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Review

Autophagy in acute leukemias: A double-edged sword with important therapeutic implications

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ABSTRACT

Macroautophagy, usually referred to as autophagy, is a degradative pathway wherein cytoplasmatic components such as aggregated/misfolded proteins and organelles are engulfed within double-membrane vesicles (autophagosomes) and then delivered to lysosomes for degradation. Autophagy plays an important role in the regulation of numerous physiological functions, including hematopoiesis, through elimination of aggregated/misfolded proteins, and damaged/superfluous organelles. The catabolic products of autophagy (amino acids, fatty acids, nucleotides) are released into the cytosol from autophagolysosomes and recycled into bio-energetic pathways. Therefore, autophagy allows cells to survive starvation and other unfavorable conditions, including hypoxia, heat shock, and microbial pathogens. Nevertheless, depending upon the cell context and functional status, autophagy can also serve as a death mechanism. The cohort of proteins that constitute the autophagy machinery function in a complex, multistep biochemical pathway which has been partially identified over the past decade. Dysregulation of autophagy may contribute to the development of several disorders, including acute leukemias. In this kind of hematologic malignancies, autophagy can either act as a chemo-resistance mechanism or have tumor suppressive functions, depending on the context. Therefore, strategies exploiting autophagy, either for activating or inhibiting it, could find a broad application for innovative treatment of acute leukemias and could significantly contribute to improved clinical outcomes. These aspects are discussed here after a brief introduction to the autophagic molecular machinery and its roles in hematopoiesis.

1. Introduction

The term autophagy (literally "self-eating") indicates some quite distinct cellular processes, which share as outcome the degradation of intracellular components by lysosomes [1]. In mammals, microautophagy represents the direct engulfment of cytoplasm via the lysosome by invagination of the lysosomal membrane [2]. In chaperone-mediated autophagy, single proteins are transported into the lysosomal lumen through the coordinated action of chaperones located at both sides of the membrane and a dedicated protein translocation complex [3]. Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved pathway that leads to self-digestion of cytoplasmic structures [4]. During autophagy, proteins, macromolecular aggregates, and superfluous/damaged organelles [e.g. mitochondria, endoplasmic reticulum (ER), ribosomes, peroxisomes] are engulfed within specific double-membrane vesicles called autophagosomes. Then, the subsequent fusion of autophagosomes with lysosomes gives rise to autophagolysosomes that are responsible for the enzymatic degradation of the engulfed material [5]. The catabolic products of autophagy are molecular building blocks such as amino acids, fatty acids, and nucleotides, which are released into the cytosol and finally recycled into bio-energetic pathways.

Autophagy plays a critical role in maintaining normal cellular homeostasis [6]. In fact, under optimal conditions, basal levels of autophagy are needed to warrant cell fitness and to maintain quality control of essential cellular components, by removing unfolded, excessive and/or aged proteins, as well as damaged or superfluous organelles. Autophagic flow can considerably increase in response to cellular stress, such as nutrient deprivation, hypoxia, DNA damage, starvation, pathogen infection, and ER stress, allowing the cell to cope with that particular "emergency" status [7–9].

As a consequence, an efficient and strict regulation of autophagy is fundamental for safeguarding cell health. Autophagy is implicated in physiological processes such as cellular renovation during development and differentiation [10,11]. However, disruption of the autophagic pathway may have severe consequences as demonstrated by the fact that dysregulated autophagy underlies different pathological conditions

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such as cancer, neurodegeneration, autoimmunity, and inflammatory disorders [12–15]. In light of these premises, it is obvious that pharmacological approaches aimed to regulate autophagy, thus inducing or repressing it, deserve considerable attention, as they represent a new arena for the development of therapeutics.

Over the last few years, numerous papers have highlighted the occurrence of autophagy in acute leukemia cells. This review shall focus on the double-edged role played by autophagy in either promoting or suppressing acute leukemia cell growth and survival, with the anticipation that both autophagy activation and inhibition, depending on the context, hold promise as a means for treating this type of hematologic malignancies and for enhancing the activity of current therapeutics. Moreover, we will also examine the roles played by autophagy in regulating the functionality of hematopoietic stem cells (HSCs).

These topics will be discussed after a brief introduction to the autophagic machinery and its regulators. For a detailed description of the autophagic process, we refer the reader to other comprehensive review articles that have been recently published [16–18].

2. The autophagic machinery and its regulation

Autophagy is a very complex multistep process that involves dozens of known proteins to assemble the required machinery. Initial studies carried out in the yeast *Saccharomyces cerevisiae*, identified a family of 'AuTophaGy-related' (ATG) proteins that form the core molecular machinery of autophagy. These proteins are hierarchically recruited for regulating each step of the autophagic process [19].

It is commonly accepted that under normal conditions autophagy rate in the cell is low. Accordingly, both an efficient mechanism aimed to inducing autophagy and a finely balanced modulation are needed to ensure a rapid adjustment of cells to conditions which require autophagy.

The autophagic pathway consists of different phases: induction, autophagosome nucleation, elongation and completion, lysosomal fusion, and degradation [20].

The first events in autophagy are the induction and the nucleation of the membrane that will become an autophagosome. This membrane has been called phagophore (or isolation membrane) and serves for sequestering portions of the cytoplasm, including unfolded protein aggregates and organelles. During expansion, the nascent membrane grows and fuses its edges to form the double membrane vacuole termed autophagosome. Hereafter, the mature autophagosome fuses with the lysosome to form an autolysosome, also called autophagolysosome (completion and lysosomal fusion steps). Finally, within the autophagolysosomes, the luminal content is degraded by acid hydrolases and recycled through permeases [12,21] (Fig. 1).

In eukaryotic cells, mammalian target of rapamycin complex 1 (mTORC1) and AMP-activated protein kinase (AMPK) represent two key regulators of the autophagy pathway, both controlling the induction phase [22].

mTORC1 is composed of mammalian target of rapamycin (mTOR), the regulatory associated protein of mTOR (Raptor), mammalian Lethal-with-Sec-Thirteen 8 (mLST8), proline-rich Akt substrate of 40-kDa (PRAS40), FK-506 binding protein 38 (FKBP38), and DEP-domain-containing mTOR interacting protein (Deptor) [23] (Fig. 1). mTORC1 collects input from up-stream signals such as growth factors, glucose, amino acids, energy status, and stress signals [24]. In particular, mTORC1 is a key down-stream target of the phosphoinositide 3-kinase (PI3K)/Akt pathway, which is aberrantly activated in many types of cancer, including hematological malignancies [25–27].

Once activated, mTORC1 transduces anti-autophagic signals by binding to the UNC51-like kinase (ULK) multi-protein complex which is essential for the initial formation of the phagophore. This complex comprises the Ser/Thr protein kinase ULK1 (or ULK2), ATG13, focal adhesion kinase family-interacting protein of 200 kD (FIP200), and ATG101 (Fig. 1). mTORC1 directly phosphorylates both ULK1 (mostly at Ser 638 and Ser 758 [28]) and ATG13 [29,30], thereby down-regulating autophagy [31]. ULK1 is also a target of Akt upon insulin stimulation, which could regulate autophagy independently from mTORC1 function [32].

On the other hand, upon nutrient deprivation or pharmacologic mTORC1 inhibition (e.g. rapamycin treatment), mTORC1 dissociates from the ULK1/ULK2 complex, leading to active forms of ULK1/ULK2, that in turn phosphorylates ATG13 [33] and FIP200 [34], which are essential for autophagic activity [31,35] (Fig. 1).

ULK1/ULK2 also phosphorylates Raptor at multiple sites and this results in mTORC1 inhibition. It has been proposed that the purpose of this negative feedback loop, that occurs upon activation of autophagy, is to maintain mTORC1 inhibition when nutrient supplies are limited [36].

