The long road: the autophagic network and TP53/ASXL1 aberrations in hematopoietic malignancies
Hilgendorf, Susan

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 27-10-2019
The multifaceted role of autophagy in cancer and the micro-environment

Susan Hilgendorf, Hendrik Folkerts, Edo Vellenga, Edwin Bremer*, Valerie R. Wiersma*

Department of Hematology, Cancer Research Center Groningen, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
* Corresponding authors
† Contributed equally

Submitted
ABSTRACT

Autophagy is a crucial recycling process that is increasingly being recognized as an important factor in cancer initiation, cancer (stem) cell maintenance as well as development of resistance to cancer therapy in both solid and hematological malignancies. Furthermore, it is being recognized that autophagy also plays a crucial and sometimes opposing role in the complex cancer micro-environment. For instance, autophagy in stromal cells such as fibroblasts contributes to tumorigenesis by generating and supplying nutrients to cancerous cells. Reversely, autophagy in immune cells appears to contribute to tumor-localized immune responses and among others regulates antigen presentation to and by immune cells. Autophagy also directly regulates T and NK cell as well as and is required for mounting T cell memory responses. Thus, within the tumor micro-environment autophagy has a multi-faceted role that, depending on the context, may help drive tumorigenesis or may help to support ant-cancer immune responses. This multi-faceted role should be taken into account when designing autophagy-based cancer therapeutics. In this review, we provide an overview of the diverse facets of autophagy in cancer cells and non-malignant cells in the cancer micro-environment. Secondly, we will attempt to integrate and provide a unified view of how these various aspects can be therapeutically exploited for cancer therapy.

Key words: Autophagy, cancer, micro-environment, stroma, immune cells, therapy
INTRODUCTION

Autophagy is an important homeostatic process in the human body that is responsible for the elimination of damaged and/or superfluous macromolecules such as proteins and lipids as well as the removal of damaged organelles like mitochondria. The successful execution of autophagy enables the recycling of nutrients, amino acids and lipids and acts as quality control mechanism to maintain organelle function\(^1\text{-}\!^4\). The importance of autophagy is evidenced by the fact that a block in autophagic flux due to knock-down of core autophagy genes is detrimental during early development in murine models\(^5\text{-}\!^{11}\). Perhaps not surprisingly, an increasing body of evidence highlights the important and multifaceted impact of autophagy in cancer. For instance, during tumor development the autophagic process appears to function as a tumor suppressor and limits tumorigenesis\(^12\text{-}\!^{15}\). In this respect, it is noteworthy that a single nucleotide polymorphism in the promoter region of the crucial autophagy gene ATG16L1, which putatively down-regulates its expression level, associates with susceptibility to thyroid and colorectal cancer and has a significant negative impact on patient survival in local and advanced metastatic prostate cancer\(^16\text{-}\!^{18}\). Further, survival of patients with advanced lung adenocarcinoma upon EGFR tyrosine kinase inhibitor treatment is significantly impacted by functional genetic polymorphisms in core autophagy genes, thus highlighting the potential clinical impact of autophagic signaling on cancer development and response to therapy\(^19\).

In established cancers autophagy activity is upregulated during and associated with resistance to cancer therapy\(^20\). Further, elevated autophagy maintains stemness in cancer stem cells. Moreover, cancer cells appear to rely more on autophagy for continued survival than normal cellular counterparts. Consequently, the inhibition of autophagy is being explored for cancer therapy particularly in combination with other cytotoxic drugs to augment cytotoxicity\(^21\text{-}\!^{23}\). Autophagy occurring in the context of cancer therapy may on the one hand be a stress response that enables cancer cells to survive and evade apoptotic elimination\(^4\). In this setting, inhibition of autophagy sensitizes cells to apoptotic cell death and may be of use to augment the efficacy of anti-cancer agents. On the other hand, autophagy may also be a driver of cytotoxic cell death and in this case inhibition of autophagy would inhibit cell death. This type of cell has been termed autophagic cell death (ACD) and has been reported e.g. for radiation therapy\(^24\text{-}\!^{28}\). Thus, depending on the type of cell death inhibition of autophagy may be warranted for combination therapy.

Thus, it is evident that autophagy is more and more emerging as a potential target for cancer therapy. However, the complex micro-environment of an established tumor comprises many different cell types in addition to malignant cells that all to a different extent utilize and rely on the autophagic process. Indeed, as will be discussed in this review autophagy not only clearly impacts on cancer (stem) cells, but also on stromal cells, endothelial cells and tumor-
infiltrated innate and adaptive immune cells. Therefore, it is crucial to understand the impact of autophagy and its therapeutic targeting in the context of this diverse cellular composition of the tumor microenvironment.

In this review, we will first briefly detail the core autophagy machinery and regulatory pathways after which we will provide an overview of current thinking on the role of autophagy in the functioning of the diverse components within the tumor micro-environment (illustrated in Figure 1). Further, we will provide directions for incorporating the sometimes opposing effects on autophagy on tumor microenvironmental components for the future implementation of autophagy-targeting drugs in cancer.

Figure 1: Review outline. This review highlights the impact of changes in autophagy within cancer cells, as well as in the context of the complex cancer micro environment. Part I describes how aberrant autophagy can contribute to cancer initiation and maintenance as well as therapy resistance (pages 7-25). Part II describes the role of autophagy in different stromal cells within the tumor micro environment, such as fibroblasts and mesenchymal stem cells (pages 26-30). Further, the impact of autophagy on anti-cancer immune responses is described (pages 30-40). Blue dapi staining; green fibronectin staining for stroma; red CD8 staining for cytotoxic T cells.
1. AUTOPHAGY SIGNALING AND REGULATORY PATHWAYS

The term autophagy defines a process that can occur in three different forms, with the most prominent form being macroautophagy, a form of autophagy that includes removal of proteins and/or organelles. In the case of mitochondria, this process is also called mitophagy. Secondly, when molecules that have to be degraded are directly invaginated by the lysosome, this process is called microautophagy. Thirdly, proteins can be degraded via chaperone-mediated autophagy (CMA). During CMA, proteins are targeted for degradation by heat shock protein hsc70 via their KFERQ-like motif\textsuperscript{29,30}. Unless specifically referred to, the term autophagy in the manuscript describes macroautophagy. In the section below, we will detail basic autophagy pathways as well as highlight regulatory hubs that are important in cancer.

1.1 The core autophagy machinery

The induction of autophagy can be subdivided into an initiation phase, elongation phase, autophagosome maturation, autophagosome-lysosome fusion and degradation of content in the autophagolysosomes (Figure 2A). The initiation of autophagy generally starts at the mTORC1 complex, the master regulator of autophagy, which under basal conditions decreases the autophagy pathway by inhibiting the ULK1 complex\textsuperscript{31}. However, upon increased nutrient demand, mTORC1 is deactivated due to reduced upstream signaling from the PI3K/Akt and the MAPK pathway, thereby enabling initiation of autophagy. In addition, the SAMP-activated protein kinase (AMPK), a key kinase regulating cellular energy homeostasis, activates the ULK1 complex when low energy levels are detected\textsuperscript{19,32}. The activated ULK1 complex, together with the Beclin-1-Vps34 complex, a complex discussed in more detail in section 1.2, initiates the formation of autophagosomes. The formation of autophagosomes can be inhibited by 3-Methyladenine (3-MA), an inhibitor of Vps34. In contrast, rapamycin, an inhibitor of mTORC1, is generally used as autophagy inducer.

Maturation of the autophagosome requires two ubiquitin-like conjugation systems. First, ATG12 is covalently bound to ATG5, a process mediated by ATG7 and ATG10. The ATG12-ATG5 conjugate is subsequently non-covalently connected to ATG16, which is required for the localization of ATG12 and ATG5 to the forming autophagosome\textsuperscript{33}. Secondly, LC3 is converted into LC3-II, which starts with the proteolytic cleavage of LC3 by ATG4 to form LC3-I. LC3-I is then bound by ATG7, which transfers LC3-I to ATG3\textsuperscript{34–36}. ATG3 subsequently catalyzes the conjugation of the lipid phosphatidylethanolamine (PE) to LC3-I, thereby yielding LC3-II. This lipidation step is enhanced by the ATG5/ATG12/ATG16 complex. Eventually LC3-II is inserted in the membrane of the elongating autophagosome. During the maturation of the autophagosome, proteins and organelles to be degraded are sequestered to the forming autophagosome by p62/ sequestosome 1 (SQSTM1). For this purpose, p62 can directly interact with LC3\textsuperscript{37}. Finally, the outer membrane of the mature
autophagosome fuses with a lysosome to form the autolysosome. The lysosome-associated membrane proteins (LAMP-1 and LAMP-2) are essential for this fusion and also maintain the integrity of lysosomal membranes\textsuperscript{38}. The macromolecules and organelles that have been entrapped in the autophagosomes are then degraded by the digestive enzymes of the lysosomes (e.g. lipases, proteases, nucleases, sulfatases), which yields amino acids, fatty acids and nucleotides for eventual reuse. The fusion of autophagosomes with lysosomes can be inhibited by chloroquine (CQ) or hydrochloroquine (HCQ), which prevent acidification of the lysosomes.

Of note, the induction of LC3-II is generally considered as marker for autophagy induction, whereas its sustained accumulation is reflective of autophagy inhibition\textsuperscript{39}. In addition, p62 is degraded during the proper execution of autophagy, and its accumulation can be used as marker for inhibition of autophagy\textsuperscript{40}.

1.2 BCL-2 family members modulate Beclin-1 dependent autophagy

Beclin-1 is an important regulatory hub to which pro- and anti-autophagic proteins can bind (Figure 2B). First, the pro-apoptotic proteins of the BCL-2 family, e.g. BCL-2, BCL-XL and MCL-1, can bind to the characteristic BH3 domain of Beclin-1, which inhibits autophagy\textsuperscript{41–43}. Secondly, non BCL-2 family proteins like UVRAG, AMBRA1, HMGB1 and VMP1 can competitively bind Beclin-1, which upon interaction induce autophagy\textsuperscript{44–47}. In addition, the hypoxia-inducible BNIP3 and BNIP3L proteins that also contain a BH3 domain can directly interact with BCL-2 family members\textsuperscript{38}. This BNIP3-BCL-2 interaction prevents BCL-2 binding to Beclin-1 and, thereby, promotes autophagy. Alterations in the pool of Beclin-1 interacting proteins can alter the balance of autophagy regulation. In line with this, gene silencing of BCL-2 using siRNA in MCF-7 cells triggered autophagy, whereas in neuron-specific MCL-1 KO mice autophagy was increased in neuronal cells\textsuperscript{49,50}. Correspondingly, treatment of various cancer cell lines with BH3 mimetics that promote dissociation of BCL-2 or BCL-XL from Beclin-1 activated autophagy\textsuperscript{51,52}. Here, autophagy was inhibited by siRNA mediated knockdown of essential autophagy proteins\textsuperscript{53}. The induction of mitophagy can also be regulated by BCL-2 members. In brief, mitochondrial depolarization promotes Parkin and PINK1-dependent induction of mitophagy, which was suppressed by transient overexpression of BCL-2 family members MCL-1 and BLC-XL\textsuperscript{54,55}. In this case, inhibition of mitophagy was independent of Beclin-1, but due to inhibition of Parkin translocation to depolarized mitochondria\textsuperscript{54}. Taken together, the elevated expression of anti-apoptotic and Beclin-1 interacting members of the BCL-2 family can reduce autophagy, including clearance of mitochondria through mitophagy.
**Figure 2: The autophagy pathway**

