Chapter 3

Autophagy Regulation of the Tumor Immunity – An Old Machinery for a New Function

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Additional information is available at the end of the chapter

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Abstract

Cancer was initially thought to be just a disease of cells with deregulated gene expression. It may be more accurate to consider cancer as a disease of the microenvironment. Despite the remarkable and fairly rapid progress over the past two decades regarding the role of the microenvironment in cancer biology and treatment, our understanding of its actual contribution to cancer resistance is still poor and fragmented. Nevertheless, the microenvironment is now considered to be of critical importance during the initiation and progression of carcinogenesis since it is involved in shaping and remodeling stroma reactivity and in reprogramming phenotypic and functional plasticity. Therefore, the tumor microenvironment represents an important hallmark of cancer, and the challenge now is to better understand how the tumor microenvironment participates in the emergence of immune-resistant tumor cell variants, which appears to be the greatest impediment to successful immunotherapy. In this context, autophagy has recently emerged as a new player in regulating the antitumor immune response under hostile tumor microenvironment. In this review, we will summarize recent data describing how autophagy activation under hypoxic stress impairs the antitumor immune response. In addition, we will discuss how tumor manages to hide from the immune attack and either mounts a "counter-attack" or develops resistance to immune cells. In particular, we will focus on the effect of hypoxia-induced autophagy in allowing tumor cells to outmaneuver an effective immune response and escape from immunosurveillance. It is our belief that autophagy may represent a conceptual realm for new immunotherapeutic strategies aiming to block immune escape and therefore providing rational approach to future tumor immunotherapy design.

Keywords: Autophagy, hypoxia, tumor immunity, immunotherapy, immune surveillance

1. Introduction

In addition to malignant cells, tumors contain cells of the immune system, the tumor vasculature, and lymphatics as well as fibroblasts, pericytes, and adipocytes. Cells of the immune system can identify and destroy tumor cells in a process termed cancer immunosurveillance.

Several types of immune cells are involved in tumor immune surveillance. Briefly, key cells of the adaptive immune system identifying cancer cells are cytotoxic T lymphocytes (CTL), which are able to recognize tumor antigens via the T-cell receptor (TCR) [1]. Some of these antigens are expressed exclusively by tumors and thus are called tumor-specific antigens [1]. Natural killer (NK) cells of the innate immune system also play an important role in tumor immune surveillance [1] by mechanisms called "missing-self" and "induced-self" recognitions [2]. In addition to CTL and NK cells, macrophages and neutrophil granulocytes are also involved in antitumor immunity [3]. Macrophages are antigen presenting cells (APCs) that display tumor antigens and stimulate other immune cells such as CTL, NK cells, and other APCs [4]. While the molecular mechanism by which CTL and NK cells recognize their target tumor cells is fundamentally different, both immune cells kill their target following the establishment of immunological synapse (IS) [5]. The formation of IS requires cell polarization and extensive remodeling of the actin cytoskeleton at various stages [6]. It is now well established that CTL and NK cells recognize and kill target cells by two major pathways: either through the release of cytotoxic granules containing perforin and granzymes to the cytosol of target cells [7] or through tumor necrosis factor (TNF) super family-dependent killing [8].

Although various immune effector cells are recruited to the tumor site, their antitumor functions are largely downregulated in response to several microenvironmental factors. Indeed, hypoxic stress in the tumor microenvironment, which is the result of an inadequate oxygen supply to the cells and tissues, is a characteristic feature of locally advanced solid tumors and is considered as the major mechanism responsible for tumor resistance to therapies [9].

Experimental and clinical evidence indicates that the majority of mechanisms suppressing the antitumor immune functions are directly evolved in the hypoxic tumor microenvironment (reviewed in [10]). Thus, NK cells and natural killer T (NKT) cells infiltrate the tumor microenvironment but are not found in contact with tumor cells [11]. It has been reported that in colorectal, gastric, lung, renal, and liver cancer NK cells appear to predict a good prognosis [12]. However, although they are present in the tumor microenvironment, NK cells may not be able to exert their tumor-killing function. A number of studies reported that NK cells in the tumor stroma have an anergic phenotype that is induced by malignant cell-derived transforming growth factor beta (TGF-β) [13]. Furthermore, immune cells in the tumor microenvironment not only fail to exercise antitumor effector functions but also co-opted to promote tumor growth [14]. In addition, it has become clear that the immune system not only protects the host against tumor development but also sculpts the immunogenic phenotype of a developing tumor and can favor the emergence of resistant tumor cell variants [15]. Thus, it has become obvious that the evasion of immunosurveillance by tumor cells is under the control of the tumor microenvironment complexity and plasticity. Reactivating the immune system for therapeutic benefit in cancer has therefore long been a goal in cancer immunotherapy.