AMPK functions as an intracellular energy sensor. Indeed, under metabolic stress conditions, the tumor suppressor liver kinase B1 (LKB1) activates AMPK that down-regulates ATP-consuming processes and up-regulates the ATP-generating ones [37]. Mechanistically, AMPK directly phosphorylates ULK1 at multiple sites (Ser 317, Ser 467, Ser 556, Thr 575, Ser 638, Ser 777), thus up-regulating ULK1 activity [38], and activates tuberous sclerosis complex 2 (TSC2), an indirect inhibitor of mTORC1 activity [31] (Fig. 1). Therefore, AMPK is an important positive regulator of the autophagic process.

During the nucleation phase, proteins and lipids are recruited for autophagosome membrane formation [39–41]. Autophagosome nucleation requires the so-called "Beclin-1 core complex" which is formed by Beclin-1, the class III PI3K/hVps34, and p150/hVps15 [31,42]. Activation of this complex generates PI 3-phosphate, which promotes autophagosomal membrane nucleation. Beclin-1 directly interacts with numerous binding partners which can lead to either activation (ATG14L, activating molecule in Beclin-1-regulated autophagy/AMBRA1, UV radiation resistance-associated gene protein/UVRAG) or inhibition (Bcl-2, Bcl-xL, Mcl-1) of autophagy [43,44] (Fig. 1).

Vesicle elongation and completion phases are accomplished by two ubiquitin-like conjugation systems. The first system, which comprises ATG7 and ATG3, regulates the lipid modification [i.e. conjugation to the lipid phosphatidylethanolamine (PE)] of Light Chain 3 (LC3) [45]. LC3 lipidation requires initial cleavage of LC3 by ATG4B protease. Unlipidated LC3 (LC3-I) is mostly cytosolic. Lipidation converts LC3-I into LC3-II which is stably associated with the autophagosome membrane [31,46] (Fig. 1).

LC3 is involved in the recruitment of cargo (proteins and organelles) into the developing autophagosome, along with p62 (also known as sequestosome 1/SQSTM1) and Neighbor of BRCA1 gene 1 protein/NBR1 [47]. These proteins function as adaptor molecules, enabling autophagy to target cargo selectively to nascent LC3-rich isolation membranes. Importantly, the presence of LC3 in autophagosomes and the conversion of LC3-I to the slowly migrating form, LC3-II, have been used as markers of autophagy [20].

The second system is composed of ATG7 and ATG10 which regulate the conjugation of ATG12 to ATG5, followed by transfer to ATG16L. Membrane binding by the ATG12–ATG5–ATG16L complex is required for efficient promotion of LC3 lipidation [22,48] (Fig. 1).

Once their formation is completed, autophagosomes fuse with lysosomes [49], forming autophagolysosomes, where resident enzymes degrade the engulfed contents which are then recycled [12]. Termination of autophagy can be achieved through reactivation of mTORC1 by nutrients (for example, amino acids) generated within the autophagolysosome. This is an important feedback mechanism which inhibits excessive activation of autophagy during periods of starvation [50].

3. The role of autophagy in hematopoietic stem cells (HSCs) and hematopoiesis

Hematopoiesis development and homeostasis are based on HSCs, a pool of rare cells characterized by the unique combination of quiescence,

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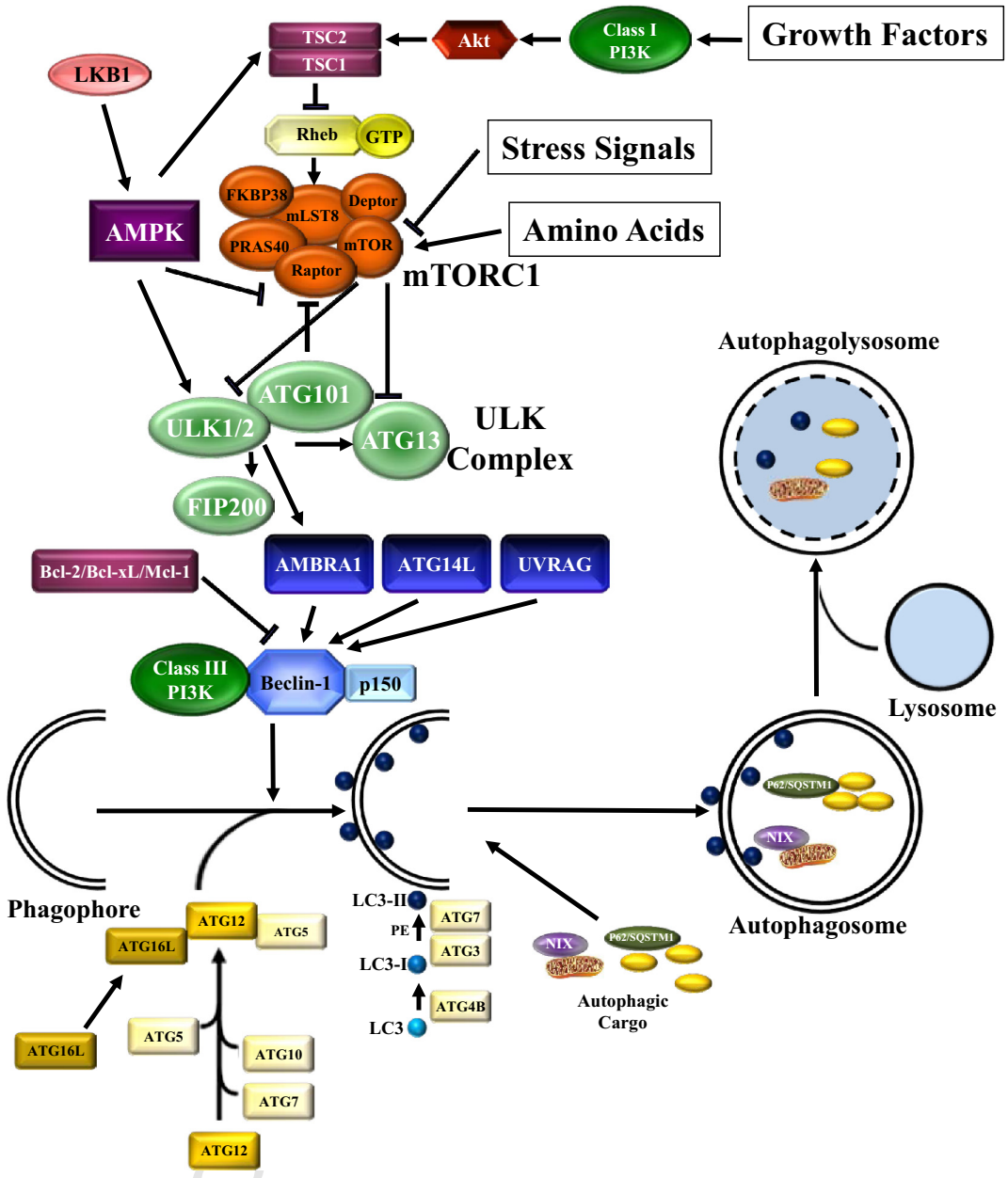

