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Autophagy and its Consequences for Platelet Biology

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Abstract

Autophagy, the continuous recycling of intracellular building blocks, molecules, and organelles is necessary to preserve cellular function and homeostasis. In this context, it was demonstrated that autophagy plays an important role in megakaryopoiesis, the development and differentiation of hematopoietic progenitor cells into megakaryocytes. Furthermore, in recent years, autophagic proteins were detected in platelets, anucleate cells generated by megakaryocytes, responsible for hemostasis, thrombosis, and a key cell in inflammation and host immune responses. In the last decade studies have indicated the occurrence of autophagy in platelets. Moreover, autophagy in platelets was subsequently demonstrated to be involved in platelet aggregation, adhesion, and thrombus formation.

Here, we review the current knowledge about autophagy in platelets, its function, and clinical implications. However, at the advent of platelet autophagy research, additional discoveries derived from evolving work will be required to precisely define the contributions of autophagy in platelets, and to expand the ever increasing physiologic and pathologic roles these remarkable and versatile blood cells play.

Keywords

Autophagic flux; autophagosome; autophagy; megakaryocytes; platelets; thrombopoiesis

Introduction

For the longest time, the research community believed that proteins are stable *in vivo* and that the degradation of cellular constituents would not be a major factor in cellular protein homeostasis [1].

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However, in 1963, C. de Duve used the term “autophagy” (from the Greek words *auto* meaning ‘self’ and *phagein* meaning ‘to eat’) for the first time in describing the process of the delivery of cytoplasmic materials via double membrane vesicles to the lysosomal degradation machinery [2]. In 1997, the identification of the first autophagy-specific gene and its gene product, *APG1* and Apg1, respectively, were described and characterized [3]. Subsequently, using yeast mutant studies, more than thirty autophagy-related (ATG) genes were identified, and a unified nomenclature was introduced [4]. Ultimately, almost all yeast ATG proteins were found to have counterparts in mammalian cells (reviewed in [5]). Furthermore, while additional proteins are required for autophagy in mammals, the basic sequence and hierarchical structure of autophagic events have been conserved when compared between unicellular and multicellular eukaryotes [5, 6].

In eukaryotic cells, autophagy is a highly conserved catabolic process. The initial process of autophagy consists of membrane remodeling, crescent-shaped phagophore formation, and subsequent building of double-membrane vesicles, coined autophagosomes (reviewed in [7]). Autophagy allows for the targeted sequestration of cellular components or even organelles into autophagosomes followed by the breakdown of such intracellular cargo once lysosomes have fused with autophagosomes, forming degrading autolysosomes [5, 7, 8] (Figure 1). Therefore, autophagy is also a primary mechanism for replenishing the pool of biosynthetic precursors and energy sources, by recycling cytosolic contents during starvation [9]. There are three primary entities of autophagy: microautophagy, macroautophagy, and chaperone-mediated autophagy, a mechanism reserved to occur in mammalian cells only. While both, micro- and macroautophagy can be non-selective (i.e. bulk) degradation processes triggered by starvation, autophagy is usually tightly regulated, allowing for the recycling of harmful or unneeded cellular material, including damaged mitochondria, protein aggregates, excess ribosomes, lipid droplets, or intracellular pathogens [10, 11]. Therefore, autophagy is considered to play a mainly protective role in cellular responses. Nevertheless, there is increasing evidence of significant crosstalk between autophagy, necroptosis, and the apoptotic pathway utilizing common signaling molecules coordinating the negative and/or positive regulation of these pathways [12, 13].

Autophagy directly contributes to the cellular metabolism; however, its functions are multifaceted, and still incompletely understood. Besides the recycling function, autophagy is actively involved in eliminating misfolded or damaged protein, therefore, playing a crucial role in protein quality control (reviewed in [1]). Autophagy was first demonstrated to be involved in tumorigenesis by Dr. Levine [14], and has since than developed into a prime research target for advanced tumor therapy approaches [15-17]. Furthermore, autophagy is known to be intricately involved in the physiology and pathophysiologic processes of the cardiovascular system [18, 19], central nervous system [20, 21], and liver functions [22, 23]. In addition, our understanding of the important functions of autophagy during inflammatory states, host infections, and immunity has dramatically increased in recent years. Autophagy can act as a defense mechanism against bacterial and viral invasion [24, 25], by counteracting microbial invasions, aiding in the active elimination of intracellular bacteria and viruses, supporting antigen presentation, and removal of damaged protein complexes or organelles to maintain homeostasis in the infected organism [26]. This demonstrates that autophagy may be a primordial form of eukaryotic innate immunity

against invading microorganisms [24, 25], and furthermore underscores the full integration of autophagy and immunity into one defense system [24, 25].

In this context it is important to highlight that autophagy is an integral part of host responses to sepsis [27], serving in a protective role by preserving mitochondrial integrity, potentially controlled by dynamin-related protein 1 (DRP1) dependent mechanisms [28], which will lead to reduced apoptotic cell death [26, 29]. In experimental animal models of sepsis, tissue autophagic activity was increased during the initial phases of sepsis [29-31], but declined several hours into the induction of sepsis [29, 31, 32]. In light of these results, autophagy is targeted for therapeutic interventions in efforts to restore dysregulated immune reactions [33-35].

