Regulation of the autophagic machinery in human neutrophils

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The induction of the autophagy machinery, a process for the catabolism of cytosolic proteins and organelles, constitutes a crucial mechanism in innate immunity. However, the involvement of autophagy in human neutrophils and the possible inducers of this process have not been completely elucidated. In this study, the induction of autophagy was examined in human neutrophils treated with various activators and detected by the formation of acidified autophagosomes through monodansylcadaverine staining and via LC-3B conversion screened by immunoblotting and immunofluorescence confocal microscopy. In addition, the expression of the ATG genes was assessed by real-time RT-PCR. We provide evidence that autophagy is implicated in human neutrophils in both a phagocytosis-independent (rapamycin, TLR agonists, PMA) and phagocytosis (Escherichia coli)-dependent initiation manner. ROS activation is a positive mechanism for autophagy induction in the case of PMA, TLR activation and phagocytosis. Furthermore, LC3B gene expression was uniformly upregulated, indicating a transcriptional level of regulation for the autophagic machinery. This study provides a stepping stone toward further investigation of autophagy in neutrophil-driven inflammatory disorders.

Key words: Autophagy · Neutrophils · Phagocytosis · ROS · TLR

Introduction

Neutrophils (PMN) are multifunctional cells, playing a central role in the innate immune system [1]. Inflammatory stimuli attract neutrophils to infected tissues where they engulf and inactivate microorganisms through the fusion of phagosomes with granules and the formation of phagolysosomes, in which antimicrobial peptides and ROS act synergistically for the clearance of pathogens [2]. In addition, neutrophil activation, degranulation and release of ROS into the extracellular medium, results in host tissue injury [3], while neutrophil apoptosis contributes to the resolution of inflammation [4].

Autophagy is a homeostatic mechanism involved in the clearance of damaged organelles or proteins and in cellular survival in periods of nutrient depletion, providing essential nutrient supply through recycling of cytosolic macromolecules and organelles [5]. Several proteins are involved in the induction of autophagy and the formation and maturation of autophagosomes, called Atg (autophagy related) proteins. Autophagy is negatively regulated by the protein kinase mammalian target of

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rapamycin (mTOR), while the PI3K hVPS34 is essential for the induction of the autophagic machinery [6]. On the other hand, it is suggested that PI3K inhibition with 3-methyladenine (3-MA), wortmannin and LY294002 inhibits the process [7, 8]. Upon activation of the autophagic process, ATG8/LC3B is lipilated (LC3B-I to LC3B-II conversion) and the lipid-modified form is translocated to autophagosomes. The LC3B-I to LC3B-II conversion is a critical marker of autophagy activation, detected by either immunoblotting or fluorescence microscopy [9].

The implication of autophagy in both innate and adaptive immunity has been previously reported. It has been suggested that the activation of the autophagic machinery in macrophages results in inhibition of survival and removal of intracellular pathogens (xenophagy) [10, 11]. Furthermore, regulation of autophagy in immune cells has been reported by activation of Toll-like receptors (TLR) and inflammatory cytokines, such as TNF-α and type I and II interferons [12–14].

The role of autophagy and potential autophagy triggers in neutrophils are not completely elucidated. Moreover, it is unclear to what extent the phagocytosis-related compartments of neutrophils contribute to the autophagic process. To answer the above questions, the behavior of the autophagic process during neutrophil activation with various agonists was investigated. A phagocytosis-independent and -dependent manner of autophagy induction in neutrophils, as assessed by monodansylcadaverine (MDC) staining, LC-3B immunoblotting and immunofluorescence microscopy was found. In addition, real-time RT-PCR expression data of various ATG genes supports the parallel regulation of the autophagic machinery at a transcriptional level.

Results

Phagocytosis-independent induction of the autophagic machinery in human neutrophils

Neutrophils under various clinical conditions or after in vitro activation are able to form vacuoles, previously characterized as autophagy-like vacuoles [15–17]. Among various agonists, neutrophil stimulation with PMA, a protein kinase C activator, resulted in cytoplasmic vacuole accumulation (Fig. 1A). This PMA-derived effect was dose and time dependent. The selected time (30 min) and dose were chosen to obtain high PMA activity and more than 98% cell viability. To identify the nature of the observed vacuoles, we performed MDC staining as a marker of acidic vacuoles and the existence of acidified autophagosomes was demonstrated (Fig. 1B). Although it was not feasible to identify the vacuoles observed by optical microscopy as autophagosomes due to their larger size compared to the size of the acidified structures detected by MDC staining, the detected acidified structures support the induction of autophagy. To further correlate the observed structures with autophagosomes, we stimulated neutrophils with rapamycin, an inducer of autophagy through inhibition of mTOR [6]. Rapamycin-stimulated neutrophils exhibited, similar to PMA, acidified structures (Fig. 1B) and vacuole formation (Fig. 1A), albeit less prominent than in PMA-treated cells. Pre-incubation with the autophagy inhibitor 3-MA completely abolished the observed vacuolization and the enhanced MDC-positive vacuole formation in both PMA and rapamycin-treated neutrophils (Fig. 1B). Similar effect was observed with the non-selective PI3K inhibitor LY294002 (data not shown).