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After decades of disappointment, cancer immunotherapy has recently emerged as a promising treatment of several cancers for which conventional therapies have failed [16]. Notably, the success of the recent proof-of-concept clinical trials of anticytotoxic T-lymphocyte-associated protein 4 (anti-CTLA4) and anti-programmed cell death 1 (anti-PD-1) based on reactivating the adaptive immune response claims that the tide has finally changed. This success is mainly attributed to an increase in our understanding of the mechanisms regulating tumor cell cytotoxicity mediated by immune cells. For several years, our group has been able to participate in this understanding by studying the mechanism responsible for the tumor escape from the immune surveillance [17, 18], which still represents the major obstacle for defining efficient cancer immunotherapeutic approaches. Therefore, it remains important to better understand how tumor cells manage to outmaneuver the immune system and evade effective immunosurveillance.

2. Hypoxic stress in the tumor microenvironment

Hypoxia in the tumor microenvironment commonly refers to a condition in tumors where the pressure of oxygen is lower than 5–10 mm Hg. The adaption of tumors to hypoxic stress is regulated by hypoxia inducible factor family of transcription factors (HIFs). It has been demonstrated in a large number of human cancer cases and/or incidents that HIFs were overexpressed and such overexpression is associated with poor response to treatment [19]. Moreover, evidence showed a clear positive correlation between enhanced hypoxic expression of HIFs and mortality [20].

2.1. Hypoxia-inducible factors

Three isoforms of HIF have been identified: HIF-1, HIF-2, and HIF-3. HIF-1 and HIF-2 (also known as EPAS1) have the same structure and are well characterized. However, HIF-3 acts as a negative regulator of HIF-1 and HIF-2 [21]. HIF-1 is ubiquitously expressed in all mammalian cells, whereas HIF-2 and HIF-3 are selectively expressed in certain tissues such as vascular endothelial cells, type II pneumocytes, renal interstitial cells, liver parenchymal cells, and cells of the myeloid lineage [22].

HIF-1 is a heterodimer composed of a constitutively expressed subunit and an O_2-regulated subunit, HIF-1β, and HIF-1α, respectively [23]. In the presence of oxygen, HIF-1α is hydroxylated on a proline residue by prolyl hydroxylase domain protein 2 (PHD2), which leads to an interaction with the von Hippel–Lindau (VHL) protein [24]. This allows the recruitment of an E3 ubiquitin ligase that catalyzes the polyubiquitination of HIF-1α and its subsequent degradation by ubiquitin proteasome system (UPS) [24]. Under hypoxia, the hydroxylation of HIF-1α is inhibited, and HIF-1α is accumulated and translocated to the nucleus where it forms a dimer with HIF-1β and activates the transcription of several genes involved in many biological processes [24]. Similar to HIF-1α, HIF-2α is also regulated by oxygen-dependent hydroxylation. While the effect of hypoxia on suppressing the activity of immune cells is relatively well defined, the mechanisms by which hypoxia educates tumor cells to escape an effective immune cell mediated killing are still largely elusive.

2.2. Hypoxia-induced autophagy

Although autophagy can be activated in response to different stimuli, including nutrient starvation and/or growth factors withdrawal, hypoxic stress is the major activator of autophagy in the tumor microenvironment [25]. Indeed, emerging recent data have showed that hypoxia-induced autophagy is an important regulator of the innate and adaptive tumor immunity mediated by NK cells and CTL, respectively. In particular, hypoxia has been described to play a central role in activating multiple overlapping adaptive mechanisms involving autophagy and leading to the emergence of resistant tumor cells able to outmaneuver an effective immune response and escape from immune cell killing. In this context, we have recently showed that the activation of autophagy in tumor cells under hypoxia dramatically decreases tumor cell susceptibility to NK- and CTL-mediated lysis [17, 26]. Therefore, autophagy activation is considered to be an important adaptive and resistance mechanism operating in tumor cells to escape the immune system. In accordance with such a role of autophagy, Lotze *et al.* showed that NK cells along with human peripheral blood lymphocytes are primary mediators in inducing autophagy in several human tumors promoting cancer cell survival [27]. Other studies showed that autophagy also plays an important role in regulating CTL-mediated antitumor immune response. While the molecular mechanisms by which autophagy impairs tumor susceptibility to NK and CTL are different, experimental evidence claims that blocking autophagy may improve tumor immunity.