Here, we will review the autophagic functions involved in thrombopoiesis, and furthermore, discuss the discovery and characteristics of autophagy in platelets. We will review the functional significance and consequences of autophagy in platelets, and lastly give an overview over common methods used to study autophagy in platelets.

Autophagy is a regulatory determinant of megakaryo- and thrombopoiesis

Megakaryopoiesis is defined as the development and differentiation of megakaryocytes from hematopoietic progenitor cells [36]. In contrast, thrombopoiesis, the development of blood platelets, represents a complicated process mostly residing in the bone marrow [37]. The current model of platelet formation is based on several elegant studies demonstrating that the large progenitor cells, megakaryocytes (MKs), release platelets by extending long, branching processes, designated proplatelets, into sinusoidal blood vessels [36, 38-40].

The interplay between several transcription factors (i.e., SCL, GATA1, RUNX1, GATA2, NF-E2, FOG1, PU.1, and Fli-1) regulate the development and differentiation of megakaryocytes [41-44]. In addition, genetic studies performed on TPO/c-Mpl, first discovered and cloned in 1994, demonstrated that the TPO/c-Mpl system is the primary rheostat for thrombopoiesis. Solar et al. showed that the elimination of either TPO or c-Mpl led to severe thrombocytopenia, due to reduced hematopoietic progenitors and mature megakaryocytes, as well as additional reduced maturation in remaining viable megakaryocytes [45].

In 1976, TEM studies performed by Lewis et al. demonstrated the presence of autophagosome-like vacuoles inside platelets isolated from patients with carcinoid syndrome, however, such intracellular structures were not detected in megakaryocytes isolated from the same patient cohort [46]. In contrast, intracellular structures in megakaryocytes resembling autophagosomes were detected in canine bone marrow samples, and attributed to cell recycling processes [47].

Subsequent research efforts utilizing megakaryocytic samples from patients with idiopathic thrombocytopenic purpura (ITP) revealed extensive cytoplasmic vacuoles, suspected to be autophagy related. Houwerzijl et al. discussed the possibility of ITP patients demonstrating a state of compensatory increased megakaryopoiesis and therefore an increased metabolic demand and relative nutrient deficiency. Therefore, in megakaryocytes under such

circumstances, autophagy was suspected to be a mechanism for recycling and generating energy to maintain the increased cellular metabolism [48]. This hypothesis was supported by the detection of extensive cytoplasmic vacuole formation in such cells. In recent years, the essential role of autophagy for megakaryopoiesis was demonstrated. Furthermore, several authors showed that autophagy is an important aspect of the pathophysiology of ITP, and the potential of targeting autophagy for therapeutic approaches will be of future interest to complement existing treatments [49, 50].

Using a hematopoietic conditional knockout mouse for *Atg7* (*Atg7*$^{f/f;}$ *Vav*$^{Cre+}$), investigators were able to demonstrate a reduced number of megakaryocytes in the bone marrow of Atg7 (autophagy related 7, an autophagy protein integral for proper functions) deficient animals [51]. Furthermore, decreased thrombopoiesis, exclusively observed in knockout animals, was reflected in reduced platelet numbers and irregular platelet size, potentially caused by a failure in abscission, leading to the accumulation of premature or large platelets. However, it is important to point out that the genetically induced defect in autophagy affected the entire hematopoietic system, and was not megakaryocyte-lineage specific. Therefore, off-target effects on other cells in the bone marrow niche could not be completely excluded. Ouseph and colleagues partially addressed this limitation by using a megakaryocytic and platelet specific Atg7 knockout mouse model (*Atg7*$^{f/f;}$*PF4*$^{Cre+}$), which deletes *Atg7* at a later stage in platelet production [52]. While not able to detect differences in platelet counts, the importance of autophagy for proper thrombopoiesis was reflected in diminished hemostasis and thrombus formation. These findings were furthermore supported by studies performed by Wang and colleagues [53]. Upon induction of autophagy by rapamycin treatment, or its inhibition by bafilomycin A1 (Baf A1) in early stages of megakaryocyte development, they were able to demonstrate a reduction in high ploidy megakaryocytes, resulting in altered proplatelet formation and subsequently decreased platelet numbers. In addition, using the aforementioned *Atg7*$^{f/f;}$*PF4*$^{Cre+}$ mouse model, the authors were able to recapitulate Ouseph's [52] observations of preserved proplatelet formation and normal platelet counts.

Based on the available evidence it seems reasonable to conclude that an abnormal level (up- or down-regulation) of autophagy causes differential effects during specific and distinct stages of megakaryocyte and hematopoietic progenitor cell differentiation. The timing of the interventional treatment needs to be carefully monitored, and results need to be interpreted in the developmental context of megakaryocytes.

Discovery and characterization of autophagy in platelets

Platelets are anucleate bodies, and the chief effector cells in physiologic hemostasis and pathologic thrombosis [54, 55]. Besides such best-known biologic functions, platelets are versatile and were demonstrated to perform additional activities in host response to injury, inflammation and infectious disease [56]. To perform such specialized tasks platelets are invested by megakaryocytes with intricate machinery involved in mRNA splicing [57, 58], protein translation [59], and the utilization of a vast transcriptome [60-63]. To further fulfill their multitude of biologic tasks, platelets use intricate regulatory pathways [57-59, 64-69] to sense and react to external cues, and to adapt appropriately.