These findings led us to further investigate the involvement of the autophagic machinery in neutrophils. Enhanced LC3B-I to LC3B-II conversion was observed in cell lysates of PMA- and rapamycin-stimulated neutrophils as detected by LC3B immunoblotting (Fig. 1C). Pretreatment with 3-MA abolished the LC3B-I to LC3B-II conversion as expected, while cells treated only with 3-MA exhibited lower LC3B-II to LC3B-I ratio compared with control neutrophils (Fig. 1C). Assessment of LY294002 resulted in a pattern similar to that observed in 3-MA pretreated cells (Fig. 1D). Next, the formation of LC3B aggregates was examined utilizing immunofluorescence confocal microscopy. Treatment with PMA resulted in the redistribution of LC3B into punctuated structures from diffuse cytosolic staining (Fig. 1E). The percentage of neutrophils with puncta was higher in PMA-treated cells compared with untreated cells and was completely abolished in neutrophils pretreated with 3-MA (Fig. 1E). Punctuated structures were also observed in cells treated with rapamycin (Fig. 1E).

The mRNA expression levels of ATG3, ATG5, ATG6, ATG7, MAP1LC3B (LC3B, ATG8), ATG12, ATG16L1 and hVPS34 were measured after stimulation with PMA and rapamycin. ATG3 and LC3B mRNA expression increased after treatment with both stimuli (Fig. 2), whilst mRNA levels of all the other genes were not significantly affected (data not shown). Pre-incubation with 3-MA resulted in comparable expression levels of both genes (Fig. 2) to control ones.

Effect of TLR activation on the regulation of autophagy in human neutrophils

To investigate whether TLR activation induces the autophagic machinery in human neutrophils, we monitored the effect of TLR stimulation on MDC staining and LC3B conversion by immunoblotting. Incubation of neutrophils with LPS, lipotechoic acid (LTA), peptidoglycan (PGN), loxorubin and R848 resulted in acidic autophagosome formation (Fig. 3A) and LC3B conversion (Fig. 3B and C). However, no statistically significant increase in the percentage of cells with acidified autophagosomes or any LC3B conversion in cells treated with poly I:C or flagellin was detected (Fig. 3A). Stimulation with LPS at the high dose of 1μg/mL resulted in non-significant changes (data not shown), while concentrations of 50 ng/mL (Fig. 3B) or 10 ng/mL (data not shown) caused a more distinctive effect. These results are summarized in Table 1. Moreover, increased LC3B gene expression was a universal finding after stimulation with the TLR agonists that were able to induce LC3B conversion (Fig. 3D and Table 1), while increased mRNA levels of ATG3, ATG4, ATG5, ATG6 and ATG7 were detected only after stimulation with LPS (Fig. 3E and Table 1).
Figure 1. Evaluation of phagocytosis-independent stimuli on the induction of the autophagic process in human neutrophils. (A) Assessment of vacuole (arrows) formation in neutrophils after stimulation with PMA (II) and rapamycin (III), compared with control cells (I: treated with PBS and serum) (magnification 1000 ×). (B) (I) Presence of late autophagosomes as observed with MDC staining in cells treated with PMA and rapamycin (RAP) compared with control cells (PBS) and cells treated with PMA and 3-MA (magnification 400 and 1000 ×). (II) Percentage of PMN with acidified vacuoles as detected by MDC staining. Data are representative of six independent experiments and are presented as mean ± SD; Wilcoxon matched-pairs test, *p < 0.05: PMA (bar 2: 86.16 ± 6.49%), rapamycin (bar 3: 65.83 ± 8.47%) treated cells, cells treated with PMA and 3-MA (bar 4: 9.17 ± 0.75%) and cells treated with rapamycin and 3-MA (bar 5: 8.5 ± 1.87%) compared with control conditions (bar 1: 15.67 ± 3.50%). The percentage of cells with acidified autophagosomes in PMN treated with PMA and 3-MA (bar 4) or rapamycin and 3-MA (bar 5) was significantly lower (*p < 0.05) compared with cells treated with PMA (bar 2) and rapamycin (bar 3), respectively. (C) Analysis of LC3B-I to LC3B-II conversion in cells treated with PMA (lane IV) and rapamycin (lane II), as observed by LC3B immunoblotting. Effect of 3-MA in both PMA (lane V) and rapamycin (lane I)-treated cells. Lane III: control cells, and lane VI: cells treated only with 3-MA. (D) Impact of PI-3K inhibition using LY294002 on LC3B conversion after PMA challenge (lane III), (lane I: control cells, lane II: PMA, lane IV: LY294002 only). One representative out of six independent experiments is shown. (E) Immunofluorescence confocal microscopy staining of LC3B. (I) Control cells treated with PBS and serum, (II) PMA-treated cells. 1. DAPI: blue, 2. LC3B: red and 3. merge. (III) Percentage of PMN with LC3B aggregates. Control conditions (bar 1: 17.7 ± 2.4%), 3-MA (bar 2: 3 ± 1.01%), PMA (bar 3: 69 ± 2.1%), PMA and 3-MA (bar 4: 4 ± 1.9%), rapamycin (bar 5: 56 ± 1.6%). Data are representative of five independent experiments and presented as mean ± SD; Student’s t-test, *p < 0.01 compared to the control (bar 1) or the indicated comparision (bar 3 vs 4).
while LC3B aggregates were also detected independently in six independent experiments and presented as mean relative expression of ATG7 mRNA levels. Representative data of ATG7 mRNA expression analysis after treatment with PMA (1.21 ± 0.42) and rapamycin (0.94 ± 0.64). Data are representative of six independent experiments and presented as mean relative expression ± SD. Wilcoxon matched-pairs test, *p < 0.05 and n.s. = not significant compared with control conditions (dot line) as indicated in the figure. Relative expression (folds) of each gene is derived from substitution of DCt values (normalized by GAPDH DCt value) in 2^−ΔΔCT equation [36].