Autophagy is a lysosomal degradation pathway that allows the cell to self-digest its own components, getting rid of excessive or damaged organelles and misfolded proteins in the cell. Such degradation process provides nutrients to maintain crucial cellular functions under nutrient deprivation, thus allowing the survival of cancer cells [28]. It has been reported that the activation of autophagy under hypoxic stress in tumor cells occurs either by HIF-1-dependent or -independent manner. In this section, we will briefly describe the different mechanisms involved in the activation of autophagy.

2.2.1. HIF-1-dependent activation of autophagy

Under hypoxia, HIF-1α is stabilized, and its heterodimerization with HIF-1β allows the binding of the transcription factor to hypoxia response elements (HREs) in target genes [9]. HRE is a *cis*-acting hypoxia response element (5′-TACGTGCT-3′), which can be located in either the 5′ or the 3′ regions of the genes [29] to confer oxygen regulation of genes expression [29–32]. The activation of HIF-1α-dependent autophagy occurs via the induction of the Bcl-2 (B-cell lymphoma 2)/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), which contains two HRE sites in its promoter region: HRE1 and HRE2. It has been demonstrated that HIF-1 directly binds to HRE2 site in order to induce the expression of BNIP3 [33], thus leading to the disruption of the autophagy inhibitory complex BECN1/Bcl-2 and the subsequent release of Beclin1 (BECN1) to promote the activation of autophagy.

2.2.2. HIF-1-independent activation of autophagy

Despite the role of HIF-1 in the regulation of autophagy under hypoxic conditions, it remains important to note the existence of other pathways that may regulate autophagy under hypoxia independently of HIF-1. Recently, two pathways that influence gene expression and tumor

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cell behavior have been described to be O_2 sensitive [34]. One of them occurs through the regulation of the mammalian target of rapamycin (mTOR) and its downstream effectors that orchestrate several biological processes, including autophagy. Indeed, mTOR signaling consists of two major pathways mediated by the specific mTOR complexes mTORC1 and mTORC2. It has been reported that mTORC1 negatively controls autophagy by the inhibition of protein kinase ATG1 involved in the formation of autophagosomes [35]. Hypoxia inhibits mTORC1 through multiple pathways; one of them is mediated through hypoxic activation of the tuberous sclerosis protein (TSC) complex, which is a heterodimeric complex formed by TSC1 and TSC2 [36]. The 5′ AMP-activated protein kinase (AMPK) is a heterotrimeric complex encoded by several genes and is the primary energy sensor in cells. Upon its activation through the increase in the AMP/ATP ratio, AMPK phosphorylates many downstream targets, including TSC2. TSC2 phosphorylation on serine residues 1270 and 1388 enhances the activity of the TSC1/TSC2 complex and thereby blocks the Ras homolog enriched in brain (RHEB)-dependent activation of mTOR [37]. Another pathway that activates autophagy in an HIF-1-independent manner is through the activation of the unfolded protein response (UPR), a program of transcriptional and translational changes that occur as a consequence of endoplasmic reticulum (ER) stress [38]. The UPR is mediated by three ER stress sensors: PKR-like ER kinase (PERK), ER to nucleus signaling 1 (ERN1), and activating transcription factor (ATF) 6 [38]. In some conditions, autophagy appears to be mediated by PERK, whereas in others, it occurs downstream of IRE1. For example, by inducing PERK-dependent phosphorylation of the eukaryotic translation initiation factor 2 alpha (elF2α), hypoxia activates autophagy by the transcriptional induction of LC3 through the expression of ATF4 [39].

