Chapter 9

Role of Autophagy in Cancer and Tumor Progression

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Additional information is available at the end of the chapter

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1. Introduction

Autophagy, which is constitutively executed at basal level in all cells, promotes cellular homeostasis by regulating organelles and proteins turnover. In tumor cells, autophagy is activated in response to various cellular stresses, including nutrient and growth factor starvation, as well as hypoxia [1]. It is now well established that autophagy can act as tumor suppressor and tumor promoter. The different roles of autophagy in cancer cells seem to depend on tumor type, stage, and genetic context. Indeed, autophagy clearly suppresses the initiation and development of tumors, however, it is considered as a key survival pathway in response to stress, and many established tumors require autophagy to survive. In this section, we will summarize the different mechanisms involved in the activation of autophagy in tumor and discuss recent reports about the dual role of autophagy in carcinogenesis and tumor progression.

1.1. Role of autophagy in tumor suppression

Several lines of indirect evidence indicate that autophagy acts as a tumor suppressor. Indeed in various cases, oncogenic transformations, such as activation of the PI3K/Akt pathway *via* activating *PI3K* mutations, *AKT* amplifications, or *PTEN* loss, are correlated, with a decreased autophagy through mTOR activation [2]. Moreover, the amplification of the apoptosis inhibitor Bcl-2 has been reported in some circumstances to inhibit autophagy through its binding to Beclin1 [3, 4]. The involvement of p53 in the regulation of autophagy seems to be more complex. Indeed, the activation of p53 by nutrient deprivation or genotoxic stress leads to the activation of autophagy through the inhibition of mTOR or by the activation of DRAM (damage-regulated autophagy modulator) [5-7]. However, consistent with the role of autophagy as tumor suppressor, the functional loss of p53 was expected to decrease autophagy or to

suppress basal autophagy. The later effect seems to depend on the cytoplasmic, not the nuclear, pool of p53 [8].

Beside the indirect evidences outlined above, there are more direct ones supporting the tumor suppressing properties of autophagy. Thus, the autophagy execution protein Beclin1 is a haplo-insufficient tumor suppressor protein. Monoallelic deletions of *BECLIN1* are found in sporadic human breast and ovarian carcinomas [9], and heterozygous deletion of *BECLIN1* predisposes mice to a variety of tumors including mammary neoplastic lesions, lung adenocarcinomas, hepatocellular carcinomas, and B cell lymphomas [10]. These results suggest that intact autophagy may be constraining tumor initiation [11]. Similarly, homozygote deletion of *ATG5* was shown to predisposed mice specifically to liver tumors with high penetrance [12].

The tumor suppressive functions of autophagy have been extensively investigated. Below we will provide mechanistic insights into the tumor-suppressive functions of autophagy.

1.1.1. Autophagy inhibits necrosis and inflammation

During the last decade, strong evidence supported that the inflammatory microenvironment plays a major role in tumor development. Indeed, chronic inflammation is a common future of early cancer development. In this regards, it has been proposed that autophagy can modulate those inflammatory reactions through different mechanisms, as autophagy-deficient tumors display an increased level of necrosis and inflammation.

First, it has been reported that activation of autophagy in tumor cells can inhibit necrotic cell death. Unlike apoptotic cell death, cells dying by necrosis stimulate a robust inflammatory response *in vivo* [13]. In 2006, a major study from the E. White's group reported that impairment of both apoptosis and autophagy promotes necrotic cell death, *in vitro* and *in vivo,* associated with an inflammatory response and an accelerated tumor growth [14]. These results suggest that autophagy takes part in the regulation of necrosis-induced cell death and thus in the subsequent inflammation.

Several studies have confirmed that autophagy is able to prevent the two forms of necrotic cell death (i) necroptosis and (ii) poly-(ADP-ribose) polymerase (PARP)-mediated cell death. Necroptosis is a form of caspase-independent cell death mediated by cell death ligands (*i.e.* TNF-α and FasL) [15, 16]. For example, Wu *et al.* have shown that autophagy is essential to overcome zVAD-induced necroptosis in L929 cells. Activation of PI3K-Akt-mTOR pathway, well-known as inhibitor of autophagy, is able to sensitize L929 cells to zVAD-induced necroptosis, while amino-acid and serum starvation offers some protection to these cells [17]. PARP-mediated cell death is another form of programmed-necrotic cell death mainly triggered by DNA damage. The cytoprotective role of autophagy in PARP-mediated necrosis was illustrated in a recent study of Muñoz-Gámez *et al.*. They have reported that DNA damages induced by doxorubicin in fibroblasts lead to PARP-1 activation and autophagy induction which protects cells against necrosis. By specific knocking down of the autophagic genes *ATG5* or *BECLIN1*, the authors were able to sensitize cells to doxorubicin-induced necrotic cell death [18].

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Autophagy acts also through different mechanisms to decrease inflammation. Autophagy is essential for the maintenance of intracellular ATP level, which in turn is required for the secretion of lysophosphatidylcholine (LPC). Secretion of LPC is associated with the acute phase of the inflammatory response and is involved in the development of chronic inflammation. It also has been shown that autophagy-deficient cells fail to generate phosphatidylserine on the outer membrane surface – an important anti-inflammatory pro-apoptotic marker. This explains how defect in autophagy can stimulate inflammatory response subsequently to insufficient clearance of dead cells [19]. Accumulation of p62 in autophagy-deficient cells activates the pro-inflammatory transcription factor NF-κB and the stress-responsive transcription factor NRF2, thus favoring inflammation and tissue injury [20]. Transcription factors of NF-κB family regulate the expression of a broad range of genes involved in the development, the proliferation, and the survival of tumor cells. Moreover, these transcription factors are important in regulation of inflammation and innate and adaptive immune responses [21]. Activation of NF-κB is mediated by the IκB kinase (IKK) complexes. It has been shown that IKK complexes are targets for degradation by autophagy when the heat shock protein 90 (Hsp90) function is inhibited [22]. Another mechanism of regulation of NF-κB by autophagy is mediated by the Kelch-like ECH-associated protein 1 (Keap1). Keap1 interacts with the kinase domain of IKKβ through its C-terminal domain. This domain is also required for the binding of Keap1 to the transcription factor NRF2, which controls expression of certain antioxidant genes. In response to tumor necrosis factor (TNF), Keap1 negatively regulates activation of NF-κB through inhibition of the IKKβ phosphorylation and induction of IKKβ degradation by autophagy pathway [23]. The E3 ubiquitin ligase Ro52 is another signaling molecule that targets IKKβ for degradation through the autophagy pathway. In response to distinct stimuli, specific interactions of Hsp90, Keap1 and Ro52 with IKKs regulate NF-κB activity through their ability to activate or repress the degradation of IKKs by autophagy [24]. It has been shown that the crosstalk between NF-κB and autophagy regulates inflammasome activity leading to the modulation of the activation of caspase-1 and subsequently the secretion of potent pro-inflammatory cytokines [25]. Overall, it appears that autophagy exerts a significant impact on the regulation of inflammation, and is an important modulator of cancer pathogenesis.