Fig. 1. The autophagic machinery and its regulation. In the presence of growth factors and amino acids, mTORC1 associates with and inhibits the ULK complex by phosphorylating ATG13 and ULK1/2. On the contrary, stress signals or AMPK lead to mTORC1 dissociation from ULK1/2 and activation of ULK complex, thus initiating the formation of the phagophore. Autophagy then proceeds with the formation of double-membrane vesicles termed autophagosomes that sequester targeted cell constituents and fuse with the lysosomes to form autophagolysosomes, where finally cargos are degraded. Arrows indicate activating events, while perpendicular lines indicate inhibitory events. Abbreviations: AMPK, AMP-activated protein kinase; ATG, AuTophaGy-related; Bcl-2, B-cell lymphoma 2; Deptor, DEP-domain-containing mTOR interacting protein; FKBP38, FK-506 binding protein 38; FIP200, focal adhesion kinase family-interacting protein of 200 kD; LKB1, liver kinase B1; Mcl-1, myeloid cell leukemia 1; mLST8, mammalian lethal-with-sec-thirteen 8; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; p62/SQSTM1, p62/sequestosome-1; PI3K, phosphoinositide 3-kinase; PRAS40, proline-rich Akt substrate of 40-kDa; Raptor, regulatory associated protein of mTOR; Rheb, Ras homolog enriched in brain; TSC, tuberous sclerosis; ULK, UNC51-like kinase; UVRAG, UV radiation resistance-associated gene protein.

self-renewal through asymmetric division, and multi-lineage differentiating potential. HSCs are responsible for blood cell production throughout lifetime. Indeed, they represent a reservoir of multipotent stem cells that provide a continuous supply of myeloid and lymphoid cells. HSCs are operationally defined by their capacity to reconstitute the entire blood system of a recipient [51,52].

Acute leukemias appear to derive not only from HSCs, but also from early progenitors. Indeed, dysregulation of fate decisions in HSCs and early progenitor cells is one of the key features of leukemic transformation [53]. Therefore, for the scopes of this review, it is useful to briefly recapitulate the findings that link autophagy with HSC functions and normal hematopoiesis.

HSCs reside in a specialized nourishing niche in the bone marrow (BM) where the majority of them lie in a quiescent state [54,55]. Although dormancy is the preferred status for HSCs, upon specific stimuli, these cells display the peculiarity of self-renewing and/or differentiating into hematopoietic progenitors that in turn give rise to the mature hematopoietic cell lineages. In this framework, many recent findings point to the likelihood that autophagy may function to balance quiescence, self-renewal, and differentiation of HSCs, both under normal and stressful conditions [56–58].

Proofs of the requirement of a functional autophagic machinery for the maintenance of the self-renewal ability of HSCs came from some recent findings obtained in mice. Conditional ablation of *Atg7* in the

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hematopoietic system (*Vav-Atg7*$^{-/-}$ mice) caused a severe impairment of the self-renewal function, as demonstrated, in vitro, by the failure to form secondary colonies in colony-forming cell assays, and, in vivo, in both competitive and non-competitive repopulation assays [59]. Importantly, immunophenotypic analysis of these *Vav-Atg7*$^{-/-}$ cells documented that they were *bona fide* HSCs, as they were Lin^{-}/Sca-1^{+}/c-Kit^{+} (LSK cells). Moreover, the number of HSCs and progenitors of multiple lineages was markedly reduced in the absence of *Atg7* [59]. Intriguingly, noncompetitive repopulation assays with fetal liver (FL) cells revealed that *Vav-Atg7*$^{-/-}$ FL cells, unlike *Vav-Atg7*$^{-/-}$ BM cells, could rescue 50% of lethally irradiated recipients, suggesting either that *Atg7* is less important for fetal than adult HSC functions or that the HSC defects resulting from the loss of autophagy exacerbated over time [59].

Autophagy impairment affected also differentiation of HSCs into mature white and red blood cells. Indeed, *Atg7*-deficient mice displayed a reduction in progenitors of both myeloid and lymphoid lineages, as well as a severe anemia (see further on). Furthermore, decreased absolute counts of T-, B-, and NK-cells in the peripheral blood were observed in these mice [59]. Likewise, it has been demonstrated that mice lacking FIP200 in hematopoietic cells (CKO mice) experienced perinatal lethality associated with a severe erythroblastic anemia. FIP200 was required for the maintenance and function of fetal HSCs. CKO embryos displayed increased HSC proliferation and myeloid expansion, which may be responsible for the depletion of fetal HSCs [60].

Mitophagy is the removal of damaged mitochondria through autophagy. In this way, it is responsible for a "quality control" of HSC mitochondria that may represent a major source of reactive oxygen species (ROS). It is well established that mitochondrial dynamics is fundamental for maintaining integrity, function, and regulation of all eukaryotic cells, including HSCs [61]. In particular, HSCs need to be protected from ROS, as elevated ROS levels cause an initial hyperproliferation of HSCs which is then followed by their depletion due to apoptosis [62,63]. Of interest, it has been demonstrated that defective autophagy is associated with an increased number of mitochondria. Indeed, HSCs from both CKO and *Vav-Atg7*$^{-/-}$ mice displayed a larger mitochondrial mass and up-regulated ROS production when compared with healthy HSC, highlighting how mitophagy is essential for HSC fitness [59,60]. Healthy HSCs have a number of mitochondria much lower when compared to more differentiated cells, and this may be due to the fact that HSCs localize to the hypoxic niche of BM, thus probably relying on glycolytic metabolism to meet energy requirements, whereas more differentiated cells prefer oxidative phosphorylation [61,64].

Autophagy protects HSCs from pro-apoptotic stress stimuli (e.g. caloric restriction, cytokine withdrawal), through a gene expression reprogramming triggered by the Forkhead transcription factor, FoxO3 [65]. It is well established that FoxO3 controls the expression of several genes involved in autophagy, such as *LC3B*, *Atg12*, *Atg4b*, *Ulk2*, *Vps34*, and *Beclin-1* [66]. Another mechanism which could be involved in the FoxO3-dependent enhancement of autophagy, is based on the observation that ROS activated FoxO factors and this resulted in increased levels of sestrin, which potentiated AMPK-mediated inhibition of mTORC1 [67].

Overall, the findings of Warr et al. [65] are in good agreement with previous reports that highlighted the key role played by FoxO3 in maintaining the HSC pool [68,69].

Interestingly, a high rate of autophagy is found in in vitro cultured human HSCs and this seems to ensure self-renewal capacity and differentiation. HSCs failed to form colonies in in vitro colony-forming assays and to differentiate into neutrophils when autophagy was blocked pharmacologically by 3-methyladenine or attenuated by ATG5 shRNA [70], implying that impairment of autophagy leads to the loss of the HSC stemness properties.

Furthermore, several lines of evidence indicate that autophagy plays a role in both erythropoiesis and lymphopoiesis.

Regarding erythropoiesis, in the mid-1980s it was hypothesized that mitochondria autophagy took place in mice erythroblasts concomitantly with nuclear extrusion and continued in BM reticulocytes [71]. More recently, these findings have been essentially confirmed in animal models, that have backed this hypothesis with molecular evidence. Indeed, *Vav-Atg7*$^{-/-}$ mice developed a severe anemia, while *Atg7*$^{-/-}$ erythroblasts accumulated damaged mitochondria with altered membrane potential, which led to cell death [72]. However, it has been shown that autophagy is induced earlier than initially thought, at the polychromatic erythroblast stage, and continues until enucleation [73].

The importance of autophagy in erythroid differentiation is also underscored by the fact that GATA-1, the master regulator of erythropoiesis, directly activates the essential autophagy component LC3B and other autophagy related genes, including *Atg4b* and *Atg12* [74]. Furthermore, in the same study it was reported that GATA-1 up-regulated the expression of the Bcl-2 family member, NIX, which critically mediates mitophagy during erythropoiesis, as NIX allows the sequestration of mitochondria by autophagosomes (Fig. 1) [75].

As to lymphopoiesis, it is emerging that autophagy is especially important for T-cell development.