A. The activation of autophagy is initiated by reduced activity of the mTORC1 complex due to activated AMPK or decreased upstream growth signaling. mTORC1 is an inhibitor of the ULK complex, therefore reduced mTORC1 activity increases the activity of the ULK complex. The ULK complex together with the Beclin-1/ VPS34 complex initiates the formation of autophagosomes. Dependent on the complex composition, Beclin-1 can act as a molecular switch between autophagy and apoptosis (see **Figure 2B**). Expansion and maturation autophagosomes is dependent on two ubiquitin-like conjugation systems, which requires multiple autophagy proteins. First, ATG12-ATG5 conjugate binds to ATG16, which stimulates LC3 lipidation. Second, LC3 is covalently conjugated to PE generating LC3-II, which is incorporated in the autophagosomal membrane. Incorporated LC3-II is required for binding and internalization of adaptor proteins such as p62. Finally, the mature autophagosome fuses with lysosomes, after which its content is broken down by digestive enzymes. Indicated in red are pharmacological agents, Chloroquine (CQ), Hydroxychloroquine (HCQ), 3-Methyladenine (3MA), and ULK inhibitors, that inhibit autophagy. In addition, rapamycin activates autophagy by inhibiting mTORC1. B. Beclin-1 is a core member of the VPS34/Beclin-1 complex, which acts as a molecular switch in controlling autophagy downstream of the ULK1 complex. Depicted in red, anti-apoptotic members of the BCL-2 family BCL-2, BCL-XL and MCL-1 can bind to Beclin-1, through interaction with its BH3 domain, thereby inhibiting autophagy. Alternatively, BNIP3 and BNIP3L (depicted in green) can competitively bind to anti-apoptotic BCL-2 members. Dissociation of anti-apoptotic BCL-2 members from Beclin-1, consequently activates autophagy. Other non BH3 proteins, also depicted in green, such as VMP1, ATG14, UVRAG and AMBRA1 can also bind Beclin-1, thereby activating autophagy.
PART I. THE ROLE OF AUTOPHAGY IN CANCER CELLS

Autophagy has a multifactorial impact on cancer and influences both cancer initiation and maintenance, as well as regulates cancer response to therapy. Alterations in autophagy levels due to mutations in key autophagy genes or aberrant activation of autophagy regulators can associate with tumorigenesis (illustrated in Figure 3A). In this respect, cancer initiation is associated with reduced autophagy levels, which leads to the accumulation of oncogenes and reactive oxygen species (ROS). In contrast, during cancer maintenance, the activity of the autophagy pathway is often upregulated, which ensures sufficient energy supply and contributes to survival during stress, e.g. hypoxia and metastasis (illustrated in Figure 3B). Also during anti-cancer therapy, autophagy is increased by which cancer cells survive and gain therapy resistance. In addition, also cancer stem cells (CSC) rely on autophagy to maintain stemness.

2.1. IMPACT OF AUTOPHAGY IN EARLY TUMORIGENESIS

Autophagy is likely important for cancer initiation as mice with mono-allelic deletion of the key autophagy regulator Beclin-1 have an increased susceptibility to spontaneous tumor development13. In line with this, mono-allelic deletions of Beclin-1 have been detected in human breast cancer, prostate and ovarian cancer, while reduced expression of Beclin-1 was detected in brain cancer56–60. Similarly, monoallelic deletion of other essential autophagy genes such as ATG5, ATG7 or total loss of ATG4C have also been implicated with an increased risk for developing of malignancies14,15. Based on this data autophagy appears to act as a tumor suppressor with reduced levels of autophagy associating with accumulation of dysfunctional organelles and proteins that may contribute to malignant transformation. Of note, a low constitutive level of autophagy is required for cell survival, as evidenced by the fact that Beclin1 or AMBRA1 knock-out is embryonically lethal in mice13,61. As described in more detail below, there are several mechanisms in cancer that may reduce autophagic flux, e.g. mutations in core autophagy genes, that may trigger cancer development. These processes and their potential impact on cancer initiation are reviewed in more detail below.

2.1.1. Mutations in autophagy genes that affect autophagy levels during tumor development

Alterations in expression of various key autophagy genes have been reported for different types of cancer, including breast, lung, pancreatic, bladder cancer and leukemia62. As also mentioned above, one of the common molecular aberrations is the loss of one of the alleles of the essential autophagy gene Beclin-1. This aberration was detected in a broad spectrum of cancers,
Figure 3: Autophagy during malignant transformation and cancer maintenance. A. Different pro-oncogenic events such as, mutation or monoallelic deletion of autophagy related genes can cause reduced autophagy activity. Reduced levels of autophagy/ mitophagy can contribute to malignant transformation due to elevated levels of ROS. B. Hematopoietic stem cells (HSCs) reside in specific bone marrow niches with low oxygen content and are characterized by high autophagy activity. During differentiation, the autophagy flux declines and mature cells leave the bone-marrow (BM) environment and enter the blood-stream. In leukemia, HSCs have acquired mutations which results in a block in differentiation and consequently accumulation of immature blasts in BM and peripheral blood of patients. C. Hypothetical model for changes in autophagy and ROS in HSCs during transformation. Normal HSCs have high autophagy flux, low mitochondrial activity and ROS levels. During cancer initiation, autophagy is repressed (although not completely inhibited), causing accumulation of mitochondria and ROS, which in turn contributes to malignant transformation. During cancer maintenance, cancer cells re-establish functional autophagy promoting tumor growth and survival. In addition, in response to drug treatment autophagy is activated and acts as a survival mechanism for cancer cells. D. Both normal bone marrow derived CD34+ and acute myeloid leukemia (AML) CD34+ cells need a certain level of autophagy to survive. Therefore, there is only a small therapeutic window of autophagy inhibition with autophagy inhibitors like HCQ.
even in breast carcinoma cell lines that are often polyploid for the Beclin-1 encoding chromosome 17. Interestingly, reduced autophagy due to allelic loss of Beclin-1 in immortalized mouse kidney cells or mouse mammary epithelial cells, led to a profound increase in DNA damage. The increased DNA damage was associated with chromosomal abnormalities, such as gene amplification and aneuploidy. For example, in immortalized mouse kidney cells the chromosome number (normally 40) was increased to an average of 56 after allelic loss of beclin-1. Moreover, mammary tissue in Beclin-1+/− mice developed benign neoplasia with hyperproliferation, whereas reintroduction of Beclin-1 expression in breast cancer MCF7 cells suppressed tumorigenesis. Similar to Beclin-1, allelic loss of the autophagy component UVRAG or reduced expression of Bif-1, both directly interacting with Beclin-1, is associated with gastric and colon cancer. In brief, UVRAG forms a complex with Beclin-1 to activate autophagy and loss of this protein resulted in impaired autophagy. Moreover, UVRAG prevented accumulation of abnormal chromosomes, although it is not clear whether this feature is autophagy dependent. Bif-1 interacts with Beclin-1 and UVRAG and also serves to activate autophagy. Consequently, loss of Bif-1 expression reduces autophagy and in knockout mice resulted in an increased number of spontaneous tumors. Together with the above-described data on Beclin-1 these findings suggest that autophagy regulation by Beclin-1 is an important hub that is targeted in cancer. Further, disruption of Beclin-1/UVRAG/BIF-1 may cause genomic instability. An additional autophagy gene, involved in maturation of autophagosomes, GABARAPL1 was found to be downregulated in breast cancer, in this case due to altered DNA methylation and histone deacetylation patterns. The functional outcome of downregulation of GABARAPL1 was a reduction in autophagic flux and increased tumorigenesis.

In a recent screening approach a more detailed picture of the mutational spectrum of 180 autophagy genes was obtained, using whole-exome sequencing of 223 cases with myeloid neoplasm. Copy number alterations or missense mutations were detected in roughly 22% of autophagy-associated genes and in 14% of the studied cases. Interestingly, the majority of mutations were nonsynonymous substitutions that associated with adverse prognosis. Clonal hierarchy analysis indicated that these autophagy mutations were predominantly secondary events. In addition to mutations in core autophagy genes, mutations in the spliceosome that are linked to aberrant autophagy gene expression in myeloid malignancy were also found. For example, the splicing factor U2AF35, which is mutated in ~10% of patients with myelodysplastic syndrome, caused abnormal processing of ATG7 pre-mRNA and consequently reduced expression of ATG7. Interestingly, complete knockout of ATG7 in HSCs in mice causes severe anemia, and in long-term atypical myeloproliferation and accumulation of myeloid blasts in organs, all characteristics associated with myeloid malignancies. How autophagic-flux is affected by these mutations remains to be functionally defined, but the likely outcome is a reduction in the level of autophagy.
Indeed, the nonsynonymous substitutions observed in leukemia are often hypomorphic, i.e. mutations that cause reduced expression, suggesting that autophagy is repressed but not completely inhibited\(^a\). In line with this, complete inhibition of autophagy due to e.g. bi-allelic deletions or premature stop codons were not observed in any of the core autophagy genes in myeloid neoplasms\(^7\). Further, in a cross-cancer unsupervised clustering analysis, autophagy-associated transcript levels significantly correlated with overall survival in leukemia, kidney cancer and endometrial cancer\(^8\). Overall, these findings suggest that mutations in autophagy genes are relevant during tumorigenesis, with autophagy generally being down-regulated but not lost.

**2.1.2. Defective mitophagy causes accumulation of reactive oxygen species (ROS)**

Down-regulation of mitophagy, the term used for the autophagic removal of dysfunctional mitochondria\(^8\), can result in an increased formation of ROS. Disruption of mitophagy by KO of essential autophagy genes such as ATG5, ATG7, ATG12 and FIP200 coincides with accumulation of defective mitochondria and increased ROS levels\(^7\). Such oxidative stress has been linked to cancer development and progression\(^8\). For instance, persistent accumulation of ROS can also damage proteins, fatty acids and DNA, which may contribute to cancer development\(^8\). Further, protein and lipid phosphatases can be inactivated upon oxidation of cysteine residues in the catalytic domain, causing changes in signaling pathways and affecting cell growth\(^9\). Interestingly, the autophagy protein ATG4 is a cysteine protease that is overexpressed in several types of cancer and is highly sensitive to ROS\(^9\). Redox modifications of cysteine residues in ATG4 prevent delipidation of LC3, thereby promoting autophagy\(^9\). In human adenocarcinoma cells, oxidative stress led to upregulation of ATG4 together with increased autophagy and increased invasion of cells though a matrigel matrix\(^9\). Another example of the interplay between ROS and autophagy is the accumulation of p62/SQSTM1, a scaffold protein for ubiquitinated cargo that is continuously cleared via basal autophagy\(^9\). Accumulation of p62 aggregates due to crippling of autophagy causes oxidative stress and triggers the DNA damage response pathway\(^9\). However, elevated ROS levels can also activate p53 mediated apoptotic cell death\(^9\). Of note, mutant p53 was shown to attenuate expression of ROS scavenger enzymes coinciding with high ROS levels, indicating that these cells are able to tolerate ROS levels to a higher degree\(^9\). The exact interplay between autophagy and ROS in cancer development is highly complex and it remains unclear how persistent elevation of ROS, due to defective autophagy can contribute to cancer development.
2.1.3. Autophagy prevents accumulation of oncoproteins

Reduced autophagy levels during tumorigenesis may also alter the intracellular levels of oncoproteins. Indeed, several oncoproteins have been shown to be a target for degradation via CMA. For example, the BCR-ABL oncoprotein was targeted to the autolysosome by CMA after treatment of CML cell lines and primary CML patient-derived cells with the chemotherapeutic arsenic trioxide. In line with this data, inhibition of autophagy prevented arsenic trioxide mediated suppression of BCR-ABL expression. Defective autophagy was similarly associated with accumulation of the oncoprotein PML/RARA, the hallmark oncoprotein of acute promyelocytic leukemia. Moreover, treatment of AML cells with internal tandem duplications in the FLT3 receptor tyrosine kinase, referred to as FLT3-ITD, with proteasome inhibitor bortezomib triggered autophagy-dependent degradation of FLT3-ITD and improved the overall survival in a xenografts. Further, the proto-oncoprotein AF1Q, which is often overexpressed in AML and MDS and associates with unfavorable prognosis, was targeted for breakdown by CMA. Thus, autophagy and specifically CMA can clear various (proto) oncoproteins and repression of this type of autophagy might contribute to tumorigenesis.