After Lewis et al. discovered autophagosome-like vacuoles inside platelets [46], it still took several decades until the research community gathered increased insight into platelet autophagy. In 2014, Jiang and co-workers reported that rapamycin treatment in a rat thrombosis model promoted platelet adhesion to endothelial cells in an autophagy dependent manner [70]. It is interesting to speculate if parts of that effect could be attributed the inadvertent induction of autophagy in platelets, and not endothelial cells only. Next, autophagy gene transcripts were shown to be present in platelets [62]. However, the first comprehensive description of the autophagic machinery being constitutively expressed in human platelets, and demonstration that autophagy is required for fundamental platelet functions was published by Feng and colleagues [71]. Using immunoblotting and immunocytochemistry/immunofluorescence techniques numerous components of the autophagy protein complexes could be detected, including LC3, SQSTM1, ATG7, ATG12-ATG5 conjugate, and BECN1. In addition, partial colocalization of LC3-positive particles with either the lysosomal marker LAMP1 or with SQSTM1 strongly suggested the detection of autophagosomes/autolysosomes [71, 72]. The list of autophagy proteins being present in platelets was subsequently expanded [52]. Human platelets were reported to also express components of the Ulk1 complex (e.g., Ulk1 and FIP200), the Beclin 1-Vps34 complex (e.g., BECN1, Vps34, Vps15, Atg14L, Nrbf2, and UVRAG), and constituents of the previously characterized [73] lysosome-autophagosome fusion machinery (e.g., EPG5 and RAB7 [72]; see Table 1 for a more comprehensive listing). In addition, complementing the *in situ* detection of endogenous LC3 in platelets [71, 72], Ouseph and colleagues utilized transgenic mice expressing GFP-fused autophagy markers (LC3, Atg5, BECN1) showing diffuse and punctate staining patterns in isolated platelets by confocal fluorescent microscopy, demonstrating the presence of autophagosomes. Using super-resolution microscopy, the accumulation of LC3 in human platelets treated with the autophagy inducing agent rapamycin, and more pronounced after Baf A1 treatment, were documented [72] (Figure 2). In several studies, double-membraned structures enclosing cellular content and organelles, most likely resembling autophagosomes, were clearly visible by electron microscopy inside human platelets under resting or stressed conditions [52, 72, 74, 75]. Mitochondria-containing autophagosomes (i.e., mitophagy) were observed in platelets isolated from mice exposed to hypoxic conditions [76]. Finally, the analysis of Vici syndrome patient platelets, a rare and severe, recessively inherited congenital disorder due to loss-of-function mutations in *EPG5* [77], revealed the accumulation of LC3-positive particles [72]. This finding suggested that the number of autophagosomes is increased in Vici syndrome patient platelets, and pointed towards platelets being similarly affected by the defect in autophagy induced by *EPG5* mutations as are other tissues, resulting in a failure to clear late-stage autophagosomes.

In summary, the descriptive data clearly display that autophagy genes and protein machinery are expressed in human and mouse platelets. Furthermore, cellular structures resembling the continuum from phagophore to autolysosome are readily detectable in resting, stimulated, stressed, or diseased platelets.

Functional consequences of autophagic activity in platelets

Nucleated eukaryotic cells constitutively express a basal activity of their autophagic machinery to maintain cellular hemostasis. Autophagic processes in nucleated cells are also tightly controlled, but are further upregulated under certain stress situations (i.e., starvation, inflammation, tumorigenesis). Here we will shed light on and summarize the known functional significance of autophagy in anucleate platelets.

In initial studies, the basally present autophagic flux in human platelets could be modified by starvation, and treatment with the MTOR complex 1 (MTORC1) inhibitor, rapamycin, resulting in accumulation of LC3-positive particles in a punctate staining pattern [71], and decrease in SQSTM1. SQSTM1 is a selective autophagic adaptor, which is incorporated into autophagosomes and is degraded alongside the autolysosomal cargo [78]. Treatment of human or mouse platelets with known and well characterized platelet agonists, namely thrombin, convulxin, PAR1 peptide, ADP, collagen, and U46619 (thromboxane A_2 (TP) receptor agonist) also resulted in alterations of the autophagic flux, indicated by a reduction in LC3II levels [52], detected by means of western blotting.

The ability to modify platelet autophagic flux by different agonists and external stressors suggested an involvement of autophagy in vital platelet functions. The use of common inhibitors of autophagy (Baf A1 or chloroquine), targeting lysosomes, weakened platelet aggregation and reduced platelet adhesion in a human blood perfusion model indicating of constitutive autophagy being required for platelet aggregation [71]. These experimental results were extended utilizing a variety of mouse models with defective autophagy, including platelet specific Atg7 knockout ($Atg7^{f/f};PF4^{Cre+}$) [52], heterozygous BECN1 disruption ($BECN1^{+/-}$) [71], platelet specific Atg5 knockout ($Atg5^{f/f};PF4^{Cre+}$) [76], and platelet specific Vps34 knockout ($Vps^{f/f};PF4^{Cre+}$) [79, 80]. All genetically modified animal models demonstrated a defect in platelet aggregation induced by a variety of commonly used agonists. The knockout of Atg7 and the disruption of BECN1 resulted in a modest defect in aggregation. In contrast, Atg5 and Vps34 knockout produced a pronounced aggregation defect in affected platelets. It is interesting to note that Vps34 knockout animals demonstrated normal tail bleeding time, while the genetic impairment of autophagy via Atg7 and BECN1 resulted in an increased tail bleeding time. The importance of autophagy in platelets for controlled hemostatic and thrombotic functions was further underscored by demonstrating reduced platelet adhesion in blood perfusion models and extended time to occlusion in a $FeCl_3$-induced carotid injury model [71, 79, 80]. Defective autophagy also resulted in morphologic changes, reflected by decreased platelet spreading [76]. Surprisingly, under starvation conditions, a state of induced autophagy in platelets, Paul and colleagues were able to also demonstrate reduced platelet aggregation [81]. This could indicate that a balanced presence of autophagy is important for proper hemostatic and thrombotic capacity of platelets.