Deletion of the essential autophagy genes *Atg5* or *Atg7* in murine T-cells resulted in decreased thymocyte and peripheral T-cell numbers, and *Atg5*-deficient T-cells had a decrease in cell survival [76–78]. The pro-survival role of autophagy in thymocytes has been also underscored by more recent findings that have highlighted how the activation of the transient receptor potential vanilloid 1 (TRPV1) induced autophagy in mice thymocytes through the ROS-regulated AMPK pathway [79]. The TRPV1-dependent autophagy was Beclin-1-dependent, and its inhibition triggered apoptosis of thymocytes. It is interesting that TRPV1 activation altered the expression of both CD4 and CD8α antigens, inducing the development of a Double Positive (DP)^{dull} thymocyte subpopulation. It was thus concluded that the DP^{dull} cell subset could represent a distinct thymocyte subpopulation involved in the homeostatic control of thymus cellularity and in the responses to chemical stress signals during T-cell maturation [79]. Furthermore, Beclin-1-deficient mice were unable to maintain normal thymic cellularity, which was most likely caused by impaired maintenance of thymocyte progenitors [80].

Regarding B-lymphocytes, both *Atg5* and *Beclin-1* were required for efficient development from pro- to pre-B cells in the BM [76,80,81].

In conclusion, a well-functioning autophagy machinery seems to be mandatory to maintain the integrity of the HSC compartment as well as for red blood cell and both B- and T-lymphocyte production.

Therefore, alterations of this catabolic process considerably influence HSC fate and hematopoietic system homeostasis, possibly laying down the basis for malignant transformation. In this connection, it is worth emphasizing here that *Vav-Atg7*$^{-/-}$ mice died within 12 weeks and displayed myeloid blast infiltrates in multiple organs including the spleen, thymus, liver, intestine, skin, pancreas, kidney, and heart. Histopathological observations revealed the presence of over 20% myeloid blasts in the BM [59]. These findings, combined with a significantly increased number of myeloid CD11b^{+}Gr1^{+}CD47^{+} cells, suggested that *Vav-Atg7*$^{-/-}$ mice developed a myelodysplastic/myeloproliferative overlapping disorder strongly resembling human acute myeloid leukemia (AML) of the myelomonocytic subtype. Importantly, this disorder was transplantable, as the myelodysplastic/myeloproliferative features were observed also in lethally-irradiated hosts reconstituted with *Vav-Atg7*$^{-/-}$ FL, BM, or LSK cells [59].

4. Autophagy and cancer

The role of autophagy in regulating either cancer cell death or survival still remains highly controversial. Due to its tumor suppressive and tumor promoting properties, autophagy, at a first glance, may seem a paradox. Indeed, the knock-down of genes involved in the autophagy process could either enhance or prevent cell cancer death [82].

Nevertheless, the only apparent paradox of this dual role of autophagy could be understood if we dissect the diverse outcomes of autophagy in different stages of the tumorigenesis. It is likely that basal autophagy

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prevents cancer initiation, thus functioning as a pro-death (tumor suppressive) mechanism. In fact, autophagy ensures removal of damaged organelles, such as mitochondria which potentially produce high amount of ROS, and protects cells against genomic instability and inflammation, thus preventing cancer initiation. Indeed, an impaired autophagic process has been related to increased DNA damage, high ROS levels, aneuploidy, aberrant accumulation of p62/SQSTM1 and ER chaperones, underscoring the key role of autophagy in preventing tumor onset [83,84].

On the other hand, when cancer is already established, autophagy can function as a pro-survival pathway. A marked increase in autophagy is observed in cancer cells subject to stress stimuli, including nutrient and growth factor deprivation, hypoxia, DNA damage, chemotherapy, and radiotherapy [85]. Here, the autophagic response is aimed to promote tumor growth and survival, helping cancer cells to cope with metabolic stress, and to escape death stimuli triggered, for instance, by chemotherapeutic agents [86–88].

In this case, autophagy represents a very attractive therapeutic target, and drugs that selectively inhibit this catabolic process may restore chemosensitivity and enhance tumor cell death. At present, chloroquine and hydroxychloroquine are the only autophagy inhibitors approved by the U.S. Food and Drug administration for clinical use [89]. They are being tested, in combination with conventional chemotherapeutics or targeted agents, in several trials completed or still in progress for the treatment of many types of tumors (http://clinicaltrials.gov/), including hematopoietic malignancies [90,91].

However, autophagy could represent a distinct mechanism of cell death, referred to as autophagic cell death (ACD), also in established tumors [89,92]. ACD induced by anticancer drugs has been described in various types of hematopoietic neoplasias, including acute leukemias [93,94]. Moreover, autophagy can promote necroptosis, a non-apoptotic form of regulated cell death [95,96].

5. Acute leukemias

Acute leukemias comprise a highly heterogeneous group of malignant hematopoietic disorders characterized by uncontrolled proliferation of clonal neoplastic cells belonging to either the myeloid (65–70% of cases) or lymphoid lineage (30–35% of cases). Acute leukemias are clinically defined by a rapid disease timing, ultimately culminating in BM failure that leads to severe anemia, leukopenia, and thrombocytopenia. Therefore, acute leukemias are typically fatal within weeks or months if left untreated. Each year, nearly 20,000 adult and pediatric patients in the U.S. are diagnosed with acute leukemia. Of these patients, 10,000 will die [97]. We will now analyze the emerging roles of autophagy in the different types of acute leukemias.

5.1. Acute myeloid leukemia (AML)

AML is the most common type of acute leukemia in adults and mainly affects elderly people, as in younger patients the incidence is two to three cases per 100,000 individuals, whereas in the seventh and eighth decades the incidence rises to 13 to 15 per 100,000 [98]. AML prognosis has improved in younger patients who can tolerate intensified treatment strategies, however there have been very limited changes in outcome among individuals who are >60 years of age [99]. Thus, the prognosis of AML remains severe, with an overall 5-year survival rate of 15–30%, while patients older than 60 years or those with an AML arising from a myelodysplastic syndrome (MDS), display an even worse prognosis (<10% survival at 5 years) [99].

It is commonly accepted that the AML phenotype results from multiple molecular, genetic, and epigenetic alterations affecting differentiation, proliferation, and apoptosis of myeloid progenitors. The majority of the AML cases (50–60%) are associated with nonrandom chromosomal translocations [100,101]. Although over 700 recurrent aberrations have been described to be associated with the AML phenotype, the more common are: t(8;21) (q22;q22)/AML1-ETO; inv(16) (p13.1q22)/core binding factor (CBF) b-MYH11; 11q23/mixed lineage leukemia (MLL)-fusion proteins; t(6;9) (p23;q34)/DEK-NUP214; inv(3) (q21q26.2) or t(3;3) (q21;q26.2)/RPN1-EVI1 [102]. Moreover, a growing number of gene mutations have been identified, including gene mutations in FLT3 (Fms-like tyrosine kinase 3) [103,104], NPM1 (nucleophosmin 1) [105], and CEBPA (CCAAT enhancer-binding protein) [106].