1.1.2. Autophagy prevents oxidative stress and genomic instability

Over the last years, the link between autophagy and suppression of cancer development has been confirmed by several *in vivo* studies using genetically engineered mice [26]. As mentioned above, autophagy-defective mice with targeted deletion of the essential autophagy gene *BECLIN1* showed an increased susceptibility to develop cancer [10] [27]. It appears that the involvement of autophagy in the management of oxidative stress and in the maintenance of the genomic integrity is related to its antitumorigenic activity. Indeed, Mathew *et al.* demonstrated that autophagy can limit DNA damage, chromosomal instability and aneuploidy, which may explain its antitumorigenic activity [28]. Several studies suggested that the ubiquitin- and LC3-binding protein p62 may play a determinant role [29] [30]. Indeed, the inability of autophagy-deficient cells to degrade p62 leads to the aberrant accumulation of this protein, which is sufficient to promote tumorigenesis [30]. Recently, two groups demonstrated

that p62 activates the transcription factor NRF2 through the direct inhibition of Keap1 [31] [32]. However, the role of NRF2 in DNA damage promotion is not clearly understood. In addition, p62 may act as an important NF-κB modulator in tumorigenesis [33]. This study highlights that the increase in DNA damage in autophagy-deficient cells was associated with high levels of damaged mitochondria and reactive oxygen species (ROS), accumulation of endoplasmic reticulum (ER) chaperones and protein disulfide isomerases. DNA alterations were suppressed by ROS scavengers, confirming the essential role of autophagy in oxidative stress management and, subsequently in protein quality control [30]. On one hand, excessive ROS exposure can directly alter the function of multiple cellular macromolecules by oxidation (*e.g.* nucleic acids, lipids, proteins). On the other hand, oxidative stress is closely linked to mitochondria dysfunction. Since autophagy is the only process allowing the mitochondrial turnover (so-called mitophagy), preventing accumulation of damaged mitochondria highly reduces the risk of oxidative stress. Moreover, mitochondria produce the bulk of ATP required for vital cellular functions (*e.g.* DNA replication, mitosis, transcription). In this regard, the ability of autophagy to control proteins/organelles quality and to maintain cellular energy homeostasis highlights its antitumorigenic activity [34]. As an illustration of this concept, the presence of damaged proteins, which are crucial in DNA replication, mitosis or centrosome function, may favor DNA damage in autophagy-deficient cells. Moreover, default in ATP production following a dysfunction in mitochondrial clearance, may also alter DNA replication or repair by leading to the arrest of the replication forks and to the generation of breakage/fusion/bridge cycles responsible for gene amplification [35]. Finally, the implication of autophagy the "normal" protein turnover may also influence the occurrence of DNA damage. For example, cell cycle progression is driven by the periodic activity of certain proteins (*e.g.* Cyclin-dependent kinases (CDKs), Cyclins, CDKs inhibitors). It is possible that a deregulation in "normal" protein turnover in autophagy-deficient cells may alter the correct sequence of the cell cycle progression [35]. Taken together, it has become clear that autophagy helps normal cells to overcome several types of stresses (*e.g.* metabolic, oncogenic), that directly limits their oncogenic transformation. By contrast, this management of cellular stresses is also observed in cancer cells, and leads in this case to cancer promotion (see section 1.2.1.) [36].

Autophagy is also able to mitigate the accumulation of genomic alteration by inducing the mitotic senescence transition. Senescence is an irreversible cell cycle arrest associated with an active metabolism, which can limit the proliferation of abnormal cells. Young *et al.* reported an accumulation of autophagosomes in Ras-induced IMR90 senescent fibroblasts suggesting that autophagy is required for tumor senescence. In addition, knockdown of *ATG5/7* delayed the senescent phenotype, while induction of autophagy clearly enhanced the protein turnover that contributed to synthesis of pro-senescence cytokines (*e.g.* IL-6, IL-8) [37]. This study suggests that autophagy not only facilitates the entry into senescence but also reinforce the senescent phenotype of cells.

1.1.3. Autophagy contributes to tumor cell death

The induction of autophagic cell death has been proposed as a possible tumor suppression mechanism. This statement is based on the observation that apoptosis occurs concomitantly

with features of autophagy [38] and that prolonged stress and progressive autophagy can lead to cell death [1].

Autophagic cell death was first described in 1973 based on the morphological features as a modality of cell death with the presence of autophagosomes and was subsequently named as type II cell death, together with apoptosis (type I) and necrosis (type III) [39]. The relevance of autophagic cell death in development has been established in lower eukaryotes and invertebrates like *Dictyostelium discoideum* and *Drosophila melanogaster* [40, 41]. There is clear evidence that mammalian development does not require autophagy as newborn mice lacking essential autophagy genes show no anatomical or histological defects and no impairment of the cell death [42]. This evidence is supported by the fact that in cultured mammalian cells (human or murine), autophagy genes depletion rather induces apoptosis than protects cell against death induced by different stresses [43, 44]. The role of autophagy in cell death induction is not clear, and needs further investigation. So far, the more convincing evidence showing autophagic cell death in mammals is from neuronal cells. Following insulin starvation, hippocampal neural stem cells undergo autophagic cell death, while suppression of autophagy by *ATG7* knockdown blocks the cell death. In this study, autophagic cell death occurs only in cells with functional apoptosis and is caspase-independent [45]. In cancer cells, recent study showed a novel anti-cancer function of the cytosolic protein FoxO1 which is able to induce the autophagic cell death, but it remains unclear if apoptosis is involved in FoxO1-mediated autophagy [46]. At present, most of experiments showing autophagic cell death in mammalian cells were mainly conducted under *in vitro* cell culture conditions and in cells with defective apoptosis machinery. It has been shown that DAPK (death associated protein kinase) plays an important role in regulation of both autophagy and apoptosis. Indeed, DAPK induces autophagy by phosphorylation of Beclin1, and is associated with the induction of apoptosis. However this type of DAPK-dependent autophagic death is caspase dependent, and it remains to be elucidated whether DAPK-mediated cell death is a real autophagic cell death, or whether autophagy only assists in the apoptosis execution phase [47]. It has been proposed by Kroemer *et al.* that cells rather die *with* autophagy, and not *by* autophagy as they showed that none of 1,400 compounds, evaluated for their ability to induce autophagic puncta and increase autophagic flux, killed tumor cells through the induction of autophagy [48]. Moreover a careful determination of the autophagic flux is needed to differentiate autophagic cell death from other forms of non-apoptotic programmed-cell death, such as necroptosis. These examples illustrate that autophagy may be involved in lethal signaling although the role of autophagy itself in cell killing remains unclear. Thus, further studies are required before the exact role and the precise mechanism of autophagic cell death will be known.