2.2 AUTOPHAGY IN CANCER MAINTENANCE

As evident from the preceding sections, autophagy can have a tumor suppressor function and is often down-regulated in cancer. However, there is also clear evidence to suggest that autophagy is required for cancer (stem) cell maintenance. Indeed, increased autophagic flux or increased dependency on functional autophagy have been reported for various types of cancer, such as melanoma, CML, AML and RAS-driven cancers. For example, in solid cancers such as breast cancer and melanoma, increased LC3 puncta positively correlated with a more aggressive phenotype. Further, autophagic flux can aid cancer cell survival during cellular stress conditions, such as hypoxia and starvation. In addition, changes in autophagy can contribute to maintenance of so-called cancer stem cells, a self-renewing subpopulation of cancer cells with stem cell properties that for certain types of cancer, such as AML, is thought to drive the disease. The various roles of autophagy in cancer maintenance are detailed below (illustrated in Figure 3B).

2.2.1. Autophagy in maintenance of cancer stem cell function

Cancer stem cells (CSCs) are characterized by elevated levels of autophagy compared to more differentiated cancer cell populations, an observation confirmed in multiple cancer types, including urinary bladder cancer as well as breast CSCs. These CSCs expressed high level of essential autophagy genes to maintain CSC properties and to remain dormant. Further, elevated autophagy was required for CSC-mediated development of tumors in vivo in...
leukemia and breast cancer\textsuperscript{113,115,116}. However, the differentiation dependent level of autophagy is not specifically linked to transformed cells. Also normal hematopoietic, mesenchymal and skin stem cells, have a higher level of autophagy as compared to more differentiated cells\textsuperscript{117,118}. Thus, primitive cells have high autophagy levels in association with low ROS levels, which might be a protective mechanism for maintaining their stem cell properties\textsuperscript{117,118}. Correspondingly, function of normal HSCs was lost in ATG7 and ATG12 knockout mice. In the long term, this loss of function did coincide with the development of myeloproliferative syndrome, which might be a consequence of a defective mitochondria clearance in association with high ROS levels\textsuperscript{80,116,119}. Also deletion of ATG5 or ATG7 in a mixed lineage leukemia murine AML model affected the survival and was associated with a decrease in number of functional CSCs and a strong decrease in leukemic blasts in the peripheral blood indicating that autophagy has a critical function in leukemia maintenance\textsuperscript{116}. Similar findings were obtained with a bladder cancer cell line, and with breast cancer mammospheres, a model of CSCs with high levels of Beclin-1 and an increase in autophagy\textsuperscript{112,113}. In summary, autophagy seems to be essential to preserve CSC function and to increase survivability.

2.2.2. Oncogenic mutations and autophagy

In established cancers, several oncogenes have been shown to induce autophagy and, thereby, contribute to cancer maintenance. For instance, oncogenic FLT3 tyrosine kinase receptor (FLT3-ITD) AMLs cells are characterized by high levels of autophagy\textsuperscript{120}. Both pharmacological as well as genetic inhibition of autophagy in FLT3-ITD in human AML cells markedly reduced cell proliferation and overcame acquired resistance to FLT3 inhibitors in mice. In addition, cancer driven by certain oncogenic RAS mutations, observed in a broad spectrum of tumors including in colon, lung and pancreatic cancers, appears to heavily depend on functional autophagy. For instance, basal levels of autophagy were increased in RAS-transformed cancer cells even under nutrient rich conditions\textsuperscript{110}. Moreover, basal autophagy was strongly increased after overexpression of both mutant HRAS and KRAS in human mammary epithelial cells\textsuperscript{121}. The underlying mechanistic reason for mutant H-RAS was found to be the activation of Beclin-1 interacting partner NOXA, thereby upregulating autophagy\textsuperscript{122}. Genetic inhibition of autophagy in cells overexpressing mutant RAS, attenuated glycolysis and inhibited proliferation\textsuperscript{121}. Similarly, ATG7 knock-out in KRAS-driven lung cancer cells increased ROS levels and triggered a striking depletion of the cellular nucleotide pool, which was rescued by supplementation with glutamine\textsuperscript{123}. In mouse models, the knockdown of ATG5 or ATG7 cells in RAS overexpressing cells triggered accumulation of dysfunctional mitochondria and reduced tumor growth\textsuperscript{107,124}. Thus, RAS-driven cancer cells exploit high levels of autophagy, which may position such cancers for autophagy inhibition.
Further, oncogenic mutations in the tumor suppressor protein p53, a protein best known for its pro-apoptotic effect upon cellular stress, also clearly affect the autophagy pathway. For instance, elevated levels of autophagy were identified in mutant p53 expressing AML cells, whereas a reverse reduction in autophagy was detected in pancreas and breast cancer cell lines that expressed mutant p53. These apparent contradictory data may be explained by the localization of p53, since p53 mutants that localized to the cytosol repressed autophagy, whereas p53 mutants localized to the nucleus did not. These clear differences in effect p53 mutants on autophagy may also impact on therapeutic response toward autophagy inhibition. Indeed, overexpression of mutant p53 in AML cells reduced the sensitivity toward HCQ treatment. Analogously, mutated p53 glioblastoma cells were less sensitive for CQ treatment. In contrast, CQ treatment impaired tumorigenesis in mutant k-Ras pancreatic tumors with wildtype p53, but augment tumorigenesis in the absence of p53. In this respect it is important to note that wildtype p53 can differentially affect autophagy, with inhibition of autophagy upon binding to proteins involved in autophagosome formation. On the other hand, wildtype p53 can promote autophagy by inhibiting mTOR or by phosphorylation of Beclin-1. Interestingly, the level of p53 is also itself regulated by autophagy. For instance, wildtype p53 is depleted via autophagy-mediated degradation in renal cell carcinoma, which allows escape from apoptotic cell death. In contrast, suppression of macro-autophagy promotes the degradation of mutant p53 via CMA, which sensitizes various human cancer cell lines for cell death. Further, a truncated p53 isoform that inhibits wildtype p53 is degraded via autophagy.

Thus, various known important oncogenic mutated proteins that are important in cancer maintenance are able to regulate autophagy, in most cases triggering elevated levels of autophagy that may aid in cancer cell survival.

2.2.3 Autophagy in cancer metabolism

Autophagy is a catabolic process whereby redundant organelles and proteins can re-enter various metabolic pathways. Cancer cells typically metabolize glucose to lactate, even when sufficient oxygen is present to support oxidative phosphorylation, a phenomenon known as the Warburg effect. Of note, pyruvate kinase (PKM2) is the final enzyme in the glycolytic pathway that controls the glycolytic flux, and is therefore important for preventing accumulation of glycolytic intermediates. In cancer, PKM2 breakdown via CMA is increased, whereby reduced PKM2 associates with accumulation of glycolytic intermediates that are rerouted to branching biosynthetic pathways to support cancer growth. Likewise, the rate-limiting enzyme hexokinase 2 (HK2) of the glycolytic pathway, was found to be selectively broken down via autophagy in liver cancer. Together, this indicates that autophagy can control glycolysis at different levels and thus impacts on cancer metabolism.
Indeed, glycolysis in MLL-ENL driven leukemia is augmented by inhibition of autophagy although the underlying mechanism remains to be determined\(^{144}\). Of note, enhanced lactate secretion due to the Warburg effect can change the extracellular microenvironmental pH, which in turn can activate autophagy\(^{145}\). For example, in breast carcinoma cells acute acidification led to an increase in LC3 puncta together with an increase in expression of ATG5 and BNIP3\(^{146}\). Thus, degradation of diverse substrates by autophagy may impact many aspects of central metabolism in cancer. Corroborating evidence hereof was obtained by labeling of wild type or ATG7\(^{-/-}\) KRAS driven lung cancer cells with heavy carbon and nitrogen isotopes, which in the autophagy-deficient cells identified a significant depletion of amino acids linked to the TCA cycle\(^{147}\). Therefore, autophagy may provide cancer cells with a mechanism to efficiently redistribute metabolites enabling metabolic rewiring, which is required for malignant transformation.

### 2.2.4. Autophagy is upregulated in hypoxic tumor regions

Autophagy is also an important regulatory pathway during adaptation of cancer cells to hypoxic stress, which likely occurs in poorly oxygenated regions of the bone marrow due to AML infiltration or in hypoxic regions of solid cancers. Indeed, in xenograft models of human head and neck cancer, autophagy was associated with hypoxic tumor regions\(^{111}\). Under hypoxic conditions, stabilization of HIF1α was detected, leading to enhanced levels of Beclin-1, increased LC3-II/LC3-I ratio and degradation of p62, e.g. upon treatment of lung cancer cell lines with cisplatin. Likewise, in adenoid cystic carcinoma the hypoxia mimetic CoCl\(_2\) activated HIF1α stabilization and induced autophagy. HIF1α activity among others upregulates expression of BNIP3 and BNIP3L expression, which can activate autophagy by shifting the balance of the regulatory Beclin-1 hub towards autophagy induction (Figure 2B)\(^{48,148,149}\). In glioblastomas, increased expression of BNIP3 or ATG9A contributed to hypoxia-associated growth, which could be blocked in vivo by HCQ\(^{150,151}\). Importantly, tumor cells in hypoxic regions proved to be particularly sensitive to HCQ treatment\(^{111}\). In a panel of cancer cell lines, hypoxia-induced cell death increased upon knockdown of Beclin-1 or ATG7, with autophagy deficient cancer cells proliferating less in mouse xenograft models\(^{152}\). Of note, xenografts of wildtype cell lines were characterized by increased LC3 and reduced p62 levels in hypoxic tumor regions, reflecting activation and execution of autophagy\(^{152}\). Therefore, in a broad spectrum of cancers induction of autophagy contributes to survival in poorly oxygenated tumor areas.

### 2.2.5. Autophagy in anoikis and metastasis

Most cancer patients succumb to their disease due to metastatic spread of the original primary tumor, an event that can occur many years after initial seemingly successful treatment of the primary tumor. During metastatic spread, autophagy is thought to be crucial for cancer...
Chapter 5

cell survival. Firstly, cancer cells that spread to distal organs have to resist cell death due to loss of contact with the extracellular matrix (ECM), termed anoikis. Cells can resist anoikis partly though activation of autophagy as shown for metastatic hepatocellular carcinoma\textsuperscript{153,154}. Similarly, transformed fibroblasts were characterized by a strong increase in autophagy after loss of ECM contact. Further, anoikis was triggered upon inhibition of autophagy in cancer cell lines driven by either RAS or PI3K\textsuperscript{121,155–157}. In an attachment-free culture model system, tumor spheroids of various cancer cell lines depended on BNIP3-associated autophagy for survival\textsuperscript{158}. Further, rapamycin-mediated activation of autophagy improved spheroid growth, while autophagy inhibition induced apoptosis\textsuperscript{158}. Correspondingly, the levels of LC3B were significantly higher in metastases compared to primary tumors in breast cancer, liver cancer and melanoma\textsuperscript{108,154,159}. Moreover, the incidence of metastases was reduced in metastatic liver cancer cells upon knockdown of beclin-1 or ATG5 in a mouse model, due to loss of resistance to anoikis\textsuperscript{154}. Thus, metastatic cells appear to be more dependent on functional autophagy to allow survival in the absence of ECM after which metastatic cells remain characterized by higher autophagy levels.

2.3. THE ROLE OF AUTOPHAGY IN CYTOTOXIC CANCER THERAPY

Treatment of cancer cells with cytotoxic drugs inevitably leads to cellular stress. Consequently, activation of autophagy is widely described, although as detailed below the impact of autophagy on cytotoxic therapy can differ depending on the type of cell death. Moreover, although the underlying cause of intrinsic and/or acquired drug resistance is likely multifactorial and often remains enigmatic, autophagy is increasingly recognized as being an important contributor to therapy resistance. In the sections below, the role of autophagy in cytotoxic cell death will be detailed, after which the role of autophagy in resistance to therapy is discussed.