Autophagy could play an important role in the evolution of MDS to AML. MDS comprises a heterogeneous group of hematologic disorders, mostly diagnosed in the elderly, which are characterized by cell morphology anomalies in the BM and peripheral blood cytopenias leading to infection, bleeding, and death. MDS is considered to be a disorder of the HSC [107], and about one-third of MDS cases progress to AML [108]. Interestingly, increased ROS levels and mitochondrial damage were observed in mononuclear BM cells from MDS patients [109]. It has been subsequently documented that erythroid precursors from low-risk MDS patients (i.e. those who are less likely to progress to AML, as compared to high-risk patients) showed an increased number of mitochondria engulfed in autophagosomes. Thus, it has been proposed that a functional autophagic machinery is essential for protecting MDS patients from ROS build-up until the cells undergo apoptosis for other causes, thus lowering the risk of MDS evolution to AML [110]. In this connection, it is important to highlight that high levels of ROS can inhibit the activity of phosphatases, including the tumor suppressor PTEN [111], a negative regulator of the PI3K/Akt/mTORC1 pathway [112], which is frequently over-active in high-risk (but not in low-risk) MDS patients [113,114]. Importantly, deletion of PTEN is leukemogenic in mice [115]. Therefore, it could be speculated that a link exists between impaired autophagy/mitophagy and MDS evolution to AML. However, validation of this hypothesis would require the demonstration that high-risk MDS patients display abnormalities in genes belonging to the autophagic machinery. However, at least so far, no changes in the expression of autophagy-related genes have been reported in MDS patients [116,117]. Nevertheless, gene expression analysis could not obviously reveal functionally deleterious mutations of genes involved in autophagy/mitophagy. Therefore, the hypothesis linking defective autophagy with malignant evolution of MDS should be further investigated, also in consideration of the fact that AML genetic abnormalities encompass chromosomal regions where key autophagy network genes are mapped (reviewed in [118]). Moreover, it is important to recall that myeloid SKM1 cells resistant to azacytidine (a hypomethylating drug used for treating high-risk MDS patients [119]), displayed an impaired apoptotic response [120] that could be circumvented using autophagy activators [121]. Therefore, there is the distinct possibility that an autophagy-activating pharmacological approach would diminish the probability of the evolution of high risk MDS to AML.

Given the overall poor prognosis of AML, great interest surrounds the development of novel and less toxic targeted therapies against signaling pathways that are aberrantly activated in AML patients and sustain leukemic cell survival and proliferation. Remarkably, most of the studies regarding the effects of autophagy modulation, have been carried out in AML cells that had been treated with novel targeted drugs. Indeed, autophagy is increasingly being recognized as a phenomenon that could have a substantial impact on the outcome of innovative therapeutic strategies (e.g. [122–124]).

Given that aberrant activation of the PI3K/Akt/mTORC1 pathway is a common event in AML patients [125], it is not surprising that this signaling network is considered an attractive target for innovative treatment of AML patients [126]. Since mTORC1 is an autophagy repressor, induction of autophagy is to be expected when AML cells are exposed to mTORC1 allosteric inhibitors, such as temsirolimus [127].

Treatment with the catalytic mTORC1 inhibitors OSI-027 or AZD-2014, resulted in induction of autophagy that functioned as a mechanism of leukemic cell resistance to mTORC1 inhibition [128]. Indeed, co-treatment with chloroquine or knockdown of ULK1 resulted in enhanced cytotoxicity, suggesting that future testing of a combination of autophagy

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inhibitors and catalytic mTORC1 inhibitors may be a promising approach for targeting AML [128]. Similar results have been reported with mTORC1 catalytic inhibitor AZD8055, which induced autophagy in AML cell lines [129]. However, autophagy could be either cytoprotective (at a high AZD8055 concentration, i.e. 100 nM) or cytotoxic (at a low concentration, i.e. 10 nM) [129], further underscoring the utmost complexity of the autophagic response, even in the same cell model.

The same group described a strong pro-survival role of autophagy in AML cells upon treatment with the anticancer agent L-asparaginase, owing to an inhibition of mTORC1 [130], a recently recognized off-target effect of L-asparaginase treatment [131].

Obatoclax (GX15-070) is an inhibitor of anti-apoptotic Bcl-2 family members, including Bcl-2, Bcl-xL, and Mcl-1, which is being evaluated alone and in combination with other drugs, in pre-clinical models of AML. It was reported that autophagy elicited by obatoclax, combined with the multiple kinase inhibitor sorafenib, played a cytoprotective role in AML pre-clinical settings, as demonstrated by a striking potentiation of the sorafenib/obatoclax-induced cytotoxicity upon the concomitant exposure to several autophagy inhibitors (3-methyladenine, chloroquine, or bafilomycin A1) [132]. However, when obatoclax was combined with the histone deacetylase inhibitors (HDACis), MGCD0103 and vorinostat, a synergistic anti-leukemic activity was observed, which was dependent on the induction of both apoptosis and autophagy. Therefore, in this case, autophagy accounted for a non-apoptotic decrease of cell viability [133].

Nevertheless, a pro-survival role of autophagy has been described in AML1-ETO-positive AML cells treated with the HDACis, vorinostat (SAHA) and valproic acid (VPA). Indeed, increased apoptosis was detected upon co-treatment with chloroquine, suggesting that coupling HDACis to autophagy inhibitors may be useful for the treatment of AML1-ETO-positive AML patients [134].

It should be underscored however, that recent findings have documented that the HDACIs, VPA, trichostatin A, and SAHA actually decreased autophagy in AML cells from patients with Down syndrome (who are at risk of developing a megakaryoblastic subtype of AML [135]), as these drugs repressed ATG7 expression both transcriptionally and post-translationally [136]. As a consequence of treatment with HDACis, AML cells displayed a dose-dependent accumulation of mitochondrial mass, increased ROS formation, and DNA damage which then led to apoptotic cell death [136] (Fig. 2A).

Another example of a pro-death role played by autophagy in AML comes from the observation that the nicotinamide phosphoribosyltransferase inhibitor APO866, killed AML cell lines independently from caspase activation [137]. Autophagy induction was detected and, in primary AML samples, both 3-methyladenine and bafilomycin A1 rescued cells from death [137].

Cytarabine, one of the mainstays of AML chemotherapy, induced both a cytoprotective and cytotoxic autophagy in AML cell lines, denoting the complex role of autophagy in response to chemotherapeutic drugs [138].

In this respect, it is worth underscoring that the high-mobility group box 1 (HMGB1) protein was released from AML HL60 cells after chemotherapy-induced cytotoxicity and activated autophagy to protect against injury. Treatment with HMGB1-neutralizing antibodies increased the sensitivity of leukemic cells to chemotherapy, whereas, exogenously added HMGB1 rendered these cells more resistant to