Implication of the inflammatory cytokines TNF-α and IL-1β in autophagy induction

Incubation of human neutrophils with TNF-α had no effect on LC3 lipidation as observed by immunoblotting and formation of acidified autophagosomes (data not shown). On the other hand, treatment with IL-1β at doses of 10 and 100 ng/mL induced the formation of acidified structures (Fig. 4A) and LC3B lipidation (Fig. 4B).

Phagocytosis-dependent induction of autophagy: The paradigm of Escherichia coli

To investigate whether phagocytosis of pathogens and autophagy are related phenomena, PMN were challenged to phagocytose opsonized FITC-conjugated *E. coli* bacteria, and phagocytosis capacity was assessed by flow cytometry. Pre-treatment with 3-MA partially impaired the ability of PMN to phagocytose *E. coli* (Fig. 5A), as did inhibition with LY294002 (data not shown). Subsequently, the induction of the autophagic process in *E. coli*-phagocytosing neutrophils was monitored. Induction of both late autophagosomes (Fig. 5B), LC3B conversion (Fig. 5C) and LC3B aggregate formation (Fig. 5E) in *E. coli*-treated cells was observed. Colocalization of LC3B aggregates with FITC-conjugated *E. coli* bacteria was found (≈40% of phagocytosed bacteria), while LC3B aggregates were also detected independently from *E. coli*-containing phagosomes (Fig. 5E). The above findings were markedly abrogated in 3-MA pretreated cells (Fig. 5C and E). To further discriminate whether the observed inhibitory effect of 3-MA in the induction of autophagy was due to impaired phagocytosis caused by PI3K inhibition or not, neutrophils were treated with 3-MA 15 min after the initiation of phagocytosis. LC3B conversion was also abolished in this set of studies (Fig. 5D). Concerning the expression of ATG genes, a pattern of induction similar to that of cells treated with LPS was observed (Fig. 6).

ROS positively regulate autophagy in human neutrophils

Recently, ROS production has been suggested to mediate LC3 recruitment to phagosomes in phagocytic cells [18]. To elucidate the possible role of ROS production in the autophagic process, human neutrophils were pretreated with the ROS scavengers butylated hydroxyanisole (BHA) or N-tert-buty1-α-phenylnitrone (BNP) prior to stimulation with PMA, a known oxidative burst stimulus. Reduced LC3B conversion was observed in cells pretreated with BHA (Fig. 7A) or BNP (data not shown). Similarly, the induction of autophagy machinery in neutrophils phagocytosing *E. coli* was attenuated in cells pretreated with BHA (Fig. 7A) or BNP (data not shown). On the other hand, the effect of rapamycin on the LC3B conversion was ROS independent, since inhibition of LC3B lipidation in cells pretreated with ROS scavengers could not be detected (data not shown). To investigate the involvement of NADPH oxidase in this process, *E. coli*-phagocytosing neutrophils were pretreated with diphenylene iodonium (DPI), a specific NADPH oxidase inhibitor. Treatment with DPI resulted in inhibition of LC3B conversion (Fig. 7B). Moreover, the percentage of neutrophils with acidified autophagosomes was markedly decreased in cells treated with DPI (Fig. 7C). Concerning TLR activation, even though they have a modest impact on oxidative burst according to previous reports [19, 20] and our observations, pretreatment of cells with BNP precluded the formation of acidified autophagosomes and the induction of LC3B conversion caused by the TLR agonists PGN (Fig. 7D and E), LPS and LTA (data not shown).