2.3.1. Autophagy has a distinct impact depending on type of cytotoxic cell death

Autophagy can be a stress response of cancer cells that enables cells to evade apoptotic elimination. An example hereof is the treatment of a triple negative breast cancer cell line with a plant-derived anti-cancer drug that induced apoptosis and activated autophagy. Here, inhibition of autophagy with 3-MA served to augment the level of apoptotic cell death\textsuperscript{160}. Similarly, in colorectal cancer cell lines a pro-apoptotic polyamine analogue simultaneously induced apoptosis and autophagy, with 3-MA co-treatment enhancing induction of apoptotic cell death\textsuperscript{161}. In another breast cancer cell line model, the novel therapeutic drug NBT was found to induce autophagy and apoptosis, with apoptosis induction being increased upon CQ treatment\textsuperscript{162}. In CML cell lines, the anti-tumor agent asparaginase induced apoptosis

126
and autophagy. Blockade of autophagy with three different autophagy inhibitors enhanced asparaginase-induced cell death. Further, inhibition of autophagy in HeLa cells upregulated expression of PUMA via FOXO3a, which upon co-treatment with etoposide or doxorubicin upregulated apoptosis as defined by enhanced activation of effector caspase 3/7. This sensitizing effect of autophagy inhibition was abolished in cells lacking PUMA, indicating that FOXO3a dependent mechanism induction of PUMA contributes to drug resistance. Interestingly, an important regulator of initiator caspase-8 activation, the anti-apoptotic protein FLIP, also can regulate autophagy activity by competitive binding to ATG3 and preventing lipidation of LC3.

Reversely, autophagy as part of ACD is required for cytotoxic cell death. An example hereof is cell death induced by a cardiac glycoside in non-small lung cancer cell lines, which was characterized by an increase in autophagic flux and was inhibited by 3-MA. Treatment with this glycoside was accompanied by activation of the JNK signaling pathway, leading to a decrease in the level of BCL-2 and a concomitant shift towards Beclin-1 mediated induction of autophagy. Of note, although glycoside treatment elevated the level of intracellular ROS, antioxidant co-treatment did not prevent glycoside-induced cell death indicating that ROS is by-product of ACD in this setting. In contrast, ROS was causal for ACD induction in triple negative breast cancer cells by the compound physagulide P (PP) purified from a Chinese herbal medicine, with co-treatment with a ROS scavenger inhibiting ACD. Several other pathways can also be involved in therapy-induced ACD. For instance, radiation treatment of breast cancer cell lines triggered ACD via activation of p53 and downstream p53 effector protein DRAM. In this case, cell viability was partially rescued upon treatment with 3-MA or by knockdown of ATG5 or Beclin-1. Further, treatment of breast cancer cells with a so-called selective estrogen receptor modulator (SERM) induced ACD via reducing ATP levels. Conversely, addition of ATP restored cell viability, coinciding with a reduction in the LC3-II/LC3-I ratio, which indicates that ACD was averted. Furthermore, treatment with the glycan-binding protein Galectin-9 triggered cell death in colon cancer cells, which was blocked by knock-down of Beclin-1 or ATG5.

In conclusion, autophagy during cytotoxic therapy can either be protective or can be instrumental for cell death induced by certain therapeutics. Thus, depending on the type of drug used in the treatment of cancer, the combination with autophagy inhibitors may be warranted or should be avoided.

2.3.2. The role of autophagy signaling in resistance to cancer therapy
As described above, autophagy during treatment may reduce sensitivity to cytotoxic therapy. Correspondingly, resistance to various types of therapy is also characterized by enhanced basal levels of autophagy, as defined by increased conversion of LC3-I to LC3-II, increased numbers
of LC3b puncta per cell, up-regulated numbers of autophagolysosomes, and degradation of p62\(^{169–171}\). For example, cisplatin resistant clones of ovarian cancer cell lines as well as an oral squamous cell carcinoma cell line were characterized by enhanced levels of autophagic flux\(^{172}\). In radiotherapy resistant breast cancer cells, ionizing radiation also elevated basal autophagy levels, indicating a protective effect of autophagy against treatment\(^{173}\). Similarly, treatment of pancreatic cancer, colorectal cancer, and AML cell lines with bortezomib was accompanied by elevated autophagic flux\(^{169,170}\). Importantly, in various cell lines and with different types of drugs, the co-treatment with autophagy inhibitors CQ or HCQ re-sensitized cells to treatment\(^{174–177}\). For instance, in breast and esophageal squamous cancer cell lines, chemo- or radiotherapy induced an autophagy response accompanied by therapy resistance\(^{177,178}\). The co-treatment with CQ did not only reduce clonogenic survival of malignant cells in vitro, but also reduced tumor burden in murine models\(^{177,178}\). Of note, overexpression of multidrug resistance pumps, such as ABCG2, not only facilitates drug resistance by increasing drug efflux but also by increasing autophagic flux\(^{179}\). In line with this, ABCG2-mediated drug resistance was strongly inhibited by knockdown of either ATG5 or ATG7\(^{179}\). In this respect, cancer stem cells are also known to overexpress ABC transporters, which may upregulate autophagy and contribute to CSC resistance to chemotherapy\(^{180}\). Further, in CSCs, autophagy was upregulated upon treatment with chemotherapy or photodynamic therapy, which contributed to CSCs survival and promoted therapy resistance\(^{181,182}\). Similarly, AML leukemic stem cells (LSCs) were characterized by elevated autophagic flux upon treatment with BET inhibitors, which contributed to resistance to therapy\(^{183}\) (Figure 3C). Of note, since both normal hematopoietic stem cells (HSC) as well as LSCs need a certain amount of autophagy to survive, there is only a relatively small therapeutic window of autophagy inhibition with HCQ (Figure 3D).

Resistance toward antibody-based therapy can also be regulated by autophagy, which has mainly been studied for cetuximab, an Epidermal Growth Factor Receptor (EGFR)-blocking antibody. For instance, cetuximab induced autophagy in various EGFR-expressing cancer cell lines by downregulation of HIF-1α and BCL-2, which promoted the association of Beclin-1 with Vps34\(^{184}\) and dose-dependently activated Beclin-1 in colon carcinoma cell lines\(^{185}\). Analogously, EGFR tyrosine kinase inhibitors activated autophagy by promoting Beclin-1-Vps34 complex formation\(^{186}\). Importantly, chemical inhibition of autophagy or knockout of Beclin-1 sensitized cancer cells for cetuximab-induced apoptosis\(^{184,185}\). Interestingly, inactive EGFR is required for the induction of starvation-induced autophagy\(^{187}\). Together, this data clearly indicates that enhanced autophagy can associate with resistance to various types of cancer therapy. Thus, it is of clear relevance to gain insight into how autophagy facilitates resistance to therapy. In the following sections, the role of key autophagy-regulating signaling pathways and cancer-associated genetic mutations will be discussed in the context of resistance to therapy.
2.3.3. Key signaling pathways associated with autophagy-dependent drug resistance

Many studies have focused on unraveling the mechanisms by which chemo- and radiation therapy induce resistance, with several key upstream signaling components being implicated. Most notably, deregulation of the upstream autophagy regulatory system of AMPK, which can both activate ULK1 and repress mTOR signaling to promote autophagy, has been reported. For instance, treatment of a colorectal cancer cell line with the drug salidroside activated protective autophagy alone as well as in combination with other anti-tumor agents via activation of AMPK\textsuperscript{188}. When AMPK activity was blocked using a kinase inhibitor, autophagy was reduced as evidenced by a decrease in LC3-II/LC3-I ratio, which synergistically enhanced the cytotoxic effects of combined salidroside and chemotherapy treatment\textsuperscript{188}. In other studies, upregulation of autophagy was attributed to direct activation of ULK1. Specifically, AML leukemic stem cells (LSCs) cells that were resistant to treatment with BET inhibitor \textit{in vitro} were characterized by ULK1 activation\textsuperscript{183}. In contrast, no ULK1 activation was detected in cells sensitive to BET inhibitor treatment. Interestingly, although ULK1 is supposed to be downstream of AMPK signaling, AMPK phosphorylation was detected in both BET inhibitor sensitive and resistant cells. Thus, resistance to treatment in these LSC appears to stem from ULK1 signaling that increases autophagic flux\textsuperscript{183}. In a follow-up study, pharmacological inhibition of AMPK did induce apoptosis in BET resistant LSCs. AMPK and ULK1 were found to have a similar cytoprotective mechanism against chemotherapeutics in primary pancreatic cancer cells as well as pancreatic cell lines\textsuperscript{189}. Further, in a t(8;21) AML model, Kasumi-1 cells survived short-term treatment with histone deacetylase inhibitors by up-regulation of autophagy. However, interactions between AMPK and mTOR were not investigated and long-term resistance was not examined\textsuperscript{190}. Resistance to therapy due to upregulated autophagy can also be acquired through repression of the mTOR pathway as demonstrated for dexamethasone treatment in various leukemic cell lines\textsuperscript{191}. Similarly, activation of autophagy in an imatinib resistant CML line and in cisplatin-resistant lung carcinoma cells was due to repression of mTOR signaling\textsuperscript{192,193}. Altered signaling of upstream regulators of mTOR caused this repression of mTOR signaling, e.g. an increase in phosphorylation/activation of AMPK or a decrease in Akt signaling\textsuperscript{191,193}. In targeted therapy, mTOR inhibitors as single agents did induce autophagy, but were ineffective anticancer therapeutics\textsuperscript{194}. However, when mTOR inhibitors were combined with autophagy inhibitors, prominent anti-leukemic effects were detected\textsuperscript{194}. In clonogenic assays, primary AML cells formed fewer colonies in combination therapy than single treatment. Similarly, knockdown of ULK1 in combination with mTOR inhibitor reduced the colony forming potential of primitive AML precursors\textsuperscript{194}. 
Another pathway involved in autophagy-mediated resistance to therapy is the MAPK pathway, with chemotherapeutic treatment of hepatocellular carcinoma cell lines leading to increased MEK and ERK activity and induction of cytoprotective autophagy. This induction of autophagy was partly blocked by MEK inhibition. In cell lines carrying the oncogenic b-Raf V600E mutation that have aberrant constitutive MAPK signaling, treatment with the specific V600E inhibitor vemurafenib resulted in AMPK-ULK1 mediated autophagosome accumulation. Autophagy was similarly upregulated in BRAF mutated primary melanoma samples treated with BRAF inhibitor compared to baseline untreated samples. Interestingly, here induction of autophagy did not occur through AMPK-ULK1 signaling, but was likely attributable to induction of ER stress response through CHOP, ATF4, and eIF2α. Similarly, in cutaneous BRAF mutated melanoma cell lines enhanced basal autophagy was observed. Oncogenic BRAF led to chronic ER-stress, which in turn activated the JNK signaling cascade and contributed to autophagy induction, leading to therapy resistance. Of note, combined treatment of vemurafenib with autophagy inhibitor CQ almost completely blocked tumor growth in a xenograft mouse melanoma model, highlighting that cytoprotective autophagy was at least partially associated with resistance to vemurafenib. Thus, various type of chemotherapy as well as targeted drugs can trigger activation of autophagy that contributes to resistance to therapy.

2.3.4. HMGB1 positively regulates autophagy, contributing to therapy resistance

Recent evidence suggests that the nuclear protein High Mobility Group Box 1 (HMGB1) is another critical regulator of autophagy that can mediate resistance during cancer treatment. Although normally in the nucleus, upon stress HMGB1 can translocate to the cytoplasm where it directly interacts with Beclin1 and displaces BCL-2. Consequently, cytoplasmic HMGB1 can activate autophagy. Many studies have linked increased HMGB1 protein levels to autophagy and therapy resistance. For instance, up-regulation of HMGB1 occurred during cisplatin treatment in non-small cell lung cancer cell lines, which associated with enhanced autophagy. Knockdown of HMGB1 reduced the levels of autophagy and increased cell death, with knockdown of HMGB1 being more efficient than treatment with well-known autophagy inhibitor 3-MA. Similarly, treatment with docetaxel upregulated HMGB1 protein, leading to enhanced autophagy levels. Upon continuous treatment with docetaxel cells became resistant to therapy, with sensitivity being restored by knockdown of HMGB1 and reducing tumor growth in a xenograft model. In an analogous fashion, treatment of leukemic cell lines with different chemotherapeutic drugs upregulated expression of HMGB1. Upregulation of HMGB1 was associated with enhanced LC3-II/LC3-I ratios and protected from treatment-induced cell death, which was prevented by knockdown of
HMGB1. HMGB1 mediated resistance to autophagy via mTOR and Beclin-1 was further reported in several different cancer cell lines. As discussed above, various other factors can induce mTOR, thereby, facilitating resistance to chemotherapy mediated by autophagy.

