process. Inhibition of autophagy reduces neutrophil degranulation, with autophagy deficiency affecting tertiary and secondary granule production most severely.

Autophagy represents a crucial mechanism in neutrophil biology and pathophysiology. The balance of autophagic responses in neutrophils is critical for cellular homeostasis and host health. Autophagy plays important roles in neutrophils during inflammatory and autoimmune diseases. Understanding the specific signaling pathways of autophagy and its effects on neutrophil function, and targeting autophagy pathways will expand new avenues for treating neutrophil-mediated inflammation, autoimmune diseases, thrombotic disorders, myeloid leukemia, or neutropenia. Therefore, there is an urgent need to design novel therapeutic drugs targeting autophagy.

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