The importance of platelet autophagic functions was further demonstrated in clinically relevant conditions and disease states.

Diabetes Mellitus:

Lee and colleagues reported significant mitophagy, the selective degradation of mitochondria by autophagy, in platelets isolated from diabetic patients [75]. They elegantly demonstrated that platelet mitophagy induction served as a platelet protective mechanism. Diabetes mellitus induced hyperglycemia leads to a pronounced oxidative stress, phosphorylation of p53, resulting in mitochondrial dysfunction and apoptosis [82]. This mechanism was reflected in platelets isolated from diabetic patients, where platelet mitophagy was induced by increased reactive oxygen species, and signaling pathways through JNK activation. The removal of damaged mitochondria by mitophagy, led to decreased p53 phosphorylation and subsequent prevention of platelets progressing towards apoptosis. Inhibition of mitophagy, using a PINK1 knockout mouse, resulted in increased vessel thrombosis in a $FeCl_3$-induced carotid injury model, further highlighting the importance of this platelet protective mechanism.

Hypoxia/Ischemia:

FUNDC1 (a mitophagy receptor located on the outer mitochondrial membrane) knockout mice and control animals were exposed to hypoxia [76]. Genetically non-modified animals demonstrated increased markers of autophagy induction in platelets (LC3 conversion) as well as enhanced signs of mitophagy, detected by immunoblot analysis and documentation of autophagosomes containing mitochondria via transmission electron microscopy. This was contrasting experiments performed with FUNDC1 knockout platelets, showing significantly reduced mitophagy activity. Autophagy was demonstrated to be mediated by direct interaction of LC3 with FUNDC1, as confirmed by co-immunoprecipitation experiments. Hypoxic induction of mitophagy in wildtype platelets significantly reduced platelet aggregation, most likely by the depletion of damaged mitochondria. In contrast, platelets from Atg5 knockout ($Atg5^{f/f};PF4^{Cre+}$) mice could not induce the hypoxia-dependent mitophagic processes, and demonstrated compromised platelet aggregation. Finally, following ischemia and reperfusion injury of the heart, animals with platelet specific inhibition of mitophagy, due to FUNDC1 knockout, demonstrated diminished cardiac function. Furthermore, hypoxic preconditioning significantly reduced the ischemia/reperfusion induced heart injury in a platelet mitophagy dependent fashion. This study underscores that having the ability to manipulate platelet mitophagy might have potential clinical implications as a cardioprotective strategy [83].

Immune Thrombocytopenia (ITP):

Autophagy has been correlated with the pathogenesis of ITP [50, 84]. It was speculated that defects in autophagy may promote ITP through induction of immune senescence of T cells, and disturbance of B cell survival [84]. This model of ITP pathogenesis was complemented by Lui and Mei in 2018 [49]. By exposing MEG-01cells to plasma isolated from ITP patients or healthy donors, they were able to demonstrate the induction of autophagy in cells exposed to ITP plasma only, a process that could be prevented by chloroquine treatment. Since platelets are the autoimmune agent in ITP, a next set of experiments focused on the presence of autophagy within ITP platelets [85]. Wang et al., showed suppressed autophagy in platelets isolated from ITP patients, and in contrast, higher levels of apoptosis in ITP

platelet, demonstrated by means of Annexin V expression. In additional studies, the authors were able to link platelet autophagy present in ITP to the PI3K-AKT-MTOR pathway activity. Finally, treatment of ITP platelets with rapamycin resulted in enhanced autophagy in such cells, reduced platelet destruction via apoptosis, and therefore increased viability.

Sepsis:

Autophagy is an integral part of host responses to sepsis [27]. The formation, maturation, and degradation of autophagosome contents counteracts microbial invasion by active elimination of intracellular bacteria and viruses. Furthermore, autophagic degradation supports antigen presentation, carrying the mounting of a robust immune response [26]. Autophagy may additionally protect host cells in sepsis by preserving mitochondrial integrity and reducing apoptotic cell death [26, 29]. Our group demonstrated in 2021 that platelets isolated from septic patients demonstrated a diminished autophagic flux, caused by decreased LC3 recognition and binding of EPG5, leading to a late-stage inhibition of autophagy [72]. This process was triggered by LPS-TLR4 signaling, with downstream signal transduction through MAPK1/ERK2-MAPK3/ERK1 and MTOR, which are part of the previously characterized TLR4-MYD88-MAP2K/MEK-MAPK/ERK-MTOR pathway, linking bacterial toxins to autophagy.