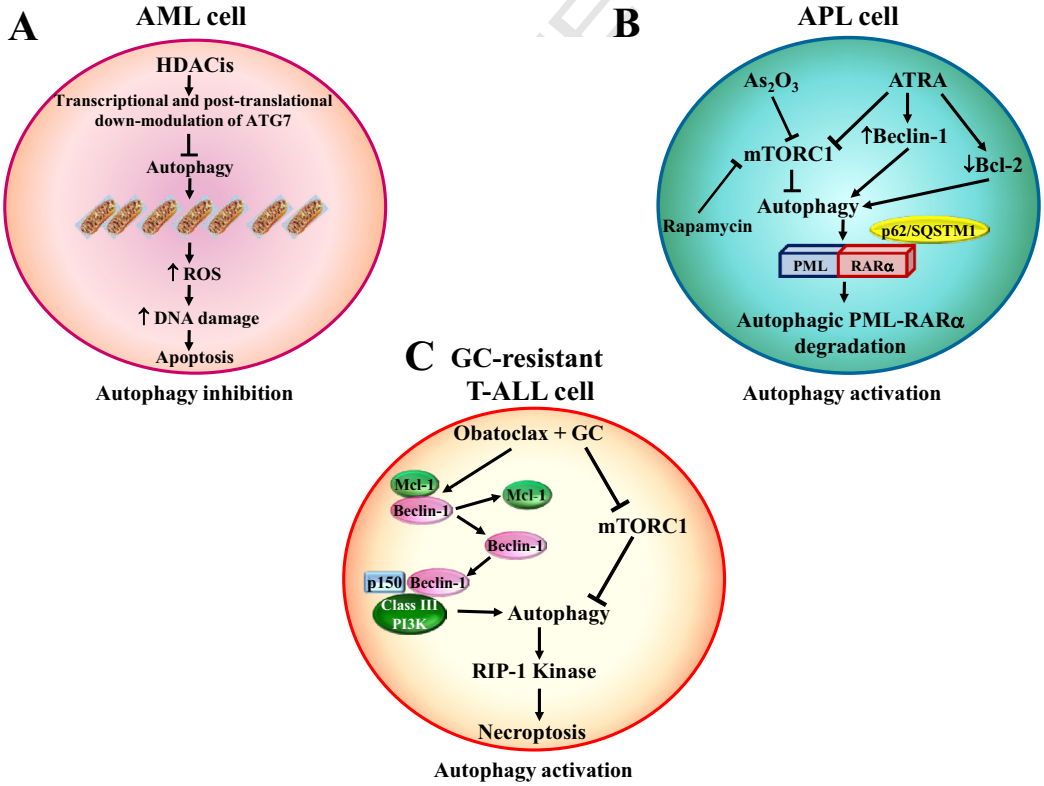

Fig. 2. Mechanisms of autophagy induction in acute leukemia cells. A: In AML cells, HDACis (valproic acid, trichostatin A, and vorinostat) induce a transcriptional and post-translational down-modulation of ATG7, which results in decreased autophagy and increased mitochondrial mass. High levels of ROS generated by mitochondria are responsible for DNA damage and apoptosis. B: In APL cells, As_2O_3, ATRA, and rapamycin inhibit mTORC1 signaling and enhance autophagy. Moreover, ATRA up-regulates Beclin-1 and down-regulates Bcl-2. Changes in the levels of these proteins contribute to increased autophagy. The PML-RARα fusion protein associates with p62/SQSTM1 and it is subsequently proteolytically degraded, thus overcoming the differentiation block which characterizes APL. C: In GC-resistant T-ALL cells, a combination treatment consisting of obatoclax plus GC leads to the dissociation of Mcl-1 from Beclin-1 and to mTORC1 inhibition. These two events up-regulate autophagy, which is converted to necroptosis via RIP-1 kinase. Abbreviations: AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; As_2O_3, arsenic trioxide; ATG, AuTophaGy-related; ATRA, all-*trans* retinoic acid; Bcl-2, B-cell lymphoma 2; GC, glucocorticoids; HDACis, histone deacetylase inhibitors; Mcl-1, myeloid cell leukemia 1; mTORC1, mammalian target of rapamycin complex 1; p62/SQSTM1, p62/sequestosome-1; PI3K, phosphoinositide 3-kinase; PML-RARα, promyelocytic leukemia-retinoic acid receptor α; RIP-1, receptor-interacting protein 1; ROS, reactive oxygen species; T-ALL, T-cell acute lymphoblastic leukemia.

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drug-induced cytotoxicity and increased autophagy [139]. Taken together, these findings suggested that HMGB1 release after chemotherapy was a critical regulator of autophagy and a potential drug target for therapeutic interventions in AML [140].

5.1.1. Acute promyelocytic leukemia (APL)

APL is a distinct subtype of AML that accounts for approximately 5–10% of all AML cases. APL is characterized by the clonal expansion and the subsequent accumulation in the BM of hematopoietic precursors blocked at the promyelocyte stage of differentiation [141]. In the vast majority of cases, APL is caused by the balanced translocation t(15;17) (q22;q12) [142] that involves the promyelocytic leukemia (*PML*) gene and the retinoic acid receptor α (*RARα*) gene [143,144]. The PML-RARα fusion protein blocks the transcription of RARα-driven genes involved in myeloid differentiation, thus resulting in a differentiation arrest [145,146]. In addition, expression of PML-RARα might induce lineage switching from committed hematopoietic progenitors into leukemia, and confer aberrant self-renewal activity to APL cells [147,148].

The prognosis of APL markedly differs from that of other AML subtypes, as a combination consisting of arsenic trioxide (As_2O_3) with all-*trans* retinoic acid (ATRA) can cure nearly 90% of patients [149,150]. This mainly depends on the ability of these two drugs to induce a proteolytic degradation of the chimeric protein. Once PML-RARα is degraded, the differentiation block is overcome [151]. Importantly, it has been demonstrated that ATRA and As_2O_3, through cooperative PML-RARα degradation, were synergistic in the clearance of promyelocytic leukemia-initiating cells, whose eradication is mandatory for the achievement of a complete remission [152].

A correlation between autophagy and therapy-induced differentiation of APL cells was reported, and it was documented that both ATRA and As_2O_3 stimulated an autophagy-dependent proteolysis of PML-RARα [153]. These findings have been subsequently confirmed by independent groups, that were able to demonstrate that knockdown of p62/SQSTM1 inhibited ATRA-induced PML-RARα degradation/myeloid cell differentiation and that ATRA up-regulated Beclin-1 [154,155] (Fig. 2B). It has been proposed that the activation of autophagy in APL cells treated with ATRA/As_2O_3 is dependent on mTORC1 inhibition, which is detected upon treatment with the drugs. Indeed, treatment with rapamycin (an mTORC1 inhibitor) increased autophagy and promoted PML-RARα degradation in APL cells similarly to ATRA/As_2O_3 [153,156] (Fig. 2).

It is worth mentioning here that As_2O_3 treatment also resulted in the autophagic degradation of the BCR-ABL1 fusion protein that is the hallmark of chronic myeloid leukemia (CML) [157,158]. Nevertheless, autophagy did not mediate the degradation of AML1-ETO in AML1-ETO-driven AML, where it had a pro-survival effect [134]. This is another demonstration of opposite functions exerted by autophagy in hematopoietic malignancies, even when they are caused by fusion oncoproteins.

Although As_2O_3 and ATRA are the mainstay of APL treatment, they are now being tested in other AML subtypes [151]. In this context, it is worth highlighting that ACD has been described to occur in response to As_2O_3 treatment in non-APL AML cell lines and primary samples [159]. Interestingly, ACD was dependent on MEK/ERK signaling, whereas mTORC1 and SAPK/JNK cascades were not involved [159]. Autophagy also played a causative role in the dasatinib-mediated differentiation of non-APL AML cells, a phenomenon which was amplified by ATRA co-treatment. Indeed, pharmacological inhibition of autophagy by 3-methyladenine or chloroquine blocked dasatinib-induced AML cell differentiation [160]. In Table 1, we have summarized the studies combining molecularly targeted or chemotherapeutic agents with pharmacologic inhibitors of autophagy performed with AML/APL cells.

5.2. B-cell acute lymphoblastic leukemia (B-ALL)

B-ALL accounts for approximately 80% of ALL cases. Although B-ALL mainly affects children where it is by far the most common malignancy, it can occur at any age. While the outcome for pediatric B-ALL patients has dramatically improved over the last two decades with survival rates of approximately 80% at 5 years, in adult patients conventional chemotherapy is less successful [161].

Table 1
Summary of studies combining molecularly targeted or chemotherapeutic agents with pharmacologic inhibitors of autophagy.