The effect of autophagy-related agents in the respiratory burst of human neutrophils was investigated using flow cytometry. It has been previously reported that PMA induces superoxide anion release by both a PI3K-dependent and a PI3K-independent signaling pathway [15]. Accordingly, treatment with 3-MA significantly inhibited the stimulation of the oxidative burst by PMA, as detected by oxidation of dihydrorhodamine 123 (Fig. 7F). Furthermore, no induction of the oxidative burst in neutrophils treated with rapamycin was observed (data not shown).

Discussion

This study aimed to elucidate the involvement of autophagy and its potential triggers in human neutrophils. Immunoblotting was used
as the standard method for detecting endogenous LC3B lipidation, due to technical limitations in transfecting these terminally differentiated and short-living cells with GFP-LC3 [21], while MDC staining for the detection of late autophagosomes was applied. In addition, the contribution of phagocytosis and oxidative burst to the autophagic process was investigated in this cell type. Our findings indicated that autophagy is induced in neutrophils by both a phagocytosis-independent and -dependent manner, thus suggesting two different mechanisms capable of triggering the autophagic machinery. Moreover, an interface between oxidative burst and induction of autophagy and the possible regulation of this mechanism at the transcriptional level was identified.

Figure 3. Effect of TLR activation on the induction of the autophagic machinery. (A) (I) MDC staining in neutrophils treated with LPS, LTA, peptidoglycan (PGN), loxoribin (LOX) and R848 (400 μM). (II) Percentage of PMN with acidified vacuoles as detected by MDC staining, LPS (50 ng/mL) (bar 2: 69.67 ± 8.21%), LTA (bar 3: 75.17 ± 9.80%), peptidoglycan (bar 4: 77.7 ± 6.96%), loxoribin (bar 5: 66.7 ± 5.61%), R848 (bar 6: 69.5 ± 7.45%), poly I:C (bar 7: 16.33 ± 3.14%) and flagellin (bar 8: 19.83 ± 5.16%)-treated cells compared with control conditions (bar 1: 14.5 ± 4.97%). Data are representative of six independent experiments and presented as mean ± SD; Wilcoxon matched-pairs test, *p<0.05, n.s. not significant. (B) LC3B lipidation status of cells treated with LPS (lane I), LTA (lane II), peptidoglycan (lane III), loxoribin (lane IV) and R848 (lane V), detected by immunoblotting. One representative out of six independent experiments is shown. (C) LC3B-II/LC3B-I ratio as obtained from integrated optical density measurements. LTA (bar 2: 1.75 ± 0.84), peptidoglycan (bar 3: 1.82 ± 1.17), LPS (bar 4: 1.9 ± 1.7), loxoribin (bar 5: 1.32 ± 0.49) and R848 (bar 6: 1.4 ± 0.77) compared with control neutrophils (bar 1: 0.76 ± 0.30). Data are representative of six independent experiments and are presented as mean ± SD. Wilcoxon matched-pairs test, *p<0.05. (D) Analysis of LC3B gene expression levels in neutrophils treated with TLR agonists. LPS (bar 1: 2.27 ± 0.43), peptidoglycan (bar 2: 2.07 ± 0.42), LTA (bar 3: 2.1 ± 0.49) and R848 (bar 4: 1.89 ± 0.35) compared with medium-treated control cells. Dotted line represents mRNA levels of medium-treated control cells. Data are representative of six independent experiments and are presented as mean relative expression ± SD. Wilcoxon matched-pairs test, *p<0.05. (E) Assessment of ATG gene expression levels in PMN after LPS stimulation (50 ng/mL). ATG3 (bar 1: 2.94 ± 0.52), ATG4 (bar 2: 1.72 ± 0.42), ATG5 (bar 3: 1.84 ± 0.34), ATG6 (bar 4: 1.8 ± 0.3), ATG7 (bar 5: 3.13 ± 0.64) and LC3B (bar 6: 2.27 ± 0.43) compared with control cells. Dotted line represents mRNA levels of medium-treated cells. Data are representative of six independent experiments and are presented as mean relative expression ± SD. Wilcoxon matched-pairs test, *p<0.05.
Formation of MDC positive autophagosomes

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<th>LTA</th>
<th>R848</th>
<th>Loxo</th>
<th>Poly I: C</th>
<th>Flagellin</th>
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LC3B conversion

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Upregulated expression of ATG genes

level was determined. The question arising, however, is whether neutrophils exhibiting these characteristics share anti-inflammatory properties or induce excess inflammation.