2.2.3. Autophagy activation under nutrient starvation

Under starvation condition, autophagy is activated by several mechanisms. One of the well described mechanisms is the regulation of autophagy by Reactive Oxygen Species (ROS) which mainly comprise superoxide (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH). Several studies have demonstrated that ROS-dependent activation of AMPK leads to the inhibition of mTOR pathway under starvation conditions, thereby activating autophagy [40].

Furthermore, recent studies showed that the upregulation of NS5ATP9 previously identified as p15PAF [proliferating cell nuclear antigen (PCNA)-associated factor] in starved HepG2 cells plays a functional role in starvation-induced autophagy and contributes to tumor cell growth. NS5ATP9 promotes autophagy by Beclin1-dependent manner under starvation condition. Indeed NS5ATP9 upregulates Beclin-1 expression at the transcriptional level, thereby inducing autophagy [41]. Another mechanism of autophagy activation under starvation conditions is MK2/MK3-dependent Beclin1 phosphorylation. MK2/MK3 are two related stress-responsive kinases members of p38 mitogen-activated protein kinase (MAPK) signaling pathway. Yobgjie Wei *et al.* showed that MK2/MK3 phosphorylate Beclin1 at serine 90, and this phosphorylation is essential for autophagy induction in response to nutrient starvation [42].

It has been reported in several studies that some microRNAs (miRNAs) are able to regulate starvation-induced autophagy. Mature miRNAs are a class of noncoding RNAs that play key roles in the regulation of gene expression by acting at the posttranscriptional level. miRNAs are short, single-stranded RNA molecules ~22 nucleotides in length. They are partially complementary to one or more messenger RNA (mRNA) molecules. By base pairing with

sequences found mainly in the 3 untranslated region (3′ UTR) of specific mRNAs, miRNAs downregulate gene expression by different manners, including translational repression. It has been shown in several studies that some miRNAs are able to regulate starvation-induced autophagy. Thus, miR376A and miR376B have been identified as regulators of autophagy under starvation condition by blocking the expression of the two key autophagy proteins ATG4C and BECN1. The inhibition of ATG4C and BECN1 by miR376A is achieved by directly affecting specific MRE (miRNA response elements) sequences in 3′ UTR region. Another autophagy-related miRNA miR181A was shown to regulate starvation-induced autophagy by regulating ATG5 level containing a MRE on its 3′ UTR region. This regulation was observed in MCF-7 breast cancer cells as well as in Huh-7 liver cancer and K562 chronic myelocytic leukemia cell lines [43].

3. Hypoxia-induced autophagy as major regulator of the antitumor immunity

Several lines of evidence highlight that hypoxia modulates both the activity of immune effectors and the response of tumor cells to these effectors. In the following section, we will summarize the effect of hypoxia-induced autophagy on the antitumor immune response mediated by CTL and NK cells. Furthermore, we will discuss how autophagy activation regulates tumor cell plasticity and leads to the emergence of resistant tumor cells able to outmaneuver an effective immune response and escape from immune cell killing.

3.1. Hypoxia-induced autophagy impairs CTL-mediated tumor cell killing

Autophagy activation not only enables tumor cells to survive stress conditions during cancer development but also provides them an intrinsic resistance mechanism against antitumor immune response. The first evidence for such a role of autophagy was provided by Noman *et al.* who demonstrated that hypoxic lung carcinoma cells can evade CTL-mediated lysis through autophagy induction [26, 44] (Figure 1). Indeed, the inhibition of autophagy using small interfering RNA (siRNA) directed against ATG5 or BECN1 restored tumor cells sensibility to CTL-mediated lysis. This was correlated with a decrease in the hypoxia-dependent induction of the phosphorylation of signal transducer and activator of transcription (STAT)-3. These results allowed the prediction that blocking autophagy would suppress pSTAT3-dependent survival mechanism making tumor cells more susceptible to CTL attack under hypoxia. Considering the degradation role of autophagy, it is difficult, however, to perceive that autophagy is involved in the stabilization of pSTAT3 under hypoxia. Focusing on the crosstalk between the adaptor protein sequestosome1 (SQSTM1/p62), UPS and autophagy, this study revealed that the induction of HIF-1α has two effects in tumor cells: (i) HIF-1α triggers the phosphorylation of Src, which subsequently phosphorylates the tyrosine residue Y705 of STAT3; (ii) HIF-1α activates autophagy by a mechanism involving the increased expression of BNIP3/BNIP3L and the dissociation of the BECN1/Bcl-2 complex. Autophagy activation results in the degradation of the p62 protein. Knowing that p62 is the receptor/adaptor protein responsible for targeting pSTAT3 to the UPS, the autophagy-dependent degradation of p62 leads to the accumulation of pSTAT3. When autophagy is inhibited in tumor cells, the