2.3.5. micro-RNAs in autophagy during treatment resistance

Several lines of evidence have emerged that indicate that micro-RNAs (miRNA), small non-coding RNAs that degrade mRNA and thereby reduce translation, may also play a regulatory role in autophagy signaling in therapy resistance. For instance, the reduced expression of miR-23b in radiotherapy resistant pancreatic cancer cell lines enhanced the level of autophagy when compared to radio-sensitive cell lines. miR-23b directly targeted and reduced ATG12 expression and overexpression of this miRNA in radiotherapy-resistant cells blocked autophagy, as evidenced by reduced LC3-II/LC3-I ratio and reduced numbers of autophagosomes per cell, and re-sensitized cells to radiation treatment. In epithelial ovarian cancer cell lines that were resistant to cisplatin treatment, a similar decrease in the level of miR-429 was detected, which was associated with enhanced levels of autophagy. Correspondingly, overexpression of miR-429 reduced autophagy via down-regulation of ATG7 and increased cellular sensitivity to cisplatin treatment. Furthermore, doxycycline treatment reduced the expression of miR-30a, a microRNA that directly targets Beclin-1 mRNA, whereas the levels of miR140-5p that targets IP3k2 mRNA were increased. In both cases induction of autophagy was enhanced and contributed to therapy resistance. In addition, treatment of colorectal cancer cells with cetuximab was associated with down-regulation of another Beclin-1 mRNA-targeting miRNA, miR-216b, again yielding elevated activation of autophagy and resistance to therapy.

In conclusion, although still in early stages the available data collectively suggests that down-regulation of various miRNAs can directly activate cytoprotective autophagy during therapy by upregulation of key components of the autophagy machinery. Thus, reduced miRNA expression appears to be causally related to autophagy-mediated resistance to therapy.

2.3.6. Hypoxia as autophagy activating signal in therapy resistance

Several studies highlight that hypoxia-induced autophagy contributes to resistance to therapy. For instance, in primary glioblastoma tissue samples, administration of the vascular endothelial growth factor-neutralizing antibody bevacizumab increased tumor hypoxia. In turn, this hypoxia associated with up-regulation of protective autophagy. Correspondingly, autophagy inhibition upon bevacizumab treatment of xenografts derived from GBM patients resulted in increased survival. In a study conducted on breast cancer cell lines, hypoxia itself did not induce autophagy. However, upon taxol treatment in hypoxic conditions cancer cells did appear to activate cytoprotective autophagy through inhibition of the mTOR pathway.
Similarly, chemotherapy resistance in triple negative breast cancer stem cells was attributed to a combination of hypoxia and upregulation of autophagy occurring in xenograft models from these patients. Overall, this data implies that hypoxia-mediated resistance to therapy is at least partly due to the induction of cytoprotective autophagy.

PART II. THE ROLE OF AUTOPHAGY IN THE TUMOR MICRO-ENVIRONMENT

The tumor microenvironment is a specialized niche created during tumor development that plays an important role in terms of cancer progression and survival and response to therapy. This micro-environment comprises many different cell types, including fibroblasts, mesenchymal stem cells (MSCs), endothelial cells and immune cells. All of these cell types to a different extent use autophagy in cellular functioning in cancer, with e.g. autophagy in stromal cells such as fibroblasts promoting tumorigenesis, whereas autophagy in immune cells such as cytotoxic T cells facilitates execution of anticancer immune responses. Thus, cells within the micro-environment may have opposing requirements for autophagy that may prove difficult to reconcile for autophagy-targeting therapy in cancer. In this section, we will attempt to capture the role and importance of autophagy and the impact of potential therapeutic targeting of autophagy for several crucial tumor microenvironmental constituents, namely cancer associated fibroblasts and MSCs, endothelial cells, innate and adaptive immune cells.

3.1. AUTOPHAGY IN THE TUMOR MICRO-ENVIRONMENT; STROMAL CELLS

3.1.1. Autophagy in stromal cells promotes cancer cell growth and survival

A positive influence of fibroblasts on cancer cell growth is well documented, with e.g. enhanced growth rates for both fibroblasts and colon cancer cell lines in co-cultures, as well as enhanced growth rates of head and neck squamous cell carcinoma (HNSCC) cells and breast carcinoma cells. Similarly, primary patient-derived AML cells survive and proliferate better in co-culture with mouse stromal cells or MSCs. In co-cultures, fibroblasts were characterized by elevated levels of autophagy as e.g. evidenced by accumulation of LC3-positive vesicles. Importantly, inhibition of autophagy markedly attenuated the beneficial impact of fibroblast in such co-cultures. Specifically, inhibition of autophagy using 3-MA treatment reduced the growth rate of colon cancer cells, whereas treatment with CQ or knockdown of Beclin-1 in fibroblasts prevented the increase in HNSCC proliferation. Together these data indicate that cancer cells induce and exploit the elevated levels of autophagy in stromal cells...
for their aberrant growth. In this respect, fibroblasts isolated from tumors indeed had higher autophagy activity than normal fibroblasts\(^{2,18}\). Thus, co-culture of cancer cells and stromal cells increases the level of autophagy in stromal cells, which apparently is used by cancer cells to support cell growth.

In addition to promoting cancer cell proliferation, there are some clues that autophagy in stromal cells also helps to promote cancer cell survival and can protect against anticancer therapy. Specifically, in co-cultures of cancer cells with fibroblasts the basal level of apoptosis in cancer cells decreased, a phenomenon reversed by inhibition of autophagy using CQ\(^{2,18,220}\). Of note, this effect on basal apoptosis was significant, yet small with the basal level of apoptosis dropping from 5% in breast cancer monocultures to 1% in fibroblast co-cultures. More importantly, fibroblasts protected breast cancer cells against treatment with tamoxifen, yielding 85% apoptosis in monocultures versus 45% in fibroblast co-cultures\(^{219}\). However, the relative importance of autophagy in this setting remains to be determined, as no autophagy inhibitors were applied to identify the impact of autophagy. Similarly, under serum deprivation conditions, MSCs were able to limit the induction of apoptosis in lung cancer cell lines through activation of autophagy\(^{221}\). Interestingly, cancer-associated fibroblasts also resist stress better than normal fibroblasts, as fibroblasts isolated from ovarian cancer patients were more resistant to oxidative stress, with sensitivity being restored by Beclin-1 or ATG5 knock-out\(^{2,18}\). Thus, autophagic signaling in stromal fibroblasts and MSCs cells can contribute to survival and growth of cancer cells.

Of note, preliminary data further indicate that autophagy can also regulate angiogenesis, with e.g. angiogenesis in B16F10 melanoma tumor being increased in heterogeneous Beclin-1 knockdown (Becn1\(^{-/-}\)) mice, yielding bigger tumors and an increased number of lung metastases\(^{222}\). In contrast, in other angiogenesis models, the inhibition of autophagy blocked angiogenesis, whereas angiogenesis was promoted by autophagy induction\(^{2,18,224}\). However, the exact impact of autophagy on angiogenesis in the cancer micro-environment is difficult to define \textit{a priori} as it will depend on specific micro-environmental conditions that likely vary even within a tumor.

\subsection*{3.1.2. Soluble factors secreted in stromal cell/cancer co-cultures affect autophagic signaling}

In many cases, the positive effect of fibroblasts on cancer cell growth was retained when cells were cultured in the absence of direct cell-to-cell contact or when conditioned medium of fibroblasts was used\(^{2,12,213,225}\). In the latter case, the conditioned medium of cancer-associated fibroblasts outperformed that of normal fibroblasts\(^{2,13,225}\). Further, the supernatant of cancer-associated fibroblasts also protected melanoma and lung cancer cells from radiation-induced cell death\(^{2,18}\). This pro-tumorigenic effect of secreted factors was due to autophagy signaling,
as conditioned medium from cancer-associated fibroblasts pre-treated with CQ failed to promote proliferation, migration and invasion\textsuperscript{213}. Thus, cancer-associated fibroblasts secrete soluble factors through autophagy that are beneficial for cancer cells. Several secreted factors were identified, including various cytokines, including IGF1, IGF2, CXCL12, all of which promoted survival of A375M melanoma and A549 lung cancer cells after radiation\textsuperscript{226}. Further, injection of cancer-associated fibroblasts at the site of tumors previously eradicated by radiation accelerated the subsequent development of tumor recurrence, which was abrogated by IGF2 knockout or 3-MA treatment\textsuperscript{236}. This finding highlights the importance of a cytokine produced by CAFs under autophagy for cancer cell survival. Importantly, IGF2 produced by cancer-associated fibroblasts also induced autophagy in cancer cells, indicating a feed-forward loop for promoting autophagy in the tumor micro-environment. In a similar fashion, IL6 and IL8 secretion by cancer-associated fibroblasts was reduced upon knockdown of Beclin-1, which decreased migration of HNSCC cells\textsuperscript{213}. Of note, direct addition of IL-6 and IL-8 to HNSCC cells promoted migration to a similar extent as co-culture with cancer-associated fibroblasts, highlighting the importance of those cytokines for the autophagy-mediated effect of fibroblasts. Cytokine production by fibroblasts was attributed to bFGF-induced autophagy, with knockdown of bFGF in HNSCC cells reducing autophagy in fibroblast and reducing cytokine secretion. Similarly, TGF-β secreted by breast cancer cells was shown to induce autophagy in cancer-associated fibroblasts\textsuperscript{227}. Thus, factors secreted by cancer cells can trigger activation of autophagy in cancer-associated fibroblasts, which concomitantly results in secretion of cytokines that elevate autophagy and have a pro-tumorigenic effect on cancer cells. Hence, inhibiting autophagy in both cancer cells and cancer-associated stromal cells likely outperforms inhibiting autophagy in cancer cells only. Indeed, simultaneous knockout of ATG7 in both MSCs and AML cells increased the sensitivity to cytarabine treatment compared to ATG7 knockout in AML cells alone\textsuperscript{228}.

3.1.3. Cancer cells trigger metabolic reprogramming of cancer-associated fibroblasts

In co-culture experiments of fibroblasts and cancer cells hypoxic stress was elevated in the fibroblast population, leading to induction of autophagy and metabolic reprogramming. For instance, in co-culture with breast cancer cells, HIF1α and NFκB signaling activated autophagy and, more specifically, mitophagy in cancer-associated fibroblasts\textsuperscript{220}. Similarly, co-culture of fibroblast and colon cancer cells induced oxidative stress in fibroblasts and elevated the level of autophagy\textsuperscript{212}. Correspondingly, expression of constitutively active HIF1α in fibroblasts also induced autophagy/mitophagy, whereas treatment with HIF1α inhibitor echinomycin reduced levels of autophagy\textsuperscript{229}. Due to elevated mitophagy, the mitochondrial mass in fibroblast co-cultured with cancer cells was strongly reduced\textsuperscript{214}. This resulted in a metabolic shift from the TCA cycle to the glycolytic pathway, also yielding increased

\textsuperscript{134}
production of ketones and lactate\textsuperscript{212}. A similar shift was detected in fibroblast engineered to overexpress the p53 inducible autophagy inducer DRAM, leading to elevated autophagy, reduced mitochondrial mass, and an increase in secretion of ketones and lactate\textsuperscript{230}. In line with this, overexpression of ATG16L1 or BNIP3L, in order to induce autophagy, reduced fibroblast mitochondrial activity and increased glycolytic pathway activity\textsuperscript{231}. Interestingly, lactate and ketones produced by fibroblasts were utilized by cancer cells leading to increasing mitochondrial mass and mitochondrial oxidative metabolism of cancer cells in co-culture with fibroblasts\textsuperscript{214}. Of note, HIF 1\(\alpha\) also directly activates the glycolysis pathway [ref]. Therefore it is unclear whether elevated autophagy is the cause of glycolysis induction or that both pathways are simultaneously induced upon hypoxic stress. Taken together, cancer cells trigger hypoxic stress in fibroblasts leading to activation of autophagy and mitophagy and a metabolic switch from TCA cycle to glycolysis. The metabolites produced by these fibroblasts are subsequently consumed by the cancer cells and contribute to cancer cell growth and survival\textsuperscript{226}.