Concerning the correlation of autophagy with PMN, the formation of “autophagy-like” double membrane vacuoles has been reported in GM-CSF-primed and anti-Siglec-9 mAb-stimulated neutrophils related to non-apoptotic cell death, while cytoplasmic vacuolization is observed in sepsis and joint fluid neutrophils from patients with rheumatoid arthritis [17]. Moreover, recent data indicating the detection of LC3B-II in phagosomes from bone marrow-derived murine neutrophils [18] and the implication of mTOR in innate immune responses [22], provide further evidence for the linkage between innate immunity and autophagy.

In this study, we provide evidence for the induction of the autophagic process after activation of human granulocytes and its implication in neutrophil biology. Along with rapamycin, we simulated granulocyte activation using PMA. PMA induces neutrophil vacuolization and superoxide anion release [15], while ROS production has been implicated in autophagy by regulating the activity of ATG4 [23]. As shown for cells treated with rapamycin, neutrophil stimulation with PMA resulted in enhanced LC3B lipidation in a PI3K pathway-dependent manner, as observed after treatment with 3-MA or LY294002. ROS inhibition partially suppressed the induction of the autophagic flux in PMA-stimulated neutrophils, providing evidence for the implication of oxidative burst in the autophagic process in this phagocytosis-independent experimental model. On the contrary, ROS inhibition did not affect the induction of autophagy in cells treated with rapamycin.

Considering that PMA and rapamycin are not natural stimuli, we next focused on the stimulation with natural agonists. LC3B recruitment has been previously observed in phagosomes of murine neutrophils in a ROS-dependent manner [18]. More specifically, LC3B-II was present on phagosomes containing IgG-coated, LPS-coated and zymozan-coated beads but not uncoated or BSA-coated beads. In agreement with this observation, we detected enhanced LC3B conversion during phagocytosis of E. coli in human neutrophils, a process that required NADPH oxidase activation and generation of ROS. Moreover, the decreased granulocyte phagocytic ability, which is in accordance with previous reports [24] and the complete abolishment of LC3B conversion after PI3K inhibition with 3-MA or LY294002, further connects autophagy to phagocytosis. TLR activation mediates pathogen recognition during phagocytosis. The involvement of TLR4 activation in the induction of autophagy during phagocytosis of E. coli was assessed in LPS stimulation studies, providing a possible linkage between pathogen phagocytosis and autophagy, mediated by TLR activation. There are controversial reports about the ability of TLR agonists to induce the autophagic process [25–28]. Even though phagocytosis of LPS-coated beads activates the autophagic machinery in murine neutrophils [18], we were unable to observe significant activation of autophagy flux in human neutrophils stimulated with high dose soluble LPS, but the results were more distinguishable in lower doses. The fluctuation in LC3B activation after stimulation with LPS could be attributed to enhanced NF-κB pathway activity, which is an inhibitor of autophagy activation [12], at these high concentrations. The inability of TNF-α to enhance LC3B conversion is also in accordance with this observation. Moreover, the screening of several other TLR agonists revealed significant induction of LC3B lipidation after stimulation with agonists of TLR2, TLR2/6, TLR7
and TLR7/8, with no effect after TLR5 and TLR3 activation. The absence of LC3B conversion after stimulation with flagellin is in accordance with a previous report [25], while it has been previously reported that TLR3 is not expressed in human neutrophils [19]. As a result, we suggest that selective TLR activation is able to induce the autophagic machinery in human neutrophils.

Autophagy is considered to be a survival mechanism preserving cell integrity in periods of cell stress. On the other hand, when autophagy is not appropriately regulated, it may lead to a
non-apoptotic and non-necrotic cell death [29]. To further implicate autophagy in cell death, it has been recently suggested that Beclin-1, a central component of the autophagic machinery, negatively regulates apoptosis [30] but what is the role of autophagy inducers in neutrophil survival? PMA has been reported to induce non-apoptotic cell death, while ROS inhibition prevented this cytotoxic effect [16]. Furthermore, neutrophils from patients with a genetic defect in membrane-associated NADPH oxidase resulting in impaired ROS production demonstrate delayed constitutive apoptosis and Fas resistance [31, 32]. Based on the correlation between ROS and cell death, we presume that the autophagic machinery is activated during ROS production in order to encounter the possible oxidative burst-dependent cell damage and death. As a result, in the case of potent ROS inducers, like PMA, cells are finally driven to cell death due to inability of autophagy to preserve cell homeostasis. On the other hand, TLR activation does not result in excess oxidative burst [19, 20]. In this case, the induction of autophagy is capable of encountering ROS production and enhances cell survival, as suggested by the previously reported inhibition of apoptosis in neutrophils after TLR activation [33]. Considering the above and the regulatory role of apoptosis in both duration and intensity of the inflammatory process [4], we propose that the pro-survival induction of autophagy in neutrophils enhances the inflammatory responses by delaying cell death and could be involved in the pathogenesis of several acute and chronic inflammatory diseases related to suppression of apoptosis, leading to tissue injury.