1.1.4. Autophagy modulates the anti-tumor immune response

The immune system plays an important role in controlling cancer progression. It is now well established that immune cells can mediate the destruction of mutated, aberrant or over-expressing self-antigens tumor cells. However, evasion of immune-mediated killing has recently been recognized as an universal hallmark of cancer [49]. It has become increasingly clear that hypoxic tumor microenvironment plays a crucial role in the control of immune

protection [50]. On one hand, tumor cells have evolved to utilize hypoxic stress to their own advantage by activating key biochemical and cellular pathways that are important for tumor progression, survival, and metastasis. Autophagy is one of these pathways activated under hypoxia that may be exploited to modulate the responsiveness of tumor cells to immune system. On the other hand, immune cells that infiltrate tumor microenvironment also encounter hypoxia, resulting in hypoxia-induced autophagy. It is now clearly established that autophagy impacts on the immune system as this process is crucial for immune cell proliferation as well as for their effector functions such as antigen presentation and T-cell-mediated killing of tumor cells [51]. In the subsequent section we will discuss the role of autophagy activation in both tumor and immune cells in the context of cancer immune response. Indeed, understanding how tumor cells evade effective immunosurveillance represents a major challenge in the field of tumor immunotherapy.

1.1.4.1. Role of autophagy in immune cells

Despite the inhospitable hypoxic microenvironment, multiple cell types within the innate and adaptive immune system are capable to recognize and eliminate tumor cells. This was attributed to the ability of immune cells to adjust their metabolic dependency once they have reached the tumor and enhance their survival by activating autophagy. Here we will discuss how autophagy impacts specific immune subsets.

Autophagy in neutrophils

The effect of autophagy induction by hypoxia was investigated in neutrophils as this type of immune cells are the first to migrate to the inflammatory site of the tumor where they promote inflammation and activate macrophages and dendritic cells (DCs) [52]. Neutrophils display high glycolytic rate making them resistant to hypoxia. Autophagy activation in neutrophils has been reported to mediate neutrophil cell death. This will decrease inflammation and ultimately lead to limit tumor growth under these circumstances [53].

Autophagy in antigen presenting cells (APCs)

In contrast to neutrophils, APCs such as macrophages and dendritic cells (DCs) must metabolically adapt to hypoxia through stabilization of hypoxia-inducible factor-1α (HIF-1α) to induce the expression of glucose transporters and glycolytic enzymes as well as limiting oxygen consuming oxidative phosphorylation [54]. As a consequence of hypoxia, macrophages and DCs have decreased phagocytosis, reduced migratory capacity, and increased production of proangiogenic and proinflamatory cytokines. While, hypoxia is involved in dampening APC activity, autophagy may contribute to survival of APCs under these conditions. It has been proposed that culturing DCs under hypoxia resulted in the stabilization of HIF-1α which initiates BNIP3 expression and promotes survival of mature DCs, possibly due to induction of autophagy [55]. It has been proposed that autophagy induction in APCs infiltrating tumor occurs *via* different signaling mechanisms such as toll-like receptor (TLR) [56, 57] and TLR4/HMGB1 [51] signaling pathways. Based on the data outlined above, we could assume that autophagy plays a role in cell death of neutrophils which may serve as an anti-inflammatory mechanism in hypoxic tumors. However, autophagy in tumor-infiltrating APCs is involved

in survival, likely by liberation of nutrients required to support the energy demands of activated cells and is important for the cell's antigen presentation capabilities [58, 59]. DCs also use autophagy to promote cross-presentation of tumor antigens on major histocompatibility complex (MHC) class I complexes for cytotoxic T-Lymphocyte (CTL) activation [60] and to facilitate antigen expression on MHC class II molecules for T-helper (Th) cell activation [59, 61]. Considering the fact that autophagy was shown to be important for the process of antigen presentation, it may be involved in positive effects of APC presence within tumors such as activation of T cells through improved MHC expression. Thus, inhibiting autophagy likely dampens cancer immune response.

Autophagy in T lymphocytes

The effect of autophagy on the activity of T cells was also investigated. Indeed, autophagy is activated in these cells upon T cell receptor engagement in both CD4+ and CD8+ subtypes [62-64]. Targeting autophagy by silencing ATG5 or ATG7 during T cell receptor stimulation leads to a significant decrease in cellular proliferation, highlighting the importance of autophagy during T cell activation [63, 64]. Evidence has been recently provided showing that autophagy is upregulated at the immunological synapse during DC and T cell contact. Suppression of autophagy in DCs resulted in hyper-stable contacts between the DC and CD4+ T cells and increased T-cell activation [65]. Autophagy is upregulated in Th2 CD4+ T cells compared with Th1 CD4+ T cells and was shown to be important for the survival of a Th2 cell line upon growth factor withdrawal [66]. In addition, cells cultured under Th1-polarizing conditions rely more heavily on autophagy for survival compared to the Th17 subset. These findings indicate that the role of autophagy is dependent on the cell type and stimuli and that blocking autophagy can skew the balance of immune subsets [67]. Once T cells mature and traffic to the periphery, autophagy is required for survival [63, 67-70]. The role of autophagy in promoting mature T-cell survival has been attributed to autophagy degrading essential components of the apoptotic cell death machinery [67] and maintaining mitochondrial turnover [68-70]. In addition, it has been demonstrated that activated CD4+ T cells exhibit reduced cytokine secretion, adenosine triphosphate (ATP) production, fatty acid utilization, and glycolytic activity when autophagy is inhibited [64]. These findings support the notion that autophagy is required for cellular function by providing metabolism through the liberation of biosynthetic precursors. It has been shown that during sustained growth factor withdrawal, autophagy supplies the metabolites necessary to generate ATP production in bone marrow hematopoietic cells [71] supporting the hypothesis that immune cells use autophagy to generate metabolites required for cell survival. More recently, it has been shown that autophagy is involved in the liberation of the ubiquitous protein puromycin-sensitive aminopeptidase epitope, thereby creating a CTL epitope that mimic tumor-associated antigens [72].

1.1.4.2. Role of autophagy in tumor cells

Autophagy has been found activated in many tumors and its inhibition can lead to either increased death or increased survival, depending on tissue type, tumor grade and any

concomitant therapy used [73, 74]. The role of autophagy induction in the anti-tumor immune response has recently received widespread attention. We have investigated the role of autophagy induction under hypoxia in tumor response to CTL-mediated lysis. Using non-small cell lung carcinoma and their autologous CTL, we clearly showed that the activation of autophagy under hypoxia in tumor cells is associated with resistance to CTL-mediated lysis (Figure 1).