Leukemia type	Cell types	Treatment	Outcome	Reference
AML	MV4-11, primary cells	AZD8055	Cytoprotective at high concentrations Cytotoxic at low concentrations	[129]
AML	MV4-11, HL-60, MOLM-14, OCI-AML3	L-asparaginase	Cytoprotective	[130]
AML	U937, HL-60, MV4-11, primary cells	Obatoclax + sorafenib	Cytoprotective	[132]
AML	HL-60, THP1, U937, primary cells	Obatoclax + HDACis (MGCD0103 or vorinostat)	Cytotoxic	[133]
AML	Kasumi-1, SKNO-1, HL-60, primary cells	Obatoclax + HDACis (valproic acid or vorinostat)	Cytoprotective	[134]
AML	U937, HEL, Kasumi-1, Kasumi-3, primary cells	OSI-027 or AZD-2014	Cytoprotective	[128]
AML	THP-1, NB4, MV4-11, MOLM-13, HEL, OCI-M1, Kasumi-1, HL60	APO866	Cytotoxic	[137]
AML	U937	As_2O_3	Cytotoxic	[159]
APL	NB4	ATRA	Cytotoxic	[153,156]
APL	NB4	As_2O_3	Cytotoxic	[153]
B-ALL	RS4;11, REH, primary cells	Dexamethasone	Cytotoxic	[169]
B-ALL	SEM, REH, RS4;11, NALM6, primary cells	Everolimus	Cytotoxic	[172,174]
B-ALL	REH, primary cells	Obatoclax + GC	Cytotoxic	[170]
B-ALL	SEM-K2, RS4;11, primary cells	Obatoclax	Cytotoxic	[171]
B-ALL	ALL1, REH, NALM6, LK63, primary cells	FTY720	Cytoprotective	[176]
B-ALL	REH, primary cells	Idarubicin	Cytotoxic	[94]
T-ALL	CCRF-CEM, CCRF-CEM VBL100, Molt-4, Jurkat, RPMI-8402, BE-13, primary cells	BEZ235	N.A.	[184]
T-ALL	CCRF-CEM, CCRF-CEM VBL100, Molt-4, Jurkat, primary cells	Triciribine	Cytoprotective	[185]
T-ALL	CCRF-CEM, CCRF-CEM VBL100, Molt-4, primary cells	MK-2206	Cytoprotective	[186]
T-ALL	CCRF-CEM, CCRF-CEM VBL100, Molt-4, Jurkat, RPMI-8402, BE-13, primary cells	Metformin (AMPK activator)	N.A.	[187]
T-ALL	CCRF-CEM, Molt-4, COG-LL-317h, COG-LL-332h	C22:0- and C24:0-dihydroceramides	None	[188]
T-ALL	Jurkat	Selenite	Cytotoxic	[189]
T-ALL	CEM-C7-14, CEM-C1-15, MEFs	Obatoclax + GC	Cytotoxic	[190]

Abbreviations: AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; As_2O_3, arsenic trioxide; ATRA, all-*trans* retinoic acid; B-ALL, B-cell acute lymphoblastic leukemia; GC, glucocorticoids; HDACis, histone deacetylase inhibitors; N.A.: not assessed; T-ALL: T-cell acute lymphoblastic leukemia.

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B-ALL is a very heterogeneous disease, characterized by recurrent karyotypic abnormalities, including aneuploidy and translocations, submicroscopic DNA copy number alterations, and mutations [161]. In particular, chromosome alterations consist of high hyperdiploidy with nonrandom gain of at least five chromosomes (including X, 4, 6, 10, 14, 17, 18, and 21); hypodiploidy with fewer than 44 chromosomes; recurring translocations, for instance: t(12;21) (p13;q22) encoding ETV6-RUNX1; t(1;19) (q23;p13) encoding TCF3-PBX1; t(9;22) (q34;q11) encoding BCR-ABL1; rearrangement of MLL at 11q23 with a wide range of fusion partners; and a number of submicroscopic alterations involving genes encoding proteins with key roles in lymphoid development like PAX5, IZKF1, and EBF1 [162,163]. Constitutive activation of PI3K/Akt/mTORC1 network is also a feature of B-ALL, and it seems to be specifically related to the presence of ETV6/RUNX1 (E/R) [164] and BCR-ABL1 fusion proteins [165].

The controversial role of autophagy in the death/survival of leukemic cells has been investigated also in B-ALL. Although the BCR-ABL1 fusion protein is the hallmark of CML, it is also detected in 25–30% of adults and in 2–10% of pediatric B-ALL cases, where it portends a poor prognosis [163]. Intriguingly, murine hematopoietic progenitor cells expressing a p185 form of BCR-ABL1 displayed low basal levels of autophagy, but were highly dependent on this process, as they rapidly underwent apoptosis in vitro upon disruption of autophagy through *Atg3* deletion or treatment with autophagy inhibitors. This dependence on autophagy extended in vivo, as *Atg3* deletion prevented BCR-ABL1-mediated leukemogenesis [166]. Therefore, cells expressing the oncogenic BCR-ABL1 kinase appear particularly dependent on autophagy for their survival and leukemogenic transformation.

Glucocorticoids (GC) are widely used for the therapy of B-ALL [167], however, GC resistance is observed in approximately 10% of pediatric B-ALL patients [168]. GC have been described to induce cell death through the autophagic machinery activation in B-ALL cell lines and primary cells [169]. Interestingly, obatoclax was able to overcome GC-resistance in B-ALL cells, where it induced caspase-dependent apoptosis, as well as autophagy. Obatoclax-induced autophagy was ATG5-dependent but Beclin-1 independent, and was not a pre-requisite for commitment to concomitant apoptosis, which was BAK-dependent [170]. Very recent studies reported that obatoclax activated a triple death mode killing (apoptosis, autophagy, and necroptosis) in cell lines and primary pediatric B-ALL cells bearing a MLL translocation, which portends a poorer prognosis [171].

The mTORC1 inhibitor, everolimus, induced autophagy in vitro in B-ALL cell lines and primary samples [172] and in vivo in NOD/SCID mice xenografted with human B-ALL cells [173,174]. Beclin-1 down-regulation by siRNA strategy, decreased the cytotoxic effects of everolimus, implying that autophagy could be a death mechanism [172]. Nevertheless, the use of the autophagy inhibitor, 3-methyladenine, could not prevent the cell death induced by everolimus in B-ALL cells [175].

The immunosuppressive drug FTY720 displayed potent anti-leukemic effects in BCR-ABL1-positive and -negative B-ALL cell lines [176]. Characterization of death modalities in both types of cell lines revealed that FTY720 caused a caspase-independent cell death and a concomitant autophagy, which, however, had a protective function [176].

An ACD triggered by APO866 has been described to occur also in B-ALL cell lines [137]. The occurrence of a cytotoxic autophagy in B-ALL REH cells has been reported in response to idarubicin [94], an anthracycline antileukemic drug broadly used for treatment of acute leukemias [177]. Idarubicin up-regulated AMPK and down-regulated mTORC1 activity, which could explain induction of autophagy. Pharmacological (bafilomycin A1 or chloroquine treatment) or genetic (siRNA down-regulation of either Beclin-1 or LC3 expression levels) impairment of autophagy partially reduced the cytotoxicity of idarubicin [94]. In Table 1, we have summarized the studies combining molecularly targeted or chemotherapeutic agents with pharmacologic inhibitors of autophagy performed with B-ALL cells.

5.3. T-cell acute lymphoblastic leukemia (T-ALL)

T-ALL is an aggressive form of leukemia characterized by uncontrolled proliferation of lymphoblasts committed to the T-cell lineage arising in the thymus from T-cell progenitors and expressing immature T-cell immunophenotypic markers [178,179]. T-ALL accounts for 10–15% and 25% of pediatric and adult ALLs, respectively. The outcome of T-ALL patients with primary chemoresistant or relapsed leukemia is still poor, even if intensified combination chemotherapies have improved the survival outcome of T-ALL, especially in the childhood [161]. However, studies of the long-term effects of chemotherapy in patients with T-ALL documented that recent gains in leukemia-free survival have been achieved at the cost of significant increases in the rates of life-threatening and debilitating toxicities [161].