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degradation of p62 is blocked and therefore p62 accumulates in tumor cells. This accumulation accelerates the UPS-dependent degradation of pSTAT3 [26, 44]. The effect of the autophagy inhibitor hydroxychloroquine (HCQ) was also evaluated *in vivo* in combination with a tyrosinase-related protein-2 (TRP2) peptide-based vaccination strategy. Using a transplantable murine melanoma B16-F10 cell line, evidence has been provided that autophagy is primarily detected in hypoxic areas of the tumor. Inhibition of autophagy in B16-F10 engrafted tumors results in a significant decrease in tumor growth by inducing apoptosis, as revealed by TUNEL staining. These results strongly argue for a role of autophagy in mediating hypoxia tolerance to the immune system. More interestingly, a significant decrease in tumor growth was observed in vaccinated and HCQ-treated group of mice as compared to control and to treatment alone. Although the subcutaneous tumor implantation models have their limits, these results strongly argue that *in vivo* inhibition of autophagy improves the antitumor effect of a TRP2-based vaccine.

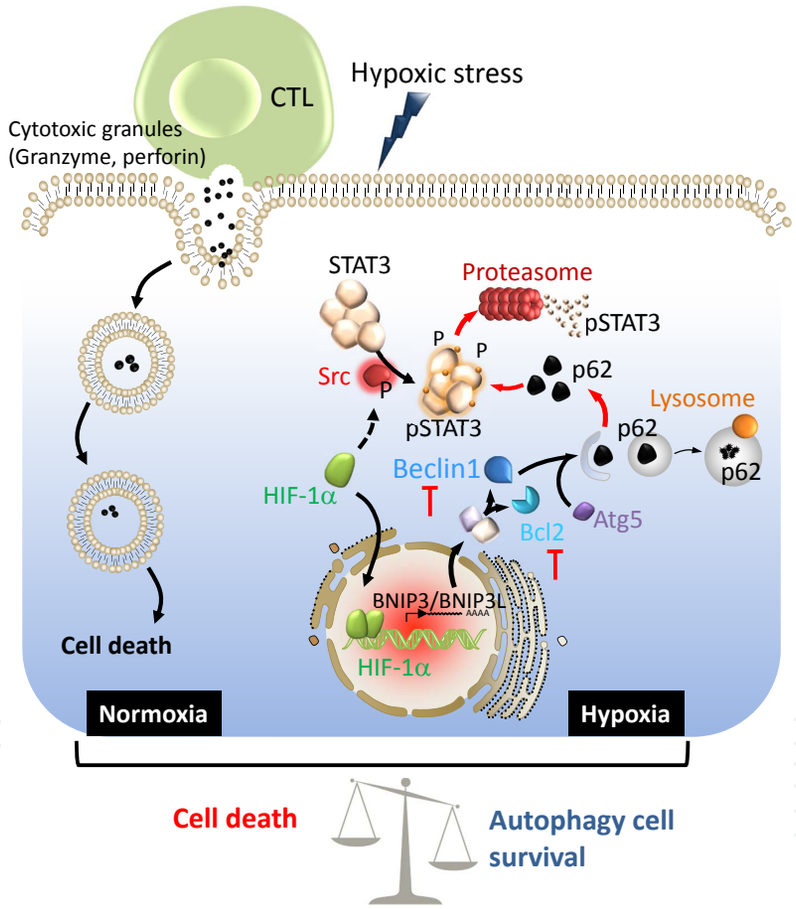

Figure 1. Regulation of CTL-mediated tumor cell lysis by hypoxia-induced autophagy. Hypoxic stress leads to the accumulation of HIF-1α. By a yet undefined mechanism, HIF-1α increases the level of phospho-Src, which subsequently phosphorylates STAT3 at the Tyr705 residue. As HIF-target gene products, BNIP3 and BNIP3L are transcriptionally upregulated and compete with the BECN1-BCL2 complex. This competition releases BECN1 from the complex and then activates the autophagic machinery by recruiting several autophagic proteins, including ATG5. As an autophagic substrate, p62/SQSTM1 is degraded in the autophagosomes following their fusion with lysosomes. In view of the fact that p62/SQSTM1 is involved in targeting pSTAT3 to the UPS, its degradation leads to the accumulation of pSTAT3 in cells. In autophagy-defective cells, p62/SQSTM1 is no longer degraded, and its accumulation accelerates the UPS-dependent degradation of pSTAT3.

3.2. Hypoxia-induced autophagy impairs NK-mediated antitumor immune response

Recent evidence described how tumor cells can escape fully functional NK-mediated immune surveillance by activating autophagy under hypoxia [17, 45] (Figure 2). Indeed, NK cells recognize and kill their targets by several mechanisms, including the release of cytotoxic granules containing perforin (PRF1) and serine protease granzyme B (GZMB). It has been recently proposed that PRF1 and GZMB enter target cells by endocytosis and traffic to large endosomes named "gigantosomes" [17, 45]. Subsequently, PRF1 is involved in the formation of pores in the membrane of the "gigantosome," leading to the gradual release of GZMB and the initiation of apoptotic cell death. The formation of amphisomes following the fusion between autophagic vacuoles and early endosomes appears to be necessary in some cases for the generation of autolysosomes. In this report [17], the authors described that the proapoptotic protein GZMB is selectively degraded upon autophagy activation in hypoxic cells thereby inhibiting NK-mediated target cell apoptosis.