Autophagy in fibroblasts has further been linked to reduced caveolin-1 (cav-1) expression in stroma of breast cancer patients, a feature associated with poor survival\textsuperscript{232,233}. Specifically, Cav-1 expression was down-regulated in fibroblasts with that were modulated to have elevated levels of autophagy\textsuperscript{214,220,229,231,234,235}. Correspondingly, cav-1 expression inversely correlated with autophagy and mitophagy in cell lines and in patient-derived human breast cancer samples\textsuperscript{220}. In mice, co-injection of breast cancer cells with fibroblasts yielded larger primary tumors and an increase in metastases, especially when fibroblasts were modulated for increased autophagic flux and reduced cav-1 levels\textsuperscript{214,234,235}.

Taken together, elevated levels of autophagy in cancer-associated fibroblasts promote cancer cell growth and survival among others due to a metabolic switch of fibroblast to glycolysis and secretion of glycolytic by-products.

\section*{3.2. AUTOPHAGY IN CANCER IMMUNITY; THE CANCER CELL SIDE OF THE COIN}

From the above paragraphs, it is clear that autophagy directly impacts on cancer proliferation and survival and thus is a target for inhibition in cancer cells. However, elevated autophagy levels in cancer cells can also have a diverse impact on anti-cancer T cell immunity. In brief, anti-cancer T cell immunity is a multi-layered and intricately regulated process, which pivots on the recognition of antigenic peptides presented on the cancer cell surface in the so-called major histocompatibility complex (MHC) class I to cytotoxic T cells (CTLs).
Upon recognition of an MHC/peptide complex, the CTLs form an immunological synapse with the cancer cell and secrete cytotoxic and tumoricidal proteins, such as granzymes and perforins. Consequently, the cancer cell is eliminated by apoptosis.

Autophagy affects many aspects of this immune response and in cancer cells for instance inhibits proper formation of the immunological synapse and reduces the cytolytic potential of CTLs and Natural Killer (NK) cells (Figure 4). In this section of the review, the role of autophagy in anti-cancer immunity is discussed in detail, with a focus on the potential impact of autophagy inhibition on the functioning of this response.

**Figure 4: Autophagy in the tumor-micro-environment impacts on anti-cancer immunity.** Autophagy in cancer cells inhibits the anti-cancer immune response by reducing the efficacy of CTL and NK cell mediated lysis by degrading granzyme B and connexin 43. However, autophagy is also required for T cell proliferation, survival and induction of T cell memory by degrading pro-apoptotic proteins and maintaining mitochondrial homeostasis. Therefore, non-selective inhibition of autophagy in the tumor-micro-environment will not only promote anti-cancer effects at the level of stroma and cancer cells, but will also dampen anti-cancer immune responses.

### 3.2.1 Autophagy in cancer cells inhibits anticancer immunity by reducing the sensitivity toward NK- and CTL-mediated lysis

Cancer cells are lysed when they express MHC-I molecules that contain tumor-derived antigenic peptides that are recognized by CTLs. To evade from recognition and elimination by the immune system, cancer cells therefore often down-regulate their MHC-I expression. Although this is mainly regulated by genetic mutations and epigenetic modifications, MHC-I molecules are also degraded by autophagy. Indeed, inhibition of
Autophagy in the cancer micro-environment

Autophagy augmented cell surface expression of MHC-I induced by treatment of melanoma cells with the immunostimulatory cytokine IFNγ. Importantly, the loss of MHC-I expression (‘missing self’) is also being recognized by NK cells, leading to elimination of the cancer cell. Several reports detail that high levels of autophagy in cancer cells reduces the efficacy of NK and CTL-mediated cell lysis. For instance, autophagy in cancer cells affects the stability of the immunological synapse generated between the cytolytic immune cell and its target cell. Specifically, formation of gap junctions, which requires connexin proteins such as connexin-43, normally facilitates the exchange of small molecules between effector and target cell and is required for NK lysis. In melanoma cells, the accumulation of connexin-43 at the immunological synapse was reduced under hypoxia, which was restored by inhibition of autophagy. Consequently, NK-mediated cell lysis was restored. Interestingly, the gap junction protein connexin-43 also transports active granzyme B into the target cell. Thus, its degradation by autophagy may affect cytolysis in various ways. In this respect, autophagy-mediated degradation of the cytotoxic effector protein granzyme B contributed to resistance to lysis of lung cancer cell lines, breast cancer cells and melanoma cells in hypoxic conditions. Correspondingly, granzyme B was predominantly detected in LC3 and Rab5 positive fractions in hypoxic cells. A similar effect was detected upon hypoxia-independent activation of HIFs, as seen in renal cancer with mutations in the von Hippel-Lindau gene. In these cells, autophagy was upregulated and cells were resistance towards NK-mediated cell lysis. Autophagy not only affect CTL lysis in hypoxia, but also in normoxia with reduced sensitivity of melanoma cells toward CTLs-mediated lysis compared to healthy cells. In all cases, CTL- or NK cell-mediated lysis was restored by inhibition of autophagy. Of note, upregulation of autophagy also confers resistance to CTL-lysis during so-called Epithelial-mesenchymal transition (EMT), a step necessary for cancer progression and metastasis, in breast carcinoma cells. Sensitivity to CTL-mediated cell lysis in this setting was partly restored by Beclin-1 knock-down. Thus, autophagy may affect cancer cell-sensitivity to immune cell lysis at multiple levels.

Taken together, increased autophagy in cancer cells negatively affects sensitivity toward NK- and CTL-mediated cell lysis through degradation of granzyme B and inhibition of the immunological synapse.

3.2.2 Autophagy in cancer cells regulates expression of immune checkpoints

Immune checkpoints are co-inhibitory receptor/ligand pairs that serve to dampen immune cell activity. Prominent example hereof is the receptor programmed cell death-1 (PD-1) and its ligand (PD-L1), which are expressed on activated T cells and APCs, respectively. This checkpoint is a crucial inhibitor of anticancer T cell responses in the tumor microenvironment. Interestingly, activation of autophagy using mTOR inhibitor rapamycin
decreased the expression of PD-L1 in lung cancer cells \textit{in vitro} and \textit{in vivo}, whereas activation of mTOR increased expression of PD-L1\textsuperscript{249}. Correspondingly, almost all human lung cancer patient samples (\textdegree ~90\%) expressing PD-L1 were characterized by increased mTOR signaling, whereas the majority (83\%) of tumors negative for PD-L1 also stained negative for active mTOR. Of note, signaling through PD-L1 itself activated mTOR signaling in melanoma and ovarian cancer cells, with PD-L1 blockade decreasing mTOR signaling in a mouse model of pancreatic cancer\textsuperscript{250,251}. Thus, PD-L1 activates mTOR and in a feed-forward loop upregulates expression of PD-L1, signaling that proceeded via mTORC1 and not mTORC2\textsuperscript{249,250}. In line with the fact that mTORC1 is a major (negative) regulator of autophagy, melanoma and ovarian cancer cells with low autophagic flux expressed higher levels of PD-L1 than cells with high autophagic flux\textsuperscript{250}. Subsequent treatment with rapamycin to induce autophagy triggered a reduction in PD-L1 expression and reactivated T cell-mediated anticancer immunity\textsuperscript{249}. Further, co-treatment with rapamycin and PD-1 blocking antibodies more effectively reduced tumor growth than single treatment and was accompanied by reduced numbers of regulatory T cells and increased CD3\textsuperscript{+} T cell numbers. Thus, low levels of autophagy signaling associate with an increase in expression of PD-L1.

\textbf{3.2.3 Autophagy in cancer cells modulates the induction of immunogenic cell death}

Autophagy can further impact on the process of immunogenic cell death (ICD), a type of apoptosis that stimulates development of anti-cancer T cell responses. ICD is induced by certain anticancer therapeutic strategies such as anthracyclines like mitoxantrone or doxorubicin, radiation therapy and photodynamic therapy (PDT)\textsuperscript{252}. ICD requires the translocation of calreticulin to the cell surface and the release of several immune-stimulating factors, among which HMGB1 and ATP\textsuperscript{253,254}. During ICD, depletion of Atg5 or Atg7 in CT26 cells (murine colon cancer) or knock down of beclin-1 reduced ATP-release upon anthracycline treatment and inhibited \textit{in vivo} anticancer immunity\textsuperscript{253,255}. In contrast, ATG5 knock-down did not reduce ATP secretion in bladder cancer and melanoma cells after hypericin-mediated PDT, although in this case knock down of ATG5 did increase cell surface exposure of calreticulin\textsuperscript{256}. In contrast, calreticulin exposure was not affected upon anthracycline treatment of autophagy-deficient and -competent CT26 cells or mouse embryonic fibroblasts (MEFs)\textsuperscript{253}.

The impact of autophagy inhibition on ICD also depends on which stage of autophagy is blocked, with calreticulin exposure being strongly reduced by blocking autophagy at early stages by silencing of ULK1, beclin1 or ATG5\textsuperscript{255}. In contrast, calreticulin exposure was increased when autophagy was blocked at a late stage using vincristine, chloroquine and Bafilomycin A1. Of note, the induction of autophagy alone using rapamycin or mTOR siRNA was not enough to induce ATP secretion or calreticulin exposure\textsuperscript{253,255}. Indeed, activation of
the pro-apoptotic effector caspase-8 is known to be pivotal for calreticulin exposure during ICD\textsuperscript{157}. Taken together, autophagy modulates the release of ATP and the cell surface exposure of calreticulin and thus contributes to ICD of cancer cells.

### 3.3. Autophagy in Cancer Immunity: The T Cell Side of the Coin

Autophagy not only impacts on the immunogenicity of cancer cells, but is also pivotal for correct functioning of APCs and T cells. For instance, the development of *de novo* T cell responses requires presentation of antigenic peptides by professional Antigen Presenting Cells (APCs), most notably Dendritic Cells (DCs). DCs present the antigenic peptides to CTLs in the context of MHC-I and further present peptides from endocytosed material to CD4\textsuperscript{+} helper T cells in the context of MHC class II. Activation of helper T cells licenses the DC to stimulate clonal expansion of CTLs and is of great importance for anticancer T cell responses\textsuperscript{258–260}. Autophagy is critically involved in antigen presentation in both MHC-I and MHC-II in DCs. Finally, autophagy is also important in functional activity of the immune cell itself, with T cell activity and generation of T cell memory requiring autophagy. Thus inhibition of autophagy in the context of cancer therapy also likely affects the activity of intra-tumoral T cells. Of note, the investigation of autophagy in the context of cancer immunity is in its infancy, but as detailed below, knowledge on core principles by which autophagy regulates T cell functioning has been gained among others in viral infection models.

#### 3.3.1 Autophagy modulates surface MHC expression and alters presentation of antigenic-peptides

Anti-cancer T cell immunity is induced upon recognition of antigenic tumor peptides presented on the cell surface of professional APCs, such as DCs. However, the surface expression of the MHC-I/peptide complex is directly affected by autophagy in DCs and macrophages (Figure 5A). For instance, expression of MHC-I on murine macrophages and DCs was upregulated upon inhibition of autophagy using various chemical inhibitors or downregulation of core autophagy genes\textsuperscript{238,261}. This upregulation was attributed to slower internalization of MHC-I\textsuperscript{261}. Thus, in the absence of autophagy, MHC-I molecules appear to be more stably expressed\textsuperscript{261}. Correspondingly, DCs from Vps34-deficient mice expressed more cell surface MHC-I as well as MHC-II\textsuperscript{262}. In contrast, surface expression of MHC-II on macrophages was down-regulated upon inhibition of autophagy using 3-MA\textsuperscript{163,263}.