T-ALL is characterized by many different rearrangements and/or mutations. The most common abnormalities found in T-ALL are summarized in some excellent reviews [179,180] and include translocations involving TCR genes on chromosomes 7q34 and 14q11, chromosomal rearrangements, aberrant expression of oncogenes, deletions, somatic gene mutations, impairments of many different signaling pathways (i.e. PI3K/Akt/mTORC1 and Notch1 signaling), as well as microRNA dysregulation [181,182].

From a historical perspective, it should be mentioned that the first description of the occurrence of autophagy in acute leukemia cells, dates back to 1997, when it was documented that TNF-α induced autophagy in the T-ALL cell line, CCRF-CEM [183].

We have reported that inhibition of either PI3K/mTOR [184] or Akt [185,186] induced autophagy in T-ALL cell lines. In case of Akt inhibitors, autophagy was cytoprotective, as its down-regulation either by chloroquine or by siRNA to Beclin-1, increased the cytotoxic effects of Akt inhibitors [185,186]. Another strategy that induced autophagy in T-ALL cell lines was activation of AMPK by metformin [187].

It has been reported that autophagy occurred in T-ALL cell lines treated with C22:0- and C24:0-dihydroceramides, however addition of the autophagy inhibitor, 3-methyladenine, neither increased nor decreased the cytotoxicity of C22:0-dihydroceramide, suggesting that autophagy was not directly linked to the T-ALL cell death mechanism [188].

Nevertheless, autophagy could also play an active role in cell death of T-ALL cells, as documented by a study in which Jurkat cells were treated with selenite, a drug with anti-tumor efficacy which is known for exerting both pro-apoptosis and pro-autophagy effects. When Jurkat T-cells were treated with autophagy inhibitors (either 3-methyladenine or bafilomycin A1) prior to incubation with selenite, a reduction of the apoptotic rate was observed, suggesting an active role played by autophagy in cell death induced by selenite [189].

A pro-death role played by autophagy has been also reported in GC-resistant T-ALL cell lines (Jurkat, CEM, MOLT-4) treated with obatoclax. Obatoclax induced dissociation of Beclin-1 from the anti-apoptotic Bcl-2 family Mcl-1 together with a decrease in mTORC1 activity. This led to a rapid activation of autophagy-dependent necroptosis, which bypassed the block in mitochondrial apoptosis, and was mediated via the receptor-interacting protein 1 (RIP-1) kinase [190] (Fig. 2C). The autophagy inhibitor, 3-methyldenine, negated the sensitizing effects of obatoclax in GC-resistant T-ALL cells. In Table 1, we have summarized the studies combining molecularly targeted or chemotherapeutic agents with pharmacologic inhibitors of autophagy performed with T-ALL cells.

6. Conclusions and future directions

From the findings discussed in this review, it is clear that autophagy is affected by and also affects diverse therapeutic treatments currently used for acute leukemia patients. However, the effects are not always the same: Indeed, they are often diametrically opposed. Sometimes anti-cancer drugs induce autophagy, whereas sometimes they inhibit it. Sometimes autophagy protects leukemic cells against cancer therapy,

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while sometimes it is required for the therapy to be efficacious. Thus, the current scenario emerging from studies targeting autophagy for clinical benefit in acute leukemia patients is still puzzling and many issues remain unresolved. Autophagy modulation, either stimulatory or repressive, can be achieved by interfering with several signaling pathways at multiple levels. Therefore, the number of compounds displaying autophagy modulating properties is extremely vast, as are the interactions between these different compounds. Nevertheless, we are starting to detect reproducible patterns and are making progress in understanding the molecular mechanisms that underlie these highly variable effects.

The results of first clinical trials in which autophagy inhibitors were combined with anti-tumor drugs have been very recently released, and they have already provided a number of valuable insights related to this kind of therapeutic approach [91,123,124,191,192].

However, several outstanding questions remains to be answered before the potential for targeting autophagy in acute leukemias could be fully understood.

The first issue is whether we can develop and validate biomarkers that will predict autophagy dependency and addiction of leukemic cells in vivo. In other words, the identification of patients who will maximally benefit from autophagy modulation is mandatory. In this sense, the recent results by Stankov et al. [136] seem important, as they clearly documented that cells with a low autophagic flux were very sensitive to drugs blocking autophagy (HDACis in this case). This cytotoxic effect could be presumably further enhanced by a combined treatment with autophagy inhibitors.

We also need better techniques to monitor the amount of autophagy and autophagic flux in humans in vivo, so that we can develop reliable pharmacodynamics markers for clinical studies of autophagy manipulation. Autophagy is, by its own nature, a highly dynamic phenomenon. Although we have sound methods to measure such a dynamic process in vitro, we have very poor ways to assess it in living organisms, especially in humans. One common misconception in the literature, even for in vitro studies, derives from the fact that an increased number of autophagosomes, could mean either that autophagy is increased (as more autophagosomes are being produced) or that it is being decreased (as fewer autophagosomes are fusing with lysosomes). In vitro, we can rigorously discriminate between these two possibilities by deliberately blocking autophagosome fusion with lysosomes, and monitoring an increase in LC3-II levels, using immunofluorescence or Western blot. However, such an approach is problematic in cancer patients, making it inherently difficult to determine whether or not drug treatment affected autophagy.

Recently published clinical trials on autophagy inhibitors, mostly relied on transmission electron microscopy (TEM) analysis of the mean number of autophagic vesicles/cell, as a pharmacodynamics marker of autophagy [91,124,191,192], although in an extremely limited number of patients other markers of autophagy induction, such as LC3-II and p62/SQSTM1 levels, were analyzed by either Western blot [91] or immunocytochemistry [124]. It should be underscored that in most cases TEM analysis of autophagic vesicle was carried out on peripheral blood leukocytes, even though in one study also the tumor tissue was analyzed [123]. Obviously, there is no proof whatsoever that leukocytes will respond to the drugs modulating autophagy in the same manner as tumor cells. However, in the study performed in multiple myeloma patients, the Authors performed their TEM analysis directly on the cancerous plasma cells [91]. In case of acute leukemias, it should be relatively easy to perform pharmacodynamics studies directly on the neoplastic cell population, obtained from either the peripheral blood or the BM. Analytical techniques requiring a limited number of cells, as reverse phase protein array (RPPA) or flow cytometry, could be used for studying the expression of key autophagic markers in patient samples. These techniques have proven their reliability in recent clinical studies on novel targeted drugs carried out in acute leukemia patients [193,194].

Another relevant issue stems from the fact that autophagy modulators (mostly inhibitors, such as chloroquine and hydroxychloroquine) do not specifically and exclusively modulate autophagy and display several off-target effects. As a consequence, these drugs could have substantial side effects, as it has emerged from the first clinical trials in which they were tested [91,124,191,192]. Hence, the identification and development of novel, specific, more powerful, and less toxic autophagy modulating agents, suitable for use in patients, are eagerly awaited. However, it should be highlighted that the first clinical trials in which hydroxychloroquine was used to inhibit autophagy have yielded preliminary but encouraging clinical results. Although no complete responses were observed in patient cohorts, some partial responses and disease stabilization were seen in most studies [91,124,191,192]. In particular, the combination of temsirolimus/hydroxychloroquine resulted in stable disease in 14/19 (74%) of melanoma patients. All the patients had evidence of progressive disease at the time they entered the study, and temsirolimus was used at a dosage that did not have any positive effects in a previous trial [123].

Additionally, we need to determine which drugs will work best when combined with autophagy inhibitors. Will drugs like mTOR inhibitors or AMPK-activating compounds that directly activate autophagy be more sensitive to autophagy inhibition than drugs that only affect autophagy indirectly?

Answers to this and other issues will be critical for moving the field forward, but there is the distinct possibility that autophagy modulators will be added to the growing arsenal we have at our disposal for treating acute leukemias.

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