To clarify the possibility of regulation of autophagy cascade at transcriptional level, analysis of ATG genes mRNA expression levels in human neutrophils was performed. A uniform upregulation of LC3B gene expression after treatment with various activators of the autophagic process was observed, suggesting that except for LC3B lipidation, LC3B gene transcription might play an additional role, independent of stimulus variations. On the other hand, we observed a stimulus-dependent regulation of several other ATG genes in a non-repeatable pattern. These data suggest that except from LC3B, it is not yet feasible to apply the measurement of certain ATG mRNA levels as a marker of autophagy in neutrophils, even though a transcriptional level of regulation is indicated. Our gene expression results were in accordance with recent reports suggesting a role for altered expression of ATG genes in the regulation of the autophagic process [34]. Thus, besides the uniform upregulation of LC3B gene expression after induction of the autophagic process in human neutrophils, further studies elucidating the regulation pathways of expression of ATG genes and the correlation between the pattern of expression of ATG genes and the regulation of the autophagic process in clinical models are needed before making any definitive conclusion regarding the role of expression of ATG genes in the induction of the autophagic mechanism in neutrophils.

In conclusion, we suggest that the autophagic machinery plays a crucial role in granulocyte functions, linked with both phagocytosis and oxidative burst and regulated by TLR activation. It is imperative to further investigate the implication of autophagy in neutrophil biology in order to better understand its role in the neutrophil-driven inflammatory process and innate immune functions. Translational research based on inflammatory kinetics of "nature experiments," like sepsis and other neutrophil-associated inflammatory syndromes, may offer additional data regarding the pathophysiological role of autophagy in the inflammatory process, thus providing a potential application for therapeutic regulation of inflammation.

Materials and methods

Neutrophil isolation from peripheral whole blood

Heparinized blood was collected from healthy donors and PMN were isolated as previously described [35]. Informed, written consent was obtained from every volunteer. The study protocol design was in accordance to the Declaration of Helsinki and the procedures have been approved by the local ethics committee. All the substances used in this study were endotoxin free, as determined by a Limulus amebocyte assay (Sigma-Aldrich).

Stimulation and inhibition studies

Neutrophils were incubated for 60 min at 37°C in a total volume of 500 μL of PBS with the addition of serum from a healthy donor at a dilution of 1/10 in order to avoid starvation-induced autophagy, unless mentioned. At this time point, the most prominent effect on LC3B lipidation after treatment with inducer of autophagy was observed. PMN were stimulated with PMA (Sigma-Aldrich, 40 ng/mL), rapamycin (Calbiochem, San Diego, CA, USA, 500 nM), E. coli O26:B6-derived LPS as a TLR4 agonist (Sigma Aldrich, 10, 50, 100 ng/mL or 1 μg/mL), R848 as a
TLR7/8 agonist (Invivogen, San Diego, USA, 5 μg/mL), poly I:C as a TLR3 agonist (Invivogen, 1 μg/mL), flagellin from *Bacillus subtilis* as a TLR5 agonist (Invivogen, 0.5 μg/mL), LTA from *Staphylococcus aureus* (Invivogen, 1 μg/mL) and PGN from *S. aureus* (Invivogen, 5 μg/mL) as a TLR2 agonist and TLR2/6 agonist, respectively, loxoribin as a TLR7 agonist (Invivogen, 1 mM), recombinant TNF-α (Sigma-Aldrich, 5 ng/mL) and recombinant IL-1β (Sigma-Aldrich, 1 and 10 ng/mL). Concerning the PI3K inhibition studies, cells were pretreated for 15 min with 3-MA (Calbiochem, 5 mM) or LY 294002 (Calbiochem, 50 μM) at 37°C, while 3-MA was administered 15 min after the initiation of phagocytosis when studying the effect of PI3K inhibition in the induction of autophagy without interrupting phagocytosis of *E. coli*. In the group of studies based on phagocytosis of opsonized *Escherichia coli*, an absolute number of 4 x 10^7 opsonized bacteria (1/20 PMN to bacteria ratio) (Phagoburst, Phagotest; ORPEGEN Pharma, Heidelberg, Germany) were used. To block the effect of ROS release, cells were pretreated for 15 min at 37°C with BHA prior to PMA or *E. coli* stimulation (lane I: PMA, lane II: PMA + BHA, lane III: *E. coli*, lane IV: *E. coli* + BHA, lane V: control cells). One representative out of four independent experiments is shown. (B) LC3B conversion status after pretreatment with the NADPH oxidase inhibitor DPI in *E. coli*-phagocytosing neutrophils (lane I: PBS, lane II: *E. coli*, lane III: *E. coli* + DPI). One representative out of four independent experiments is shown. (C) Percentage of PMN with acidified vacuoles as detected by MDC staining. *E. coli*-phagocytosing PMN (74.83 ± 9.09%) and PMN treated with DPI before incubation with opsonized *E. coli* (77.17 ± 8.33%). Data are representative of six independent experiments and presented as mean ± SD; Wilcoxon matched-pairs test, *p* < 0.05. (D) Effect of NADPH-oxidase inhibition with DPI in the formation of acidified autophagosomes in *E. coli*-phagocytosing neutrophils. Percentage of PMN with acidified vacuoles as detected by MDC staining. *E. coli*-phagocytosing PMN (77.17 ± 8.33%) and PMN treated with DPI before incubation with opsonized *E. coli* (21.67 ± 4.37%). Data are representative of six independent experiments and presented as mean ± SD; Wilcoxon matched-pairs test, *p* < 0.05. (E) Alterations in LC3B conversion in BNP pretreated, peptidoglycan-stimulated neutrophils (lane III), (lane I: medium-treated cells, lane II: peptidoglycan-treated cells). One representative out of four independent experiments is shown. (F) Effect of PI3K inhibition with 3-MA in the percentage of PMA-induced oxidizing cells. PMA (99.41 ± 0.33%) and PMA + 3-MA (15.82 ± 3.97%) compared with control conditions (14.67 ± 3.20%). Data are representative of six independent experiments and presented as mean ± SD; Wilcoxon matched-pairs test, *p* < 0.05.