Linking this finding with previously described dynamics of the autophagic system in clinical and experimental settings of sepsis shows some remarkable parallels. Cecal ligation and puncture-induced sepsis was demonstrated to result in initial clearance of autophagosomes, but accumulation of SQSTM1 at later stages of the disease (8 h) [29, 31]. Furthermore, TEM analysis of tissues from septic animals did reveal an increased number of autophagosomes, but only few autolysosomes [33], also supporting the hypothesis of reduced autophagosomal and lysosomal fusion during certain stages of sepsis. This dynamic autophagy host response is also reflected by the increased tissue autophagic activity during the initial phases of sepsis in experimental animal models [29-31], and a decline in autophagic activity and late-stage inhibition several hours into the induction of sepsis [29, 31, 32], findings reflected by the platelet data [72]. Therefore, this disturbed maturation of autolysosomes in platelets could represent a generalized therapeutic target for sepsis.

Together, the evidence is clear that autophagy occurs within platelets and is essential, not only for hemostasis and thrombosis, but for numerous other pathophysiologic events. The reviewed data and studies highlight the importance of autophagy in platelets, and merits future investigations. Platelet autophagy should also be viewed as a viable therapeutic target, and might open several new interventional avenues in thrombosis, hemostasis, as well as inflammatory and infectious disease.

Methods used to study autophagy in platelets

Visualizing autophagic structures:

TEM: Numerous platelet studies utilized TEM as an important tool to reveal the morphology of autophagic structures at the nm scale, and below the diffraction limit of light [52, 72, 74, 75]. The approach using electron microscopy enables the investigator to

demonstrate autophagic structures in platelets in their natural environment [86] (Figure 3). Additional 3D-spatial information can be garnered by using scanning transmission electron microscopy (STEM) as demonstrated for the interaction of the canalicular system and α-granules [87]. The combination of TEM and immune-detection (immune-gold technique) can also be utilized to specifically detect autophagy proteins in the subcellular context. However, careful image analysis needs to be performed, since alternative intracellular structures can be enclosed by a double membrane, and therefore being misinterpreted as autophagic structures [8].

Confocal Microscopy: Autophagy can be monitored by carefully performed immune-detection of punctate LC3 staining in platelets [72] (Figure 4). Additional proteins involved in autophagy can also be revealed by using specific antibodies. However, antibodies should be tested for non-specific binding, and off-target recognition should be carefully monitored by utilizing the appropriate control experiments.

Super-resolution Microscopy: This technique is primarily aimed at defining a location instead of the intensity of a fluorophore, and therefore the accumulation of a labeled protein at a specific morphologic equivalent. Nevertheless, studies aiming at localization and colocalization of autophagy proteins benefit from the resolution below the diffraction limit of light (Figure 2).

Transgenic expression of fluorescently labeled autophagy proteins: *GFP-LC3/+*, *Becn1-EGFP/+* and *EGFP-Atg5/+* mice were successfully used to visualize dynamics of autophagy proteins [52]. It is important to note that both EGFP transgenic mouse strains express the target genes under the control of the respective endogenous promotors and regulatory elements, providing molecular insights without the potential interference introduced by overexpressing the target protein.

Knockout mouse models:

A variety of platelet-specific mouse models with defective autophagy have been developed and utilized, including platelet specific Atg7 knockout ($Atg7^{f/f;}PF4^{Cre+}$) [52], heterozygous BECN1 disruption ($BECN1^{+/-}$) [71], platelet specific Atg5 knockout ($Atg5^{f/f;}PF4^{Cre+}$) [76], and platelet specific Vps34 knockout ($Vps^{f/f;}PF4^{Cre+}$) [79, 80].

It seems to be appropriate to speculate that future mouse models utilizing platelet-specific knockout approaches (see Table 1 for functional reference), might provide further insights into megakaryocytic and platelet specific regulation of autophagy. Targeted elimination of EPG5 or ULK1 could directly address recent findings in regards of platelet autophagy function during sepsis, and open new research avenues.

When using such model organisms, or generating a *de novo* platelet-specific knockout mouse model of an autophagy target, appropriate controls should be implemented to control for off-target effects. This is especially important since platelets originate from megakaryocytes, thus disturbed autophagy in megakaryocytes might have effects on thrombopoiesis and leading to defective platelet generation.

Autophagy inhibitors and inducers:

It is worthwhile to mention that most chemical inhibitors of autophagy are not entirely specific, and it is therefore important to control and test for additional dose- and time-dependent off-target effects. Commonly used inhibitors (Table 2) as bafilomycin A1 (inhibits the V-ATPases, elevates the lysosomal pH, resulting in a block in fusion of autophagosomes with lysosomes), or chloroquine should be used with caution, since the resulting accumulation of autophagosomes strongly depends on the initial autophagic flux. Furthermore, chloroquine may initially stimulate autophagy leading to potential misinterpretations of experimental results.

Fewer compounds are known to act as inducers of autophagy. The most commonly used chemical is rapamycin, an allosteric inhibitor of MTORC1. One caution is that MTOR is an integral part of several signaling cascades in platelets, and it thus controls other platelet pathways and processes [88, 89].