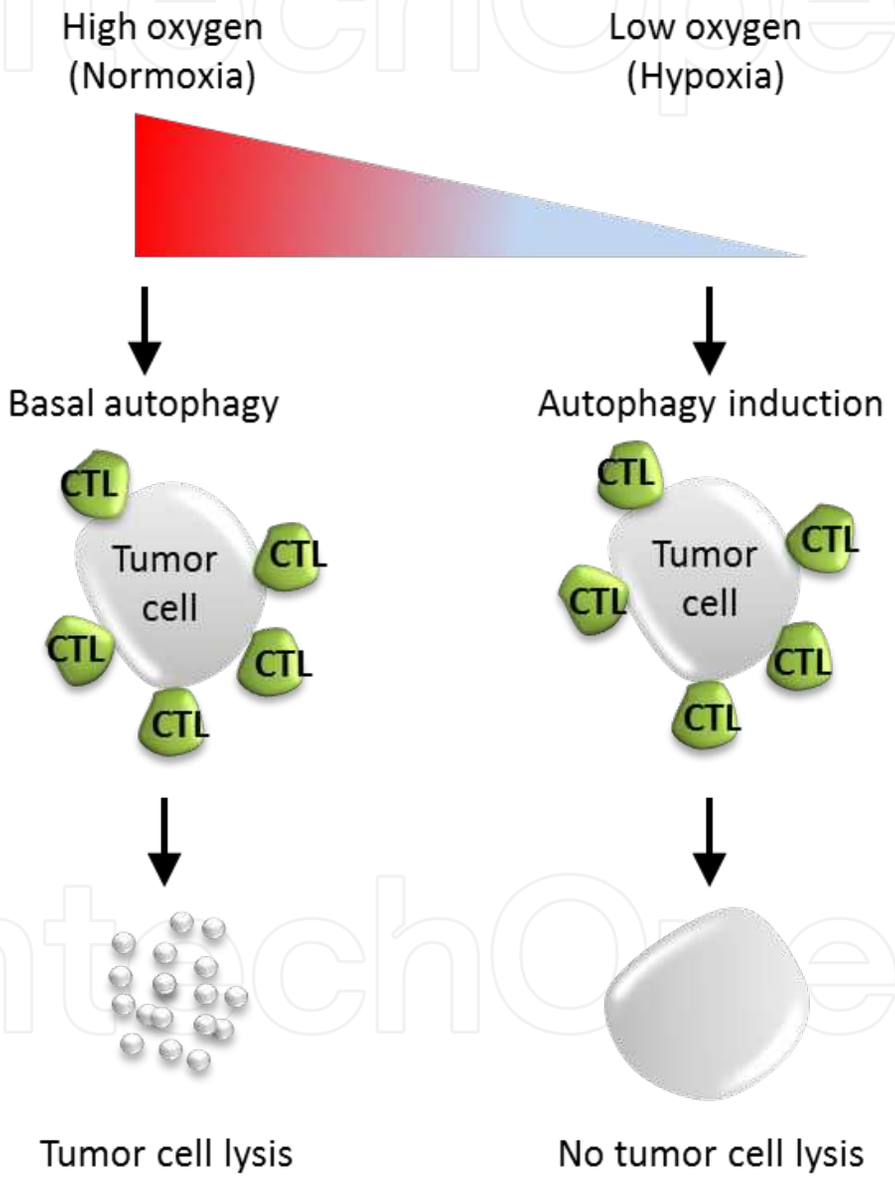

Figure 1. Effect of hypoxia-induced autophagy in CTL-mediated tumor cell killing

Targeting autophagy in hypoxic tumor cells restores CTL-mediated killing [75]. The mechanism by which hypoxia-induced autophagy leads to tumor resistance to CTL was investigated. We provided evidence that hypoxia-inducible factor (HIF)-1α and autophagy coordinately

operate to induce and stabilize a survival pathway involving the activated signal transducer and activator of transcription-3 (STAT-3) [76]. Furthermore, we also showed that targeting autophagy *in vivo* enhances the anti-tumor effect of tumor vaccine. These findings extend the notion that simultaneously boosting the immune system and targeting autophagy could enhance the therapeutic efficacy of cancer vaccine and may prove beneficial in cancer immunotherapy [75].

Since autophagy can also promote the survival of tumor cells through nutrients recovered from degrading and recycling damaged organelles, it has been recently proposed that chemotherapy-induced autophagy causes the release of ATP from tumor cells, thereby stimulating antitumor immune response. Targeting autophagy blunted the release of ATP by tumor cells in response to chemotherapy without affecting that of other damaged signals. Autophagy-dependent extracellular ATP recruits DCs into tumors and activates a T cell response to tumor cells [77]. Based on this study, it seems that the activation of autophagy in the context of DNA damage-induced apoptosis, causes ATP release which subsequently recruits immune cells.

It is now well established that immune effector cells integrate signals that define the nature and magnitude of the subsequent response. In this context, it has been shown that at high effector-to-target ratios, autophagy was induced in several human tumors by natural killer (NK) cells. Importantly, cell-mediated autophagy promoted resistance from treatment modalities designed to eradicate tumor. Thus, the lymphocyte-induced cell-mediated autophagy promotes cancer cell survival and may represent an important target for development of novel therapies [78].

The complexity of cancer immune response is related to the fact that different immune subsets cooperatively and coordinately act through the secretion of cytokines and other soluble factors. Thus, it stands to reason that antitumor immune responses are not entirely dependent on the presence or absence of any particular subset, but rather on the stoichiometry of immune effectors versus immune suppressors. As a result, any anti-cancer therapies that skew the immune effector to suppressor ratio by impacting autophagy may exert a large effect on overall patient survival [79]. While mounting evidence suggest that autophagy induction enhances immune cell function, autophagy seems to operate as a tumor cells resistance mechanism against immune response. In spite of this, inhibition of autophagy in the clinic can behave as a double-edged sword because it can enhance or suppress cancer immune response. Thus, therapeutic strategies targeting autophagy in tumor cells must consider the potential negative impact on antitumor immunity. The key question that emerged is: what is the net outcome of the autophagy inhibitor in clinic? There are numerous studies supporting that immunotherapy of cancer should focus on inducing and reprogramming cells of the innate and adaptive immune system. Therefore, it is tempting to speculate that combined therapy based on autophagy inhibitor and reprograming immune cells could significantly improve cancer immunotherapy.

1.1.5. Autophagy inhibits metastasis

Metastases are responsible for most cancer-related deaths. Metastatic cascade involves several steps, including: i) invasion from the primary tumor site, ii) intravasation and survival in the

systemic circulation, iii) extravasation at the secondary tissue site and, iv) colonization of this target tissue [80]. Autophagy has been found to either promote or prevent the metastatic progression, depending on the step in which it is activated (Figure 2 adapted from [81]). In this section, we will focus on the anti-metastatic activity of autophagy, while its pro-metastatic properties will be overview in the section 1.2.2.