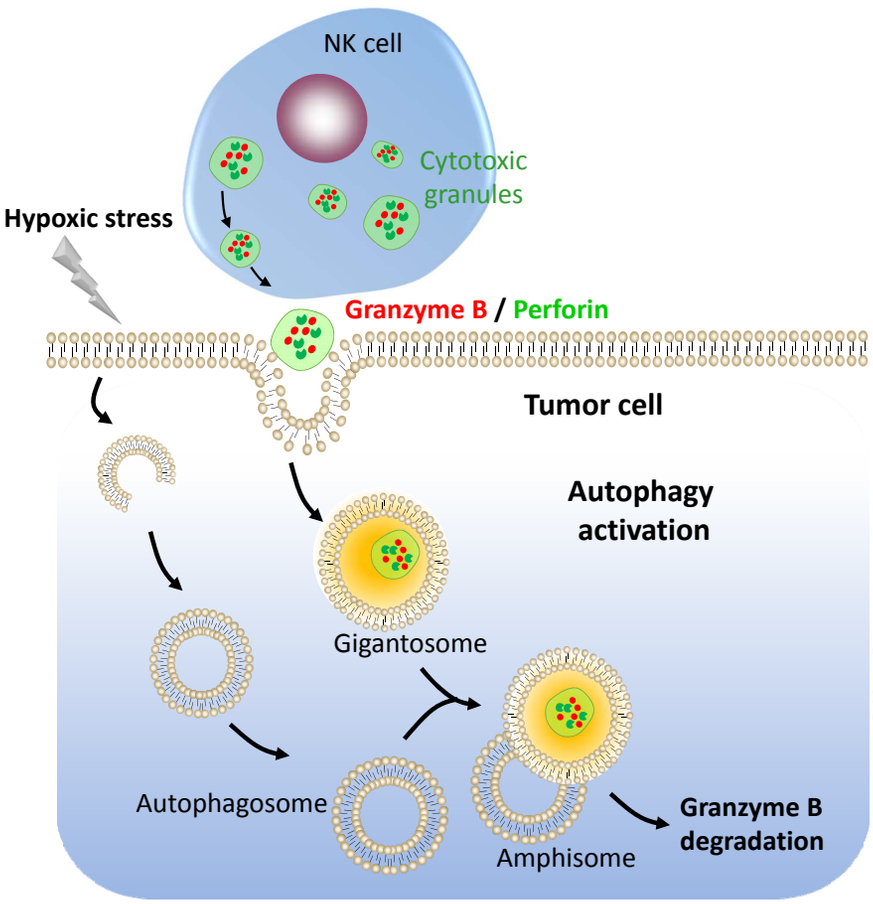

Figure 2. Regulation of NK-mediated tumor cell lysis through selective degradation of NK-derived GZMB by autophagy in hypoxic tumor cells. Following the recognition of their targets NK cells secrete cytotoxic granules containing perforin, granzyme B, and other hydrolytic enzymes to the target cells. These granules enter target cells and traffic to enlarged endosomes called "gigantosomes." Under hypoxia, excessive autophagy in target cells leads to the fusion of autophagosomes with gigantosomes containing perforin and granzyme B and the formation of amphisomes. The selective degradation of granzyme B by autophagy leads to hypoxic tumor cell escape from NK-mediated killing.

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In light of the *in vitro* observations, they investigated whether targeting autophagy enhances *in vivo* NK-mediated antitumor immune response. BALB/c and C57BL/6 mice were transplanted with syngeneic murine 4T1 breast adenocarcinoma and B16-F10 melanoma tumor cells, respectively. They first demonstrated that NK cells control *in vivo* B16-F10 and 4T1 tumor development as the depletion of host NK cells significantly increases tumor growth. There is a significant decrease of autophagy-defective B16-F10 and 4T1 tumors volumes presumably as a consequence of potentiation of tumor cell killing by NK cells. Overall, this study underlines the inhibition of autophagy as a cutting-edge approach to overcome the suppressive effect of the hypoxic tumor microenvironment on the antitumor immune response.

More recently, the role of autophagy in regulating the NK-mediated immune response was extended to other tumor models. The clear cell renal cell carcinoma (ccRCC) is frequently associated with tumor suppressor VHL gene mutations. Such mutations lead to the stabilization and accumulation of HIF-1α and HIF-2α and their target genes (Figure 3) Using VHL-mutated-786-O renal carcinoma cells, it has been reported that the subsequent stabilization of HIF-2α was strikingly associated with the resistance of 786-O cells to NK-mediated lysis. Targeting HIF-2α or reconstitution of wild-type VHL in 786-O cells (hereafter referred to as WT-7 cells) significantly decreased the level