Thus, inhibition of autophagy upregulates surface expression of MHC-I, although the impact on surface expression of MHC-II is less conclusive. However, with one notable exception in an influenza model, functional studies highlight that despite an increase in surface MHC-I...
expression the inhibition of autophagy actually weakens T cell responses\textsuperscript{261}. This weakening can likely be attributed to an alteration in the pool of immunogenic peptides presented in MHC, which has been mostly determined in the context of so-called cross-presentation in DCs. Cross-presentation is a process which enables loading of MHC-I on DCs with extracellular antigens, which e.g. is important for activation of CTL responses in melanoma\textsuperscript{264}. DCs with cross-presentation capacity are characterized by increased levels of autophagy compared to DCs that do not cross-present, with inhibition of autophagy reducing MHC-I mediated cross-presentation of OVA and human Cytomegalovirus (CMV) peptides\textsuperscript{265,266}. Antigen presentation in MHC-II was similarly altered upon inhibition of autophagy, with reduced DC-mediated processing of an immunodominant mycobacterial peptide, reduced presentation of herpes simplex virus (HSV) antigens and vaccinia virus Ankara antigens\textsuperscript{267–269}. Consequently, antigen-specific T cell responses were down-regulated. Thus, autophagy inhibition modified the peptide pool presented in MHC and appears to reduce presentation of immunodominant epitopes.

In line with this role of autophagy in presenting appropriate antigenic peptides, knock-out of the autophagy-regulator vsp34 in DCs abrogated the induction of B16 melanoma-specific CTLs \textit{in vivo}, yielding a significantly higher incidence of lung metastases\textsuperscript{270}. In this setting, cross-presentation of OVA peptides derived from apoptotic cells was strongly reduced, although Vps34-deficient DCs did present higher levels of cytoplasmic OVA-derived antigens\textsuperscript{271}. In addition, mice with autophagy-deficient DCs failed to efficiently develop protective Th1 cell immunity and hence died faster upon challenge with a lethal dose of HSV\textsuperscript{268}. Further, starvation-induced autophagy increased loading of intracellular and lysosomal-derived peptides on MHC-II molecules in Human B-lymphoblastoid cells\textsuperscript{272}.

Taken together, most studies highlight that inhibition of autophagy negatively affects MHC-dependent presentation of tumor antigens by APCs and thereby negatively affects T cell immunity (Figure 5B).

3.3.2. Autophagy is required for T cell proliferation, survival and generation of protective T cell memory

Autophagy plays a crucial role in normal T cell functioning as evident from the severe reduction in basal peripheral T cell counts, especially CTLs counts, in autophagy knock out mouse models\textsuperscript{87,262,262–262}. Naïve resting T cells have only minimal numbers of autophagosomes, but T cell receptor (TCR)-mediated activation triggers a strong increase in autophagosome content in activated CD8\textsuperscript{+}CD28\textsuperscript{+} CTLs and in activated helper T cell subsets\textsuperscript{273,283–287}. Induction of autophagy was augmented by T cell co-stimulation with the cytokine IL-2 or by 4-1BB signaling, which was accompanied by an increase in lysosomal content and colocalization of lysosomal LAMP staining with autophagosomal LC-3 staining\textsuperscript{274,285,286–288}.
Moreover, in activated T cells transfected with an LC3-GFP-mCherry construct the LC3-GFP signal was lost, indicative of fusion of autophagosomes with lysosomes. Thus, T cell activation increases autophagosome and lysosome content and increases autophagic flux.

Figure 5: Autophagy contributes to the formation of antigenic-peptides in antigen-presenting cells. Professional antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages, display antigenic peptides in the context of MHC-I or MHC-II molecules to T cells, which will trigger an immune response. Autophagy reduces MHC-I surface levels, which is converted upon autophagy inhibition. However, autophagy is also required for the generation of antigenic peptides. The inhibition of autophagy will therefore skew the peptidome, yielding less diversity in the antigens presented to T cells. Indeed autophagy, inhibition limits T cell activation by APCs.
Chapter 5

The importance of autophagy activation for T cell functioning became apparent upon knock-out of various core autophagy genes and upstream autophagy regulators in T cells. In all cases, this resulted in poor proliferation of T cells upon TCR-activation, which was not improved by addition CD28 or IL-2 co-stimulation\textsuperscript{87,262,278–281,289,290}. Indeed, in T cell specific ATG5 or ATG7 knock-out mouse models a strong decline in reactive CTLs was detected in murine models of lymphocytic choriomeningitis virus (MCMV), influenza virus or mouse cytomegalovirus\textsuperscript{275,291}. Notably, autophagy-deficient T cells did express equal levels of the activation markers CD69 and CD25 upon TCR-stimulation, suggesting that downstream TCR signaling does occur\textsuperscript{87,273,278}. Thus, it currently remains unclear whether the defect in proliferating capacity of autophagy-deficient T cells is on the level of the TCR or more downstream.

In addition to impaired cell proliferation, CTLs from ATG5-KO chimeras were ~50% more apoptotic than control T cells, whereas viability of helper T cells from Beclin-1 KO mice was also strongly reduced\textsuperscript{273,279}. Further, spontaneous apoptosis or apoptosis after TCR-activation in T cells was increased when core autophagy genes and regulators were knocked out\textsuperscript{87,262,275,276,278,280,282}. Indeed, ATG3 and ATG7 KO T cells contained more active caspase-9 indicative of elevated levels of apoptosis, with viability being partly restored upon pan-caspase inhibition\textsuperscript{279,282}. In addition, upon TCR-activation, autophagy-deficient T cells secreted less pro-inflammatory cytokines, i.e. IL-2 and IFN-γ, which may also negatively impact their survival\textsuperscript{277,285}. Taken together, inhibition of autophagy at multiple levels has a negative effect on T cells.

Importantly, autophagy is also pivotal for the development of T cell memory responses, with no protective T cell immunity in ATG5 KO mice upon a rechallenge with influenza. Similarly, ATG7 KO mice vaccinated against MCMV failed to generate a T cell response upon re-infection\textsuperscript{275}. Also the inhibition of CMA in T cells by knock down of LAMP-2A impaired the control of Listeria monocytogenes in mice upon re-challenge\textsuperscript{288}. Of note, residual peripheral T cells detected in autophagy-deficient mouse models displayed a CD44\textsuperscript{high}CD62\textsuperscript{low} phenotype, a phenotype typically associated with effector and effector memory T cells\textsuperscript{87,262,275,282}. Although this finding is in apparent contrast with the impaired induction of T cell memory, a similar ‘memory-like’ phenotype has been reported in lymphopenia in T cell-depleted mice\textsuperscript{292}. Similar to autophagy deficiency, such ‘memory-like’ T cells were in fact incapable of generating effective T cell immune responses. Thus, autophagy is pivotal for normal T cell function and, crucially, for development of memory T cells that provide protective immunity.
3.3.3 Autophagy-dependent degradation of mitochondria and pro-apoptotic proteins maintains T cell homeostasis

The inhibition of autophagy negatively impacts on T cells, likely due to deregulated clearance of organelles and proteins, with transcriptional profiling of ATG5 wildtype vs. KO T cells among others suggesting a key involvement in mitochondrial homeostasis. In line with this analysis, knock-out of ATG5 or other core autophagy genes was associated with a bigger mitochondrial mass compared to wildtype T cells, with a notable exception being Beclin-1 KO. Further, mitochondrial mass increased over time in inducible autophagy KO models and was seen only in ‘aged’ T cells in the periphery. In autophagy-deficient T cells, the increase in mitochondrial mass was accompanied by elevated levels of ROS, whereas activation of autophagy using autophagy-inducer torin-1 reduced ROS levels and increased survival. Correspondingly, treatment of hypoxic ATG5 KO T cells with ROS inhibitor n-acetyl-cysteine (NAC) restored T cell proliferation and prevented cell death.

Autophagy also directly regulated expression of pro- and anti-apoptotic proteins in T cells. For instance, caspase-8 and -3 protein levels were strongly increased in beclin-1 KO T cells, whereas no increase in mRNA levels was detected. Further, activation of autophagy using rapamycin triggered co-localization of LC3 and caspase-3 and was accompanied by down-regulation of caspase-3 levels. Similarly, the level of several other pro-apoptotic proteins like BCL-2, BIM, BCL-xl, Bax, cytochrome C and AIF were increased upon interference with autophagy, although the impact of autophagy inhibition on these proteins varied among studies, possibly related to timing of measurements especially in inducible models.

Finally, autophagy also impacts on several key cell cycle regulators in T cells. For instance, TCR-activation of T cells normally induces the autophagic degradation of cyclin-dependent kinase inhibitor 1B (CDKN1B), an inhibitor of cell cycle progression. Inhibition of autophagy prevented CDKN1B degradation upon TCR-stimulation and, thereby, inhibited proliferation. Similarly, autophagy ensures degradation of BCL-10, a mediator of TCR-to-NF-κB signaling and Itch and Rcan-1, two inhibitors of TCR signaling.

Taken together, the autophagy pathway is important for T cell survival and proliferation as it retains mitochondrial homeostasis and ensures the degradation of pro-apoptotic and anti-proliferative proteins. An interesting exception to this rule was recently reported for a specific T cell subset, the so-called Th9 T cell, which is reported to have potent anticancer immunity. In this case, inhibition of autophagy prevented degradation of PU.1, the master transcription factor for TH9 cells. Hence, inhibition of autophagy enhanced differentiation of helper T cells to Th9 cells.
**3.3.4 The role of autophagy in Treg functioning**

Regulatory T cells (Treg) are a subpopulation of CD4 T cells that inhibit effector T cell responses, with increased Treg infiltration in cancer associating with poor survival [ref]^{297,298}. Compared to naïve CD4\(^+\) cells, Tregs contain more autophagosomes and have higher LC3-II levels indicating the presence of high levels of autophagy^{299}. Knockout of autophagy genes induced apoptosis of Treg cells and blocked Treg-mediated suppression of effector T cell responses. This subsequently yielded higher percentages of tumor infiltrating CTLs and smaller tumors in a mouse model of colon adenocarcinoma^{299}. Further, animals with autophagy-impaired Tregs were more prone to develop autoimmune diseases and adoptive transfer of Tregs from Vps34 KO mice failed to protect against colitis^{262}. Thus, autophagy is important for Treg functioning. Autophagy inhibition may thus alleviate Treg immunosuppressive activity in cancer.

**4. CONCLUSIONS AND PERSPECTIVES**

Autophagy has a multifaceted impact on the cancer micro-environment and is an interesting target for cancer therapy. In established cancers, autophagy acts as a survival mechanism, for example in conditions of elevated nutrient demand or low oxygen availability. Importantly, cancer cells elevate their autophagic flux during treatment to gain resistance toward (chemo) therapy. In addition, tumors activate autophagy in adjacent stromal cells to benefit from cytokines, growth factors and nutrients secreted upon induction of this pathway. Further, although the autophagy pathway is often repressed during early tumorigenesis, every cell requires a basal level of autophagy. Therefore, emerging cancer cells with down-regulated levels of autophagy might be more reliant on remaining autophagy activity.

Interestingly, many types of malignancies have high prevalence in the aging population, while it has been suggested that a reduction in autophagy may be a contributor to the aging process. In line with this, autophagy was decreased in two-third of HSCs of aged mice compared to young mice, with aged HSC with higher autophagy activity having better long-term regenerative potential^{85}. Further, aging of the hematopoietic compartment is associated with myeloid malignancies, with a lineage skewing of HSCs due to upregulation of myeloid-specific genes and down-regulation in lymphoid genes^{300–304}. Intriguingly, similar myeloid skewing was detected upon mono allelic knockout of ATG7 or ATG12 in mice, which associated with development of myeloproliferative syndrome^{80,85}. Interestingly, treatment with autophagy inducer rapamycin also enhanced influenza specific CD8\(^+\) T cells responses in aged vaccinated mice, but not in aged mice with ATG7 KO^{275,305,306}. Thus, reduced autophagy...
during ageing may increase susceptibility to tumorigenesis as well as negatively impact on T cell immunity. In this respect, activation of autophagy may even be a strategy for rejuvenation, with treatment of mice with the autophagy inducer rapamycin extending their lifespan307.