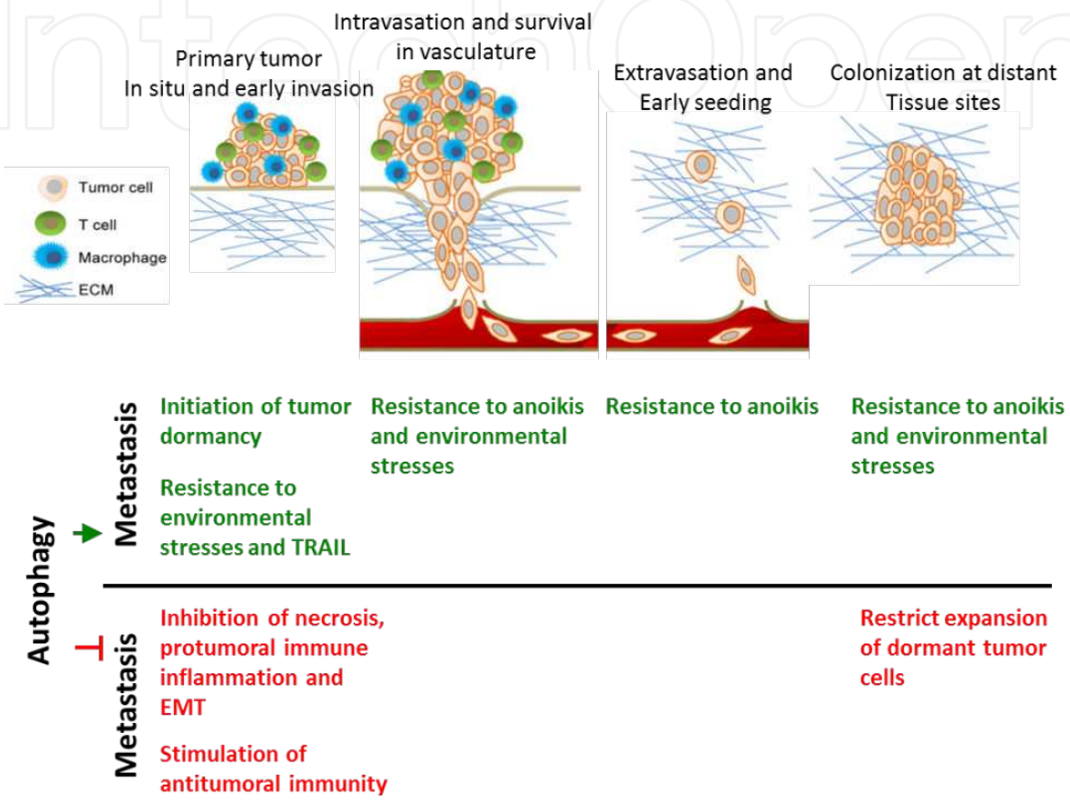

Figure 2. Dual role of autophagy in metastasis (adapted from [80])

1.1.5.1. Autophagy modulates the inflammatory response

At early steps, autophagy is able to limit the metastatic progression from the primary tumor site by restricting inflammatory response. Indeed, infiltrated immune cells can supply some signals within the tumor microenvironment that influence tissue remodeling, angiogenesis, tumor cell survival and spreading. Clinical and experimental data have confirmed the dual role of the immune system in tumor metastasis. As example, Lin *et al.* have demonstrated that macrophages depletion into the primary tumor site in transgenic CFS-1 null mice (colony-stimulating factor-1 is a cytokine involved in the proliferation, differentiation and survival of macrophages) delayed the development of metastasis [82]. While certain immune cells (*e.g.* macrophages, B cells, granulocytes, mast cells) may favor cancer development, others (*e.g.* Natural killer cells and T lymphocytes) may preferentially inhibit it [83] [84]. However, the tumor-promoting or tumor-suppressive properties of immune cells are not clearly defined and seem to be closely dependent on the tissue context and the cellular stimuli [85].

Autophagy can modulate inflammation during metastasis by different ways. On one hand, autophagy may lead to a direct activation of antitumor immunity through the release of high-mobility group box protein 1 (HMGB1) from tumor cells that are destined to die [86]. When released, HMGB1 stimulates the Toll-Like Receptor 4 on dendritic cells and, subsequently, promotes the tumor cell killing by inducing T-cell immunity [87]. On the other hand, autophagy can indirectly attenuate the macrophage infiltration by inhibiting tumor cell necrosis (see section 1.1.1.). Indeed, tumor-associated macrophages (TAMs) are important components of the leukocyte infiltrate and their involvement in metastasis progression have been extensively studied. TAMs positively influence tissue remodeling, angiogenesis, tumor invasion and intravasation through the production of growth factors, cytokines and matrix metalloproteases [88] [85] [89].

1.1.5.2. Autophagy alters the epithelial to mesenchymal transition (EMT)

Many studies have shown that the acquisition of mesenchymal feature by carcinoma cells promotes cancer invasion and metastasis. Epithelial to Mesenchymal Transition (EMT) is a process that leads to the complete loss of epithelial characteristics to achieve a mesenchymal cell phenotype. Initiation and completion of EMT requires the expression of specific transcription factors, microRNAs, cell surface proteins and matrix-degrading proteases [90]. Once undergoing EMT, cancer cells acquire invasive properties that enhance their ability to detach from the primary tumor site and to colonize distant tissues. Recently, two studies have pointed out that autophagy may modulate EMT. Lv *et al.* have shown that the expression of the Death-effector domain-containing DNA-binding protein (DEDD) is inversely correlated with the metastatic phenotype of breast cancer cells. Ectopic expression of DEDD in metastatic MDA-MB-231 cells leads to the autophagy-mediated degradation of the two major EMT inducers Snail and Twist, and subsequently to the loss of the metastatic phenotype. Conversely, knock-down of DEDD in non-metastatic MCF-7 cells reduces autophagy and leads to EMT promotion [91]. Earlier, Sun *et al.* have suggested the implication of the Bcl-2 anti-apoptotic protein, which is also known as an inhibitor of Beclin-1-dependent autophagy, in EMT induction. Under hypoxia condition, Bcl-2 and Twist are coexpressed in hepatocellular carcinoma and physically interact to form a complex that promotes EMT [92].

1.1.5.3. Autophagy restricts expansion of dormant tumor cells

Cancer recurrence is a determinant element for patient life expectancy because this disease presents a high risk of relapse after therapy or a long period of remission. Presence of residual dormant cells in the primary tumor site or in distant organs is one of the major causes of cancer relapse. Tumor dormancy is characterized by a prolonged, but reversible, growth arrest in G0-G1, by which tumor cells survive in a quiescent state. However, dormant tumor cells have to re-activate their proliferative activity to allow the development of micro- or macro-metastasis. Lu *et al.* have reported that induction of aplasia Ras homologue member I (ARHI) gene in ovarian cancer xenografts in mice induced autophagy, led to tumor dormancy and significantly inhibited xenograft growth. Interestingly, a proliferative recovery was obtained when the ARHI-induced autophagy was not maintained supporting the fact that autophagy is

required for the establishment of the dormant state [93]. These results illustrate how autophagy can maintain the dormant phenotype of tumor cells and thus inhibit the entry into an active dividing state. By this way, autophagy either prevents the expansion of isolated dormant tumor cells and the development of macrometastases.