LC3 detection by western blot and measurement of autophagic flux:

LC3 (LC3-I) is a ubiquitin-like protein that can be conjugated to PE (LC3-II). LC3-II is the only protein marker that is reliably associated with completed autophagosomes [8]. LC3 can be detected via western blot, however, it is important to remember that changes in LC3-II amounts are tissue- and cell context-dependent. Furthermore, since LC3-I (16-18 kDa) and LC3-II (14-16 kDa) are close in size, the use of gradient gels is recommended. In addition, a series of exposure times should be documented to be able to detect even trace amounts of LC3-II, but also to ensure the detection of the appropriate housekeeping protein (actin is the first choice) within its linear range. Finally, using the LC3-II/I ratio does not necessarily address the issue of autophagic flux. LC3-I might be less sensitive to detection, depending on the used antibodies. The increased sensitivity to degradation of LC3-I after repeated freeze-thaw cycles might interfere with experimental results, and translational processes might mask the robustness of LC3-I conversion or LC3-II accumulation [8].

Autophagic flux should not be addressed by LC3 conversion/LC3-II turnover or accumulation analysis only [8]. Other protein markers should be used in combination with LC3. SQSTM1 becomes incorporated into the formed autophagosome and is degraded in the autolysosomes. Thus, measuring LC3 II turnover and paralleled changes in SQSTM1 can be used to quantify dynamics in cellular autophagic flux. Using autophagy inducing and inhibiting agents, or the combination of both will further help in delineating the extend of autophagic flux being present under specific conditions [8, 72].

Summary

In summary, it is widely accepted that abnormal levels (up- or down-regulation) of autophagy can cause differential effects during specific and distinct stages of megakaryocyte and hematopoietic progenitor cell differentiation. Furthermore, compelling evidence can be presented demonstrating expression of autophagy genes and protein machinery, as well as the presence of cellular structures resembling autolysosomes and respective precursors in human and mouse platelets in health and disease. The functional importance of platelet

autophagy during such diverse physiologic and pathophysiologic events was recently established using human *ex vivo* approaches, cell culture, and mouse models. Nevertheless, future research will be of importance to create new mechanistic insights into the regulatory pathways involved in autophagy in megakaryocytes and platelets, and therefore, paving avenues towards potential specific therapeutic interventions.

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Abbreviations

ADP	adenosine diphosphate
ATG5	autophagy related 5
ATG7	autophagy related 7
ATG14L	autophagy related 14
Baf A1	bafilomycin A1
BECN1	Beclin 1
DRP1	dynamin-related protein 1
EPG5	ectopic P-granules autophagy protein 5 homolog
FIP200	focal adhesion kinase family interacting protein of 200 kDa
Fli-1	Fli-1 Proto-Oncogene, ETS Transcription Factor
FOG1	Friend of GATA1
GATA2	GATA Binding Protein 2
ITP	immune thrombocytopenia
LAMP1	lysosomal associated membrane protein 1
MAP1LC3/LC3	microtubule associated protein 1 light chain 3
MAPK1/ERK2	mitogen-activated protein kinase 1
MAPK3/ERK1	mitogen-activated protein kinase 3
MTOR	mechanistic target of rapamycin kinase

MYD88	MYD88 innate immune signal transduction adaptor
NF-E2	Nuclear Factor, Erythroid 2
NRBF2	Nuclear Receptor Binding Factor 2
PE	phosphatidylethanolamine
PU.1	Spi-1 Proto-Oncogene
RAB7	Member RAS Oncogene Family
RUNX1	RUNX Family Transcription Factor 1
SQSTM1/p62	sequestosome 1
STEM	scanning transmission electron microscopy
TLR4	toll like receptor 4
TPO	thrombopoietin
c-Mpl	thrombopoietin receptor
Ulk1	Unc-51 Like Autophagy Activating Kinase 1
UVRAG	UV Radiation Resistance Associated
Vps15	Phosphoinositide-3-Kinase Regulatory Subunit 4
Vps34	Phosphatidylinositol 3-Kinase Catalytic Subunit Type 3

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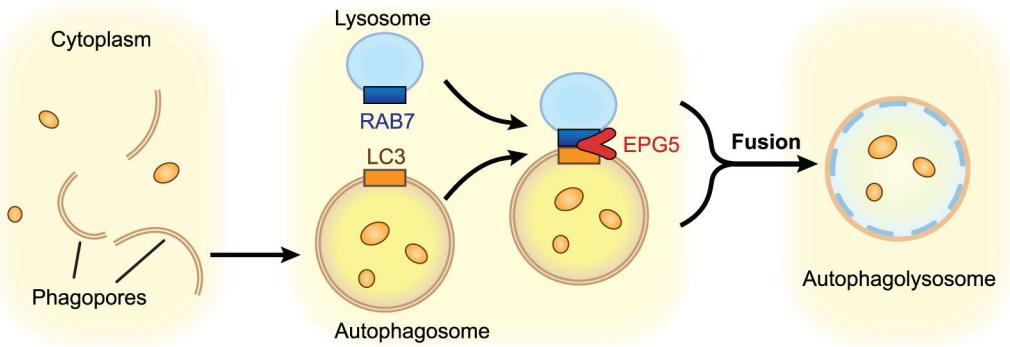

Figure 1.

Schematic overview of macroautophagy. The initial phagophore starts forming from double-membrane. The phagophore expands, enclosing cytoplasm, macromolecules and organelles, and forms an autophagosome. EPG5 is recruited to lysosomes by associating with RAB7 and LC3, leading to fusion of the autophagosome with a lysosome. The fused compartment where the cellular contents are degraded is called an autophagolysosome/autolysosome.