Figure 7. ROS inhibition studies and their impact on neutrophil autophagic activity. (A) LC3B conversion status after pretreatment with the ROS scavenger BHA prior to PMA or *E. coli* stimulation (lane I: PMA, lane II: PMA + BHA, lane III: *E. coli*, lane IV: *E. coli* + BHA, lane V: control cells). One representative out of four independent experiments is shown. (B) LC3B conversion status after pretreatment with the NADPH oxidase inhibitor DPI in *E. coli*-phagocytosing neutrophils (lane I: PBS, lane II: *E. coli*, lane III: *E. coli* + DPI). One representative out of four independent experiments is shown. (C) Percentage of PMN with acidified vacuoles as detected by MDC staining. *E. coli*-phagocytosing PMN (77.17 ± 8.33%) and PMN treated with DPI before incubation with opsonized *E. coli* (21.67 ± 4.37%). Data are representative of six independent experiments and presented as mean ± SD; Wilcoxon matched-pairs test, *p* < 0.05. (D) Effect of NADPH-oxidase inhibition with DPI in the formation of acidified autophagosomes in *E. coli*-phagocytosing neutrophils. Percentage of PMN with acidified vacuoles as detected by MDC staining. *E. coli*-phagocytosing PMN (77.17 ± 8.33%) and PMN treated with DPI before incubation with opsonized *E. coli* (21.67 ± 4.37%). Data are representative of six independent experiments and presented as mean ± SD; Wilcoxon matched-pairs test, *p* < 0.05. (E) Alterations in LC3B conversion in BNP pretreated, peptidoglycan-stimulated neutrophils (lane III), (lane I: medium-treated cells, lane II: peptidoglycan-treated cells). One representative out of four independent experiments is shown. (F) Effect of PI3K inhibition with 3-MA in the percentage of PMA-induced oxidizing cells. PMA (99.41 ± 0.33%) and PMA + 3-MA (15.82 ± 3.97%). Data are representative of six independent experiments and presented as mean ± SD; Wilcoxon matched-pairs test, *p* < 0.05.
with DPI (Sigma-Aldrich 10 \mu M) and BHA (Sigma Aldrich, 100 \mu M) or BNP (Sigma Aldrich, 50 \mu M). ROS production was assessed by flow cytometry-based assay using Phagoburst (ORPEGEN Pharma).

**RNA isolation, cDNA synthesis and quantitative real-time PCR**

RNA isolation and cDNA synthesis was performed as previously described [35]. PCR primers and conditions are included in Table 2. Relative expression levels were determined using the 2-DDCT method [36].

**Western blot analysis**

Western blotting was performed as previously described [35]. Overnight incubation at 4°C with LC3B pAb (1/1000; L7543 Sigma) was performed, followed by probing with HRP-conjugated secondary antibody (1/2000; HAF008 RnD Systems) for 1 h at room temperature.

**Quantitation of phagocytosis in blood neutrophils**

The ability of blood neutrophils to conduct phagocytosis was assessed by commercially available flow cytometry-based assay (Phagotest; ORPEGEN Pharma). Cells were analyzed in a FACScan flow cytometer (BD Biosciences). MFI was applied in order to quantify phagocytic activity.