Based on the evidence reviewed here, the inhibition of autophagy in not only cancer cells but also in cancer-supporting stromal cells may represent a potentially promising strategy to increase cancer cell death, especially in combination with other therapeutic approaches. Indeed, several phase I/II clinical trials are currently being conducted in various cancers with established autophagy inhibitors, such as 3-MA, CQ or HCQ (Table 1). On the other hand, an effective anti-cancer immune response appears to require autophagy at multiple levels. For instance, autophagy promotes the generation of antigenic-peptides to be presented in MHC-I and MHC-II molecules on APCs. Further, T cells rely on an active autophagy pathway for their proliferation and survival. Hence, systemic application of autophagy inhibitors would likely inhibit anti-cancer immune responses. Indeed, the in vitro treatment of T cells with chloroquine reduced T cell-dependent cell lysis, inhibited T cell proliferation and reduced cytokine secretion308. Further, accumulation of autophagic vacuoles was observed in PBMCs of patients treated with HCQ, suggesting inhibition of autophagy occurred in these immune cells21–23,309. In line with this, the inhibition of autophagy was associated with lymphopenia in 31% of the patients in the temsirolimus study, whereas mTOR inhibitor everolimus negatively affected multiple immune cell subsets in renal carcinoma patients21,22. Thus, autophagy inhibition likely occurs with current therapeutics, but should be avoided in cancer-associated immune cells.

Of note, in a phase II study in glioblastoma patients 600 mg/d HCQ was found to be the maximum tolerated dose, a dose at which autophagy inhibition was not consistently achieved in the tumor310. Doses used in other early clinical cancer studies range from 800-1000 mg/d (Table 1). Thus, the therapeutic window for the clinical use of CQ seems to be quite small. Indeed, as also recently reported by us, there is only a small therapeutic window of autophagy inhibition with HCQ between CD34+ AML cells and healthy normal bone marrow derived CD34+ cells (Figure 3D)106. Moreover, current autophagy inhibitors such as CQ have a poor biodistribution profile, with levels of CQ in the blood being much higher than in the tumor311.

Taken together, it is clear that development of therapeutic strategies that inhibit autophagy more selectively in cancer cells appears warranted. Here to, the development of novel autophagy inhibitors that have an increased activity profile in vivo, with limited cytotoxicity may help improve the therapeutic window for autophagy inhibition. For instance Lys05, a bisaminoquinoline and synthetic derivative of CQ, was ten-fold more potent than HCQ through better accumulation and deacidification of lysosomes and was effective in inhibiting autophagy in xenograft studies312. Another inhibitor of interest is ARN5187,
### Table 1

<table>
<thead>
<tr>
<th>Malignancy</th>
<th>Patient numbers</th>
<th>Treatment</th>
<th>Response</th>
<th>Autophagic response</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastroesophageal</td>
<td>27</td>
<td>Vandetanib (HDAC) + HCQ</td>
<td>Dose-limiting toxicity: 800mg/day HCQ</td>
<td>Autophagic changes: no significant changes in AV accumulation at day 49, IHC revealed increased MAP1LC3B at day 49</td>
<td>Mahalingam et al., Combined autophagy and HDAC inhibition. Autophagy. 2014</td>
</tr>
<tr>
<td>Melanoma</td>
<td>37</td>
<td>Temozolomide + HCQ</td>
<td>Dose-limiting toxicity: 1200mg/day HCQ</td>
<td>Autophagic changes: with 1200mg/day HCQ significant AV accumulation at 6 weeks, increased AVs with temozolomide + HCQ compared to single HCQ treatment</td>
<td>Ranevski et al., Combined MIF and autophagy inhibition. Autophagy. 2014</td>
</tr>
<tr>
<td>Glioblastoma multiforme</td>
<td>16</td>
<td>Radiation therapy + Temozolomide + HCQ</td>
<td>Dose-limiting toxicity: 800mg/day HCQ</td>
<td>Autophagic changes: increased AVs/cell and LC3-II/I at 3 weeks</td>
<td>Rosenfeld et al., A phase II trial of HCQ in conjunction with radiation therapy and concurrent and adjuvant temozolomide in patients with newly diagnosed glioblastoma multiforme. Autophagy. 2014</td>
</tr>
</tbody>
</table>

AV = autophagic vacuole; DFS = disease-free survival; HCQ = hydroxychloroquine; IHC = immunohistochemistry; MR = minor response; OS = overall survival; PR = partial response; SD = stable disease; VGPR = very good partial response.
### Serum levels of M. paratuberculosis

<table>
<thead>
<tr>
<th>Study</th>
<th>Serum Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study A</td>
<td>100 ng/mL</td>
</tr>
<tr>
<td>Study B</td>
<td>50 ng/mL</td>
</tr>
<tr>
<td>Study C</td>
<td>150 ng/mL</td>
</tr>
</tbody>
</table>

### References

1. Scott et al., Double autophagy stimulation using chemotherapy and mTOR inhibition combined with HCQ for autophagy modulation in patients with relapsed or refractory MM. Haematologica. 2017
2. Chi et al., Double autophagy modulators reduce 2-deoxyglucose uptake in sarcoma patients. Oncotarget. 2015
3. Nti et al., Frequent subclinical macular changes in combined BRAF/MEK inhibition with high dose HCQ in treatment for advanced metastatic BRAF mutant melanoma. Retina. 2017
5. Mahalingam et al., Combined autophagy and HDAC inhibition using HCQ in Patients With Metastatic Pancreatic Adenocarcinoma. The Oncologist. 2014
6. Scott et al., Double autophagy stimulation using chemotherapy and mTOR inhibition combined with HCQ for autophagy modulation in patients with relapsed or refractory MM. Haematologica. 2017
7. Chi et al., Double autophagy modulators reduce 2-deoxyglucose uptake in sarcoma patients. Oncotarget. 2015
8. Nti et al., Frequent subclinical macular changes in combined BRAF/MEK inhibition with high dose HCQ in treatment for advanced metastatic BRAF mutant melanoma. Retina. 2017
9. Scott et al., Double autophagy stimulation using chemotherapy and mTOR inhibition combined with HCQ for autophagy modulation in patients with relapsed or refractory MM. Haematologica. 2017
10. Chi et al., Double autophagy modulators reduce 2-deoxyglucose uptake in sarcoma patients. Oncotarget. 2015
11. Nti et al., Frequent subclinical macular changes in combined BRAF/MEK inhibition with high dose HCQ in treatment for advanced metastatic BRAF mutant melanoma. Retina. 2017
which blocks the final step of autophagolysosome maturation, with superior cytotoxic activity over HCQ in various cancer cell lines\textsuperscript{311}. Several other approaches to inhibit autophagy that target upstream regulatory events are currently under investigation in leukemia and solid cancer treatment. These include the PI3K inhibitor buparlisib, which selectively targets Vps34\textsuperscript{314}. First promising results in phase I single agent studies were observed in solid cancers as well as advanced leukemias, although toxicity was detected\textsuperscript{315,316}. Other upstream targets being investigated include ULK\textsuperscript{317–319}. One of the more potent inhibitors of ULK1, SBI-0206965, had prominent anti-cancer activity in human non-small cell lung cancer and adenocarcinom\textsuperscript{319,320}. All of these new autophagy-targeted drugs are in early stages, with future clinical trials being awaited to learn on toxic effects and potential efficacy.

Further, increasing the tumor-selectivity of autophagy drugs can be pursued, e.g. by use of liposomal formulations. Such formulations have been widely used to enhance drug retention and alter biodistribution by passive or active targeting. In this respect, encapsulation of chemotherapeutic drugs such as doxorubicin in liposomes has yielded more effective formulations with less side-effects\textsuperscript{321}. Such formulations can be further optimized by using antibody-conjugated and tumor-targeted liposomes\textsuperscript{322}. Of note, chloroquine-liposomes have already been generated in the context of CQ as malaria drug and were also suitable to simultaneously deliver CQ and a tumoricidal drug\textsuperscript{323–328}. The co-delivery of CQ and doxorubicin improved the anti-tumor activity compared to liposomal-doxorubicin\textsuperscript{328}. Alternatively, drugs such as CQ may be directly targeted to cancer cells using an antibody-drug conjugates\textsuperscript{329}.

Future studies will have to determine whether selective targeting of cancer cells can prevent the adverse effect of inhibition of anti-cancer immunity. Concerning in this respect is the reported upregulation of PD-L1 upon inhibition of autophagy, which serves to activate this important immune checkpoint in cancer. If further proven, combined treatment with autophagy and PD-1/PD-L1 checkpoint inhibitors may prove a straight-forward approach to circumvent this issue. Nevertheless, inhibition of autophagy also alters the composition of peptides presented in MHC and thus may still impact on cancer immunity. Studies that elucidate this potential effect of autophagy inhibition in more detail are urgently needed to steer design of future clinical studies.

As it evident that autophagy can steer the pool of antigenic peptides that are presented in MHC-I and MHC-II on the cell surface, attempts have been made to exploit autophagy for the purpose of therapeutic vaccination. For instance, fusion of NY-ESO-1, a cancer testis antigen frequently overexpressed in melanoma, to LC-3 resulted in autophagosome targeting and augmented NY-ESO-1-specific anti-melanoma helper T cell responses\textsuperscript{330}. An additional approach to exploit autophagy for induction of immunity is through autophagosome-based
vaccination. Here, autophagosomes are isolated from cancer cells treated with a proteasome inhibitor. Such autophagosomes contain tumor-associated antigens and on the surface express CLEC9A ligands that facilitate endocytosis by APCs. DCs pulsed with such autophagosomes load content into MHC-II or MHC-I and were more efficient in inducing OVA-specific T cell responses compared to soluble protein. This autophagosome vaccination approach reduced B16F10 melanoma cell growth, eliminated 3LL Lewis lung tumors and protected mice from a challenge with sarcoma. Of note, tumor cells may also release autophagosomes themselves and modulation of autophagy could therefore trigger release of autophagosomes from tumor cells and positively impact on anticancer T cell immune responses.

Of note, an important issue that needs to be addressed for any new type of therapy to enter clinical practice is the identification of appropriate patient stratification criteria. In this respect, current clinical trials do not have inclusion/exclusion criteria that take autophagic activity in the tumor into account. Further, appropriate testing of autophagy inhibition in patients will have to be developed, with current trials mainly monitoring autophagy in peripheral blood mononuclear cells as a surrogate marker of response or in tumor biopsies. However, the level of autophagy in PBMCs does not seem to correlate with autophagy inhibition in the tumor micro-environment. Therefore, PET/CT and MRI probes for ATG activity or currently being developed. Thus, research in the upcoming years should focus on not only identifying optimal inhibitors of autophagy in patients, but also on the identification of appropriate patient selection criteria and monitoring tools in order to position autophagy targeting for clinical use.

In conclusion, a host of evidence has emerged on the importance of autophagy in cancer cells and its validity as target for this disease. However, the various cell types in the tumor micro-environment differ in their reliance on autophagy, making it hard to predict the exact outcome of autophagy inhibition in cancer. Further detailed investigations into the specific impact in this complex milieu are needed to steer rational design of therapeutic targeting autophagy in specific cancer subtypes and combination strategies.

ACKNOWLEDGEMENTS

The authors want to acknowledge Dr. Marco de Bruyn for his help with the figures.
5. REFERENCES


55. Vives-Bauza C, Zhou C, Huang Y, et al. PINK1-dependent recruitment of Parkin to mitochondria in...
Chapter 5


84. Lemasters JJ. Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. Rejuvenation Res 2005;8:3–5.


237. Garrido F, Aptsiauri N, Doorduijn EM, Garcia Lora AM, van Hall T.
Autophagy in the cancer micro-environment


261. Loi M, Müller A, Steinbach K, et al. Macroautophagy Proteins Control MHC Class I Levels on Dendritic Cells and Shape Anti-viral