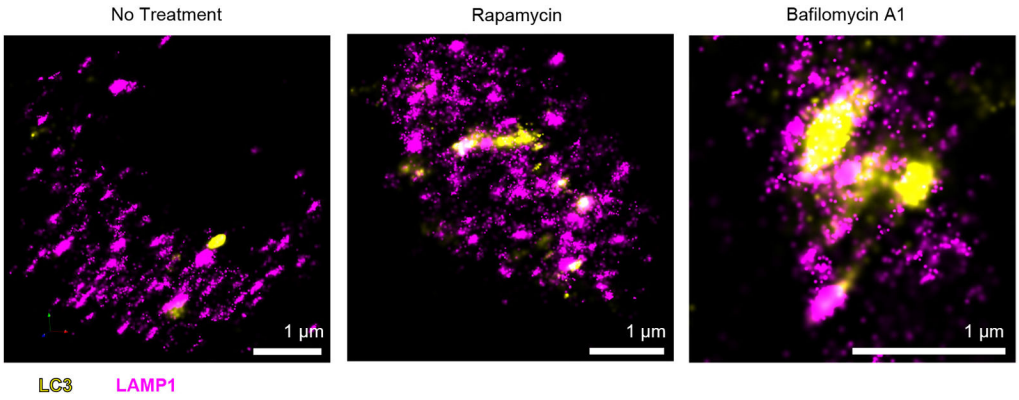

Figure 2.

Autophagosomal dynamics can be visualized using super resolution microscopy in human platelets. Platelets isolated from healthy individuals were left untreated or treated with rapamycin or bafilomycin A1, and immunostained with an anti-LC3 (yellow), and an anti-LAMP1 (magenta) antibody. Cells were subsequently analyzed using super resolution microscopy. Scale bars: 1 µm.

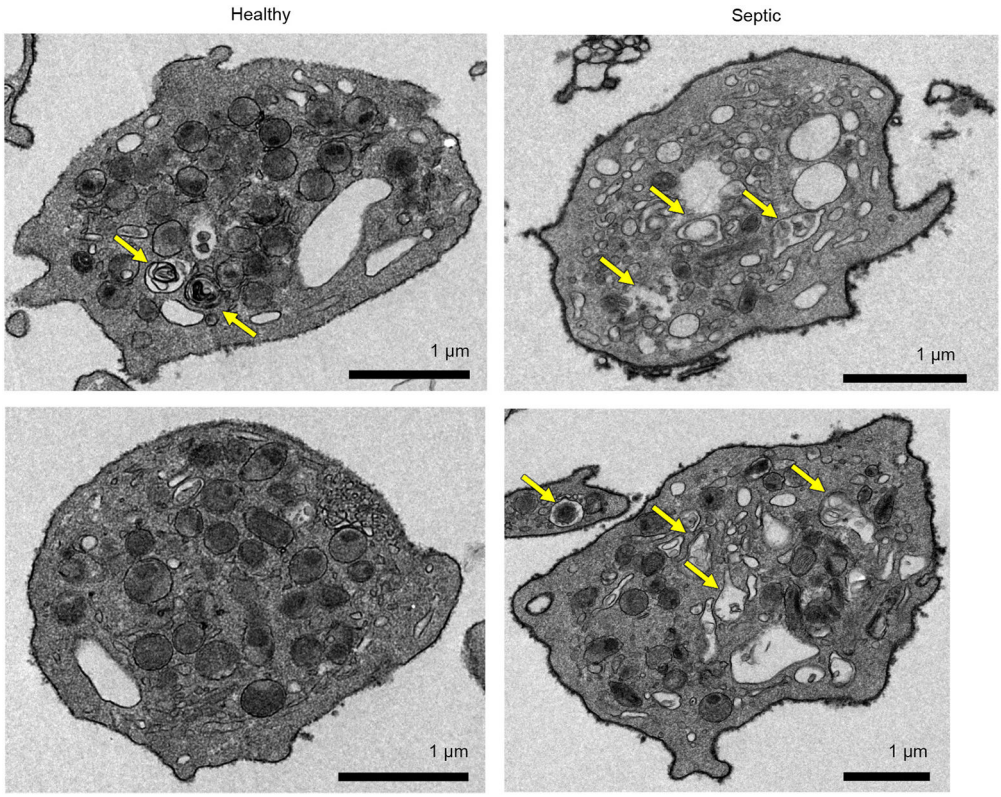

Figure 3.

Platelets isolated from septic patient demonstrate increased autophagy when compared to platelets isolated from healthy individuals. Transmission electron microscopic analysis of healthy platelets (left) and platelets isolated from septic patients (right, scale bars: 1 μm). Yellow arrows indicate autophagosomes of various content.