**Densitometric analysis**

Densitometric analysis was performed using Gel Pro Analyser 3.1 on the scanned films after immunoblotting. The integrated optical density of each blot lane was measured.

**Fluorescence and confocal microscopy**

To observe acidified autophagosomes (autophagolysosomes), MDC staining was used as previously described [37]. Neutrophils were cultured with 0.05 mM MDC at 37°C for 30 min. The number of vacuolated neutrophils, MDC or LC3B positive cells was determined by examining 100 cells/sample. The analysis was performed in duplicate with samples blind coded.

To observe LC3B aggregate formation, treated cells were attached to glass slides prior to microscopy using Shandon cytofix III and stained for LC3B. Briefly, cells were washed and fixed with 3.7% paraformaldehyde and ice cold MetOH, blocked in 4% serum and incubated in 1/50 anti-LC3B pAb (Abgent, San Diego, CA, USA), detected by 1/60 Cy3 conjugated donkey anti-goat 2nd antibody and counterstained with DAPI for nuclear staining. Slides were viewed and images were acquired on a Zeiss LSM510 laser scanning confocal microscope coupled to a Zeiss 200 M axiovert with a 63× Apochromat oil lens (Zeiss).

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**Table 2.** Primer sequences\(^a\) and real-time RT-PCR conditions\(^b\)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence of primers</th>
<th>rt RT-PCR conditions</th>
</tr>
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<tr>
<td>hVPS34</td>
<td>FRD: 5'-TGGAACTGGAATGAATGGC-3’</td>
<td>(i) 52°C for 5 min</td>
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<tr>
<td></td>
<td>REV: 5'-GCATCCCTTGGCCGAAAC-3’</td>
<td>(ii) 95°C for 2 min</td>
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<tr>
<td>ATG16L1</td>
<td>FRD: 5'-TCCAGGGAGGGCGCCAAG-3’</td>
<td>(iii) 40 cycles of:</td>
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<td></td>
<td>REV: 5'-ATCAGAATTCTATCCACATTG-3’</td>
<td>• 95°C for 15 s</td>
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<tr>
<td>ATG12</td>
<td>FRD: 5'-CTCTATGAGTTTGGCCAGT-3’</td>
<td>• 52°C for 40 s</td>
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<tr>
<td></td>
<td>REV: 5'-ATCAGATCTTTAATCTTCTG-3’</td>
<td>• 52°C for 5 min</td>
</tr>
<tr>
<td>MAP1LC3B</td>
<td>FRD: 5'-CGGTGATAATAGAAGCATCAAGG-3’</td>
<td>(iv) melting curve</td>
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<td></td>
<td>REV: 5'-CTGAGATTGGTGGAGAGG-3’</td>
<td>analysis</td>
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<tr>
<td>ATG7</td>
<td>FRD: 5'-AGGAGATTCAACCAGAGACC-3’</td>
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<td>REV: 5'-GCACAGAGCCCAAGAGAG-3’</td>
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<td>BECLIN1 (ATG6)</td>
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<td>REV: 5'-GCCCTTTCCATCCATCC-3’</td>
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<td>ATG5</td>
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<td>ATG4B</td>
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<td>REV: 5'-TCACCAGCGACATCATG-3’</td>
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<td>GAPDH</td>
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<tr>
<td></td>
<td>REV: 5'-CATCAGCCCCACTTTGTTTGG-3’</td>
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</table>

\(^a\) Designed by Beacon Designer™ ver. 4.0.

\(^b\) Real-time PCR was performed using SYBR Green qPCR Master Mix (2-) gene expression master mix (Fermentas, St. Leon-Rot, Germany) on a Chromo4™ Real-Time Detector (Bio-Rad, CA, USA).

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Microimaging, Thornwood, NY, USA). Images were processed using LSM Image Browser (Zeiss, Version 4.0.0.241). Data are presented as the percentage of LC3B positive cells [38].

### Statistical analysis

Values are presented as mean±SD. Statistical analyses were performed using Wilcoxon matched-pairs test and Student's t-test for paired samples to compare differences in means. The significance level was set to p<0.05. Data were processed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA, USA) and OriginPro8.

### Acknowledgements

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### Conflict of interest

The authors declare no financial or commercial conflict of interest.

### References


Abbreviations: 3-MA: 3-methyladenine · Atg: autophagy related · BHA: butylated hydroxyanisole · BNP: N-tert-butyl-a-phenylnitrone · DPI: diphenylene iodonium · LTA: lipotechoic acid · MDC: monodansylcadaverine · mTOR: mammalian target of rapamycin · PGN: peptidoglycan

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