1.2. Role of autophagy in tumor progression and metastasis

1.2.1. Autophagy induces survival of tumor cells under a variety of stresses

It has been well documented that tumor cells activate autophagy in response to stress, which enables long-term survival when apoptosis is defective [94]. Autophagy must be a highly selective process to allow extensive cellular degradation while retaining functional integrity. This section will address how autophagy confers tumor cells with superior stress tolerance, which limits damage, maintains viability, sustains dormancy and facilitates recovery.

Cancer cells need to adapt their metabolism to ensure the demands of proliferation enhanced in the microenvironment. The oncogenes affect signaling pathways important in regulation of metabolism, which support cancer growth and proliferation [95]. Autophagy is activated in response to multiple stresses, such as hypoxia, nutrient starvation, and the endoplasmic reticulum (ER) stress [96], during cancer progression. Under metabolic stress, inhibition of autophagy could lead to accelerated apoptosis, thus limiting further tumor progression. In this section, we discuss the role of autophagy regulation in tumor microenvironment and tumor growth [97].

1.2.1.1. Autophagy as adaptive metabolic response to hypoxia

Tumor cells are subjected to elevated metabolic stresses (*i.e.* lack of nutrition, oxygen deprivation) due to a defect in angiogenesis and inadequate blood supply. High levels of hypoxia resulted in an alteration of metabolism, enhanced invasiveness and resistance to therapy. Metabolic stress in tumors is mainly caused by the high metabolic demand required for cell proliferation and the impairment of ATP production [98]. Autophagy acts as an alternative source of energy and promotes tumor cell survival in hypoxic microenvironment. White *et al.* first showed that induction of autophagy in the hypoxic core of tumors promotes cancer cell survival. Hypoxia within tumors can be generated by hypoxia-inducible factor-1 alpha (HIF-1α)-dependent or -independent manner [99]. On one hand, autophagy in hypoxia can be induced through HIF-1α-dependent expression of the BH3-only protein Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) and the related protein, BNIP3L [100]. These proteins are downstream targets of HIF-1α and are able to induce mitophagy in hypoxia to manage ROS production.

Mechanistically, Bellot *et al.* showed that induction of BNIP3 and BNIP3L in hypoxic cells disrupts the Beclin1-Bcl-2 complex leading to Beclin1 release and subsequently autophagy induction as an adaptive survival response during prolonged hypoxia [101]. We have shown that the acquisition of TNF-resistance in breast cancer cells is correlated with constitutive activation of HIF-1α, even under normoxia, and with the induction autophagy by several

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signaling pathways highlighting the important role of autophagy in tumor cell adaptation to hypoxia [102] (Figure 3).

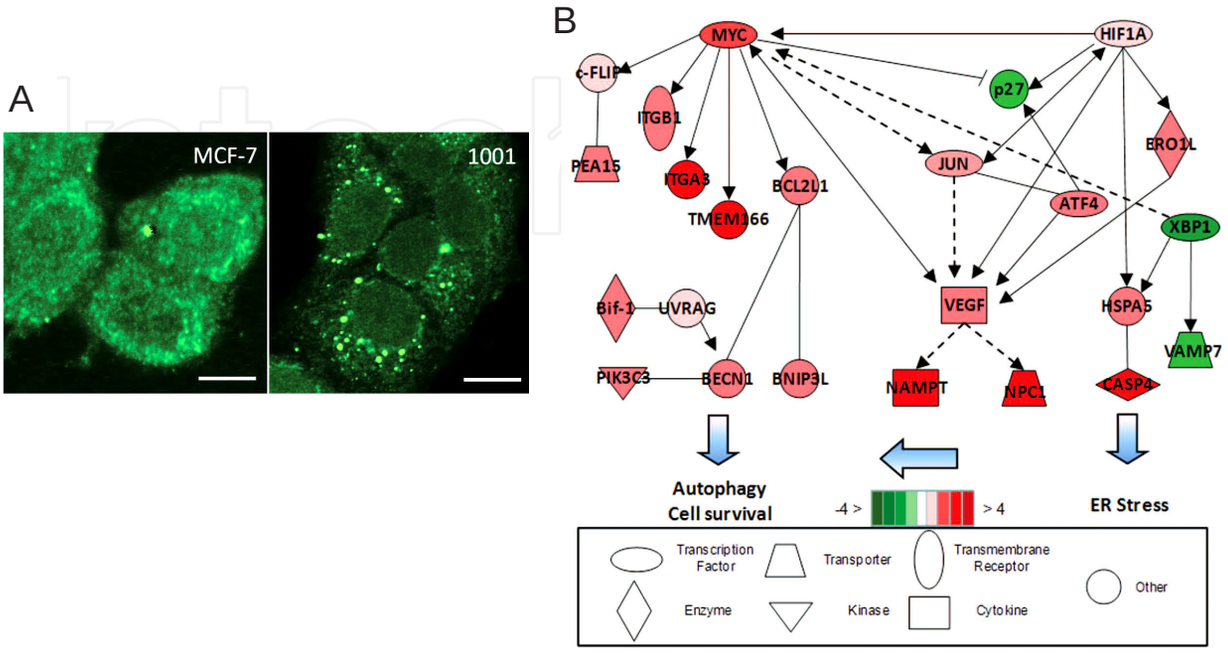

Figure 3. Induction of autophagy in TNF-resistant breast cancer cells. (A) Immunofluorescence analysis of autophagosomes formation in TNF-resistant MCF-7 clone (1001). MCF-7 and 1001 cells were labeled with anti-LC3 primary antibody and Alexa-Fluor 488-conjugated secondary antibody. No autophagosomes were observed in MCF-7 (diffuse green staining) and several autophagosomes (green dot-like structures) were observed in 1001 cells. (B) Data mining of autophagy microarray results performed by Ingenuity software highlights the involvement of MYC and HIF1-α downstream pathways in the activation of autophagy in TNF-resistant cells. Solid lines indicate a direct interaction and dotted lines indicate an indirect interaction; arrows indicate that protein A acts directly (solid line) or indirectly (dotted line) on protein B. Green represents downregulation while red depicts upregulation in 1001 compared to MCF-7 cells. The intensity of color represents the average of log2 fold change from three independent experiments. Symbols affected to each gene reflect cell functions.