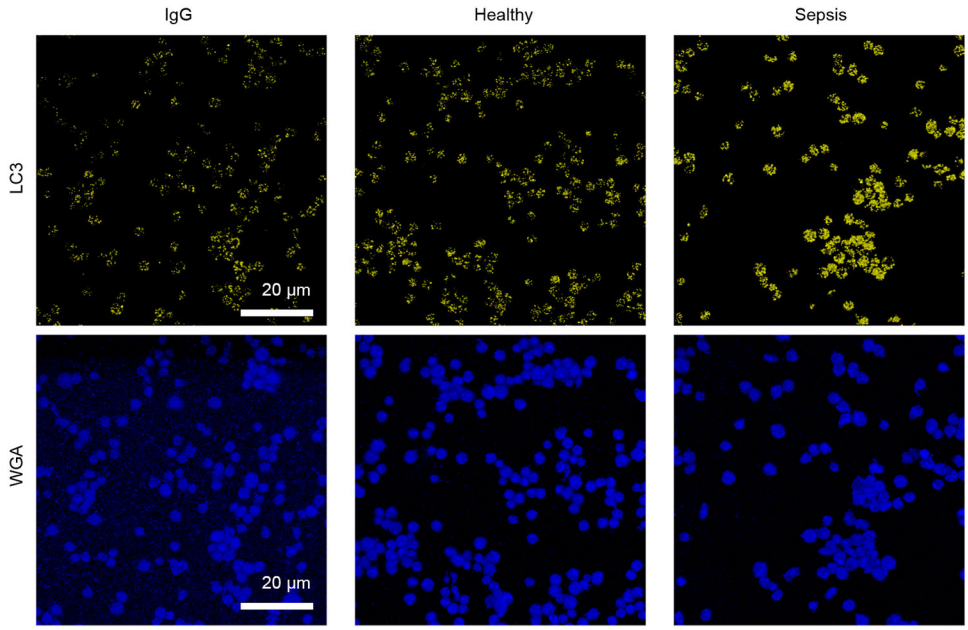

Figure 4.

LC3 expression is demonstrated in human platelets isolated from septic patients (right) and healthy platelets (middle, left IgG control). Freshly isolated platelets from healthy subjects and septic patients were fixed in suspension immediately after isolation. Immunofluorescence staining with an anti-LC3 (yellow) antibody demonstrates increased expression of LC3 platelets isolated from septic patients (scale bars: 20 μm). Cells were co-stained using WGA (blue).

Table 1.

Mammalian autophagy proteins are present in platelets [52, 62, 71, 72, 97, 98].

Functional Unit	Mammalian Autophagy Genes/Proteins	Reported Function in Autophagy
ULK1 complex	ULK1/2/3/4 FIP200 ATG13 ATG101	ULK1 complex drives the formation of the phagophore, the initial autophagosomal precursor membrane structure [90].
VPS34 complexes: ATG14L-Beclin 1* UVRAG-Beclin 1# UVRAG-Beclin 1-Rubicon§	BECN1*#§ VPS34*#§ VPS15*#§ ATG14L* NRBF2* UVRAG#§ RUBCN (Rubicon)§	VPS34 complex is responsible for the production of the phospholipid phosphatidylinositol 3-phosphate (PI3P) at the site of forming the autophagosome [90-92].
Atg12–Atg5-Atg16L complex	ATG7 ATG10 ATG12 ATG5 ATG16L1/2	This complex is thought to mediate the lipidation reaction by recruiting ATG3-LC3 to the membrane and to facilitate the transfer reaction to PE [93].
LC3-PE complex	ATG4A/B/C/D ATG7 ATG3 MAP1LC3A/B/C GABARAP GATE-16	LC3 is covalently bound to PE through a ubiquitin-like conjugation system. (E1 – Atg7, E2 – ATG3) [94].
PtdIns3*P* effectors	WIPI1/2/3/4 ATG2A/B	WIPI2 and WIPI4 function as essential and non-redundant PtdIns3*P* effectors. WIPI2 recruits the Atg12–Atg5-Atg16L complex, and in addition, WIPI4 interacts with ATG2 [95].
Only transmembrane protein in the autophagy core machinery	ATG9A/B	ATG9 proteins supply and direct membranes from donor organelles for autophagosome formation [96].
Autophagy receptor	P62/SQSTM1	SQSTM1 links ubiquitinated substrates with LC3 [8].
Autophagosome-Lysosome fusion	RAB7 EPG5	EPG5 is a RAB7 effector, binds LC3, and determines the fusion specificity of autophagosomes with late endosomes/lysosomes [72, 73].

Table 2.

Commonly used pharmacologic regulators of platelet autophagy [8]. This table is not meant to be complete, as new compounds are routinely being added to the platelet research portfolio.

Pharmacologic regulator	Target protein or structure	Effect
3-methyladenine	Class III phosphotidylinositol-3-kinase inhibitor	Inhibits autophagy at an early stage [71, 75, 81, 85]
ABO (6-amino-2,3-dihydro-3-hydroxymethyl-1,4-benzoxazine)	Modulates intracellular Ca^{2+} concentration via ANXA7 [99]	Induces autophagy [85]
Ammonium chloride	Neutralizes acidic compartments by being protonated	Inhibits the clearance of autophagosomes [52, 81, 85]
Bafilomycin A1	Inhibitor of Vacuolar-ATPase	Inhibits autophagy by introducing a block in fusion of autophagosomes with lysosomes [53, 71, 72]
Chloroquine	Raising the lysosomal pH	Inhibits autophagy by introducing a block in fusion of autophagosomes with lysosomes [52, 71, 85]
Lithium	Inhibits inositol monophosphatase (IMPA)	Induces autophagy by increasing the levels of BECN1-containing PIK3C3/VPS34 complexes [81]
Rapamycin (Sirolimus)	Allosteric inhibitor of Mammalian target of rapamycin (MTOR)	Induces autophagy [53, 71, 72, 85]
Suberoylanilide hydroxamic acid (SAHA, Vorinostat)	Histone deacetylase inhibitor	Induces autophagy via p53-dependent pathway [81, 100]
Wortmannin	Class III phosphotidylinositol-3-kinase	Inhibits autophagy