of HIF-2α and restored the resistance of 786-O cells to NK-mediated lysis. These results highlight the critical role of HIF-2α in activating an intrinsic mechanism that makes renal cell carcinoma (RCC) less sensitive to NK cell attack. To gain further insight into the mechanism by which HIF-2α regulates RCC susceptibility to NK-mediated lysis, global gene expression profiling was performed on control and siRNA-HIF-2α-transfected 786-O cells. The result showed that the gene inositol 1,4,5-triphosphate receptor, type I (ITPR1) was overexpressed in 786-O as compared to HIF-2α-defective cells. Interestingly, targeting ITPR1 in 786-O was sufficient to dramatically restore NK-mediated lysis of these cells. These findings predict that the accumulation of HIF-2α in VHL-mutated 786-O cells leads to the overexpression of ITPR1 which subsequently alters the susceptibility to NK cell attack. Chromatin immunoprecipitation experiment further showed an HIF-2α enrichment of the ITPR1 promoter fragment containing HRE-7 in 786-O compared to WT-7 cells indicating that ITPR1 is a direct target of HIF-2α. Interestingly, immunochemistry analysis showed a positive correlation between ITPR1 and HIF-2α expression in RCC patients. They next analyzed whether the accumulation of ITPR1 in 786-O cells was associated with the induction of autophagy. The authors were not able to detect any difference in the activation of autophagy in VHL-mutated 786-O and VHL-corrected WT-7 cells cultured without NK effectors. However, when co-cultured with NK cells, only VHL-mutated 786-O cells were able to activate autophagy. These data strongly argue that the expression of ITPR1 is prerequisite for the induction of autophagy in RCC by a signal derived from NK cells. This was further supported by our data showing that targeting ITPR1 in 786-O cells abrogates the ability of NK cells to activate autophagy [46, 47].

As discussed above, the activation of autophagy in target tumor cells impairs NK-mediated tumor cell killing by degrading NK-derived GZMB. In accordance with this, higher level and activity of NK-derived GZMB were detected in WT-7 as compared to 786-O cells exhibiting increased level of autophagy. Targeting BECN1 in 786-O cells significantly restored GZMB