Furthermore, Denko *et al.*, showed that autophagy in hypoxia can be induced independently of nutrient deprivation, HIF-1α activity, and expression of BNIP3. The HIF-1 independent hypoxia-induced autophagy involves the activity of the 5′-AMP-activated protein kinase (AMPK) [103]. Recent reports suggest that in addition to its role in the regulation of normal metabolism, AMPK can also regulate cellular energy homeostasis through autophagic degradation of intracellular components. Decrease in the ATP/AMP ratio and the activation of AMPK promotes catabolic pathways instead of anabolic processes. The major downstream pathway activated in HIF-1-independent hypoxia depends on tuberous sclerosis complex (TSC) and mammalian target of rapamycin (mTOR) – a master regulator of cell growth, cellular metabolism, and autophagy [104]. Recently, it has been shown that this pathway can be triggered before any detectable decreases in intracellular ATP levels [105]. Another signaling pathway induced by hypoxic stress and involved in the activation of HIF-1 independent-autophagy is the unfolded protein response (UPR), an evolutionarily conserved pathway activated in response to ER stress. The UPR is activated by three distinct ER stress sensors on

the ER membrane: PKR-like ER kinase (PERK), IRE-1, and activating transcription factor 6 (ATF6). PERK kinase phosphorylates the eukaryotic initiation factor 2α (eIF2α) leading to inhibition of the initiation step of mRNA translation [106]. In addition to the inhibition of protein synthesis, PERK phosphorylation of eIF2α enables a selective translation of the ATF4 transcription factor which has a key role in autophagy regulation in response to ER stress. In response to activation of UPR, PERK stimulates autophagy to clear protein aggregates generated by ER stress [107]. Various ER stress–inducing agents have been shown to induce autophagy in yeast and mammalian cells. Additionally, both the PERK/eIF2α and IRE-1 from UPR system have been implicated in regulation of autophagy [108]. Autophagic degradation of cellular components provides the energetic balance governed by AMPK, and suppression of autophagy in cancer cells can increase both resistance to hypoxic stress and tumorigenicity.

1.2.1.2. Autophagy in nutrient starvation

It is now well known that the metabolic stress induced by starvation in tumor microenvironment activates autophagy. Moreover, this metabolic stress is also dependent on autophagy as it allows organelles and proteins recycling in order to provide energy for cell survival. It has been shown that cancer cell lines with Ras activation display elevated levels of basal autophagy essential for survival through starvation and tumor growth [109]. Autophagy induced by starvation (*i.e.* glucose, L-glutamine, pyruvate, serum or amino acids) is mediated by ROS [110]. Indeed, autophagy can be regulated by both superoxide radical and hydrogen peroxide [111]. Another pathway implicated in regulation of autophagy in starvation conditions is dependent on AMP Kinase (AMPK). Specifically, under starvation conditions, activated AMPK inhibits the mTOR pathway and leads to induction of autophagy flux [112]. A new mechanism of regulation of starvation-induced autophagy by microRNAs (miRNAs) has been recently proposed by Gouzacik *et al*..They have shown that miR-376b was able to attenuate starvation- and rapamycin-induced autophagy in MCF7 and Huh-7 cells. Two direct functional targets of this miRNA were characterized; ATG4C and Beclin1. This finding underlines the importance of miRNAs in tumor microenvironment and as a new regulator of the autophagy pathway [113].

1.2.2. Autophagy promotes tumor cell metastasis

As mentioned in the section 1.1.5., autophagy may also promote different steps of metastatic cascade, mainly by favoring the survival of cancer cells in inhospitable environments (*e.g.* systemic circulation, target organs) (Figure 2). Induction of autophagy allows cancer cells to survive under a variety of stresses (*e.g.* hypoxic, metabolic), that subsequently favors tumor progression (for details, see the section 1.2.1). Several lines of evidence also suggest that autophagy-induced resistance to apoptosis plays a crucial role in cancer progression. It is well documented that metastatic cancer cells are more resistant to apoptosis than their poorly-metastatic counterparts [114]. As example, autophagy is upregulated in tumor cells that are resistant to TRAIL-induced apoptosis [115]. Moreover, blocking specific autophagy actors (*e.g.* Beclin1, ATG7) efficiently restores TRAIL-induced apoptotic cell death, highlighting the cytoprotective role of autophagy in TRAIL-resistance [115] [116].

During the metastatic progression, cancer cells activate mechanisms to resist to anoikis. Anoikis is a form of apoptotic cell death induced by the detachment from the surrounding extracellular matrix (ECM) [117]. Activation of autophagy during anoikis may be a survival strategy developed by the cells to overcome the stress of ECM detachment. Fung *et al.* have pointed out that autophagy is induced in both non-tumorigenic epithelial cell lines and in primary epithelial cells following the ECM detachment [118]. They demonstrated that autophagy protects cells from anoikis as RNAi-depletion of autophagy regulators (ATGs) promotes apoptosis and reduced clonogenic viability upon reattachment. ECM-detached cells activate the PERK-eIF2α-ATF4-CHOP pathway, which is responsible for both autophagy induction and oxidative stress limitation [119]. However, only few studies have confirmed the ability of autophagy to inhibit anoikis in cancer cells. As example, Chen *et al.* demonstrated that loss of autophagy in oncogenic-transformed mammary epithelial cells (PI3K-H1047R) promotes survival and proliferation in 3D organotypic culture [120].

Although autophagy prevents cancer progression by maintaining tumor cells in a dormant state, initiation of dormancy may also promote tumor progression by favoring survival of cancer cells. In this regard, it has been shown that breast cancer cells that lack β1 integrin are in a dormant state, suggesting that dormancy may help cancer cells to overcome the stress of ECM detachment, and subsequently resist to anoikis [121].

1.2.3. Upregulation of autophagy promotes resistance to cancer therapy

Autophagy may function to remove proteins or organelles that are damaged by cancer treatments or, through the degradation of cellular components, may provide nutrients for the rapidly growing cells. Indeed, inhibitors of autophagy can produce different outcomes: cell survival or cell death. Obviously, autophagic cell survival confers tumor cells with superior stress tolerance, which limits damage, maintains viability, sustains dormancy, and facilitates recovery. The dual role of autophagy highlights the need to carefully define its role in tumor cells before applying autophagy-based therapy. It will be important for clinical oncologists and cancer researchers to determine which cancer cell types most commonly undergo autophagy in response to therapy, and whether increased autophagy is a sign of responsiveness or resistance.

Nevertheless, several studies have shown that tumor cells can survive anti-cancer treatment by activating autophagy. This statement was validated using genetic or pharmacological inhibitors of autophagy which led to sensitize tumor cells to cancer therapies. In this context, it has been reported that inhibition of autophagy sensitizes cancer cells to DNA damaging anticancer agents. Evidence has been provided that inhibition of autophagy by 3-methyladenine (3-MA) or by targeting Atg7 enhances the cytotoxicity of 5-fluorouracil in human colorectal cancer cells [122]. Autophagy inhibition also enhances the therapeutic efficacy of cisplatin and 5-fluorouracil in esophageal and colon cancer cells, respectively [122, 123]. Targeting autophagy by genetic approaches using Beclin1, Atg3, and Atg4b siRNA sensitizes resistant cancer cells to ionizing radiation [124]. These studies strongly argue that autophagy operates as a mechanism through which cancer cells acquire resistance to radiotherapy and chemotherapy. There are numerous studies supporting the involvement of autophagy in

cancer stem cells resistance to ionizing radiation and other anti- cancer treatments [125]. Thus, in malignant gliomas, the CD133+ cancer stem cells express higher levels of the autophagic proteins LC3, Atg5, and Atg12. In addition, ionizing radiation seems to induce autophagy only in CD133+ cancer stem cells compared to CD133- counterpart [126]. Furthermore, glioma cells treated with autophagy inhibitors exhibit more extensive DNA double-strand breaks than cells treated with radiation alone [127]. We have recently demonstrated that autophagy induction in tumor cells under hypoxia decrease the tumor cell killing by cytotoxic T lymphocytes. Furthermore, we provided evidence that simultaneously boosting the immune system by vaccination and inhibiting autophagy may improve cancer immunotherapy [75, 76].

While the general consensus is that autophagy inhibition is an effective strategy for cancer therapy, some drugs that are being used in the clinic induce autophagy. In most cases, however, it has not been proven that these drugs induce death *via* the autophagy pathway. Indeed, for many of these drugs it is hypothesized that combining them with autophagy inhibitors may improve their efficacy.

2. Autophagy as a target for anti-cancer therapies

Evidence indicated that the modulation of autophagy is an important component of tumorigenesis, making it a possible therapeutic target. Pharmacological inhibitors of autophagy can be broadly classified as early- or late-stage inhibitors of the pathway. Early-stage inhibitors include 3-methyadenine, wortmannin, and LY294002, which target the class III PI3K (Vps34) and interfere with its recruitment to the membranes. Late-stage inhibitors include the antimalarial drugs chloroquine (CQ), hydroxychloroquine (HCQ), bafilomycin A1, and monensin. Bafilomycin A1 is a specific inhibitor of vacuolar-ATPase [128], and monensin and CQ/HCQ are lysosomotropic drugs that prevent the acidification of lysosomes, whose digestive hydrolases depend on low pH. Since autophagosomes and lysosomes move along microtubules, microtubule-disrupting agents (taxanes, nocodazole, colchicine, and vinca alkaloids) can also inhibit the fusion of autophagosomes with lysosomes. Other inhibitors of autophagy that block autophagosome degradation include the tricyclic antidepressant drug clomipramine and the anti-schistome agent lucanthone [129, 130]. Of the known autophagy inhibitors outlined above, only CQ and HCQ have been evaluated in humans, because they are commonly used as antimalarial drugs and in autoimmune disorders. These drugs cross the blood-brain barrier, and HCQ is preferred to CQ in humans because of its more favorable side-effects profile [131]. Quinacrine, which also has been used in patients as an anti-malarial, has been shown to inhibit autophagy similarly to CQ. In fact, quinacrine showed greater cytotoxicity in gastrointestinal stromal tumor (GIST) cell lines treated with imatinib than CQ [132], and therefore this may be a promising anti-autophagy agent for future clinical trials.

Currently, there are nearly 20 clinical trials registered in the National Cancer Institute (www.cancer.gov/clinicaltrials) exploring anti-autophagy strategies in a variety of human cancers. Most of these trials are ongoing, with minimal published results available, and nearly all use HCQ. It is worthy to note that CQ or HCQ are lysosomotropic agents that act at the

level of the lysosome by inhibiting acidification, thereby impairing autophagosome degradation. These clinical trials were initiated based on the fact that autophagy is induced in a variety of tumor cells and preclinical models by several types of chemotherapeutic agents as a survival mechanism. Because only a subpopulation of tumor cells undergo autophagy, it is unlikely that autophagy inhibitors are used in cancer therapy as single agent. Indeed, most of these clinical trials used HCQ in combination with other anti-cancer therapies. While these preclinical data are generally supportive of incorporating anti-autophagy therapies in cancer treatment trials, it has been observed, in some circumstances, that inhibition of autophagy decreases therapeutic efficacy. Understanding the circumstances in which autophagy inhibition impairs the therapeutic effect will be of great importance. Importantly, while CQ and HCQ are effective inhibitors of autophagy *in vitro*, whether they will do so at doses used in current clinical trials is still uncertain. An important issue related to the use of these autophagy inhibitors concerns the micromolar concentration that is required to inhibit autophagy and show anti-tumor efficacy in preclinical models. While this is theoretically achievable at tolerated doses after prolonged dosing, it should be better optimized in clinic [133, 134]. Trials combining HCQ as neoadjuvant treatment will provide tumor tissues available for analysis both before and after HCQ treatment. However, the effectiveness of HCQ in the inhibition of autophagy still proves difficult, as HCQ is often combined with other therapies (chemotherapy and radiotherapy) that are also known to modulate autophagy. Alternative biomarkers to predict for autophagy activation as well as autophagy dependence are currently an area of intense investigation [135]. A recently reported phase I trial of HCQ in combination with adjuvant temozolomide and radiation in patients with glioblastoma found that the maximum tolerated dose of HCQ was 600 mg per day, and this dose achieved concentrations of HCQ required for autophagy inhibition in preclinical studies. In this trial, investigators observed a dose-dependent inhibition of autophagy, as indicated by increases in autophagic vesicles (revealed by electron microscopy), and detected elevations in LC3-II in peripheral blood mononuclear cells. In addition, in a phase I trial of 2-deoxyglucose, an agent that blocks glucose metabolism, autophagy occurred in association with a reduction in p62/SQSTM1 in peripheral blood mononuclear cells [136]. These data suggest the potential interest of such biomarkers in the evaluation of autophagy modulation during therapy and in the correlation with treatment outcome [137].

CQ inhibits the last step of autophagy at the level of the lysosome, thereby impacting lysosomal function. Therefore, its effects are not entirely specific to autophagy. Currently, there is a great deal of interest in developing new inhibitors of autophagy. In this regards, and given the complexity of the autophagic process, multiple proteins involved in this process could be good candidates for developing others autophagy inhibitors. It is likely that kinases would be prime candidates for inhibition such as Vps34, a class III PI3K, which has a critical early role in autophagosome development. This is particularly attractive, as there has been significant success in designing effective class I PI3K inhibitors [138]. However, one potential issue which needs to be considered is that Vps34 has roles in other aspects of endosome trafficking, and this may lead to unwanted effects and toxicity [139]. The mammalian orthologs of yeast ATG1, ULK1/2, which acts downstream from AMPK and the TOR complex, have been recently shown as critical proteins for autophagy activation [140-142]. Others potential targets for autophagy

inhibitors would be LC3 proteases such as ATG4b, which are necessary for LC3 processing. However, whichever approach is taken, the delicate balance between potency and toxicity must be determined to achieve a clinical success. While there are still uncertainties of how autophagy inhibition will fare as an anti-cancer therapy, the preclinical data generally support this approach. The current clinical trials will hopefully provide insight into whether this will be a viable therapeutic paradigm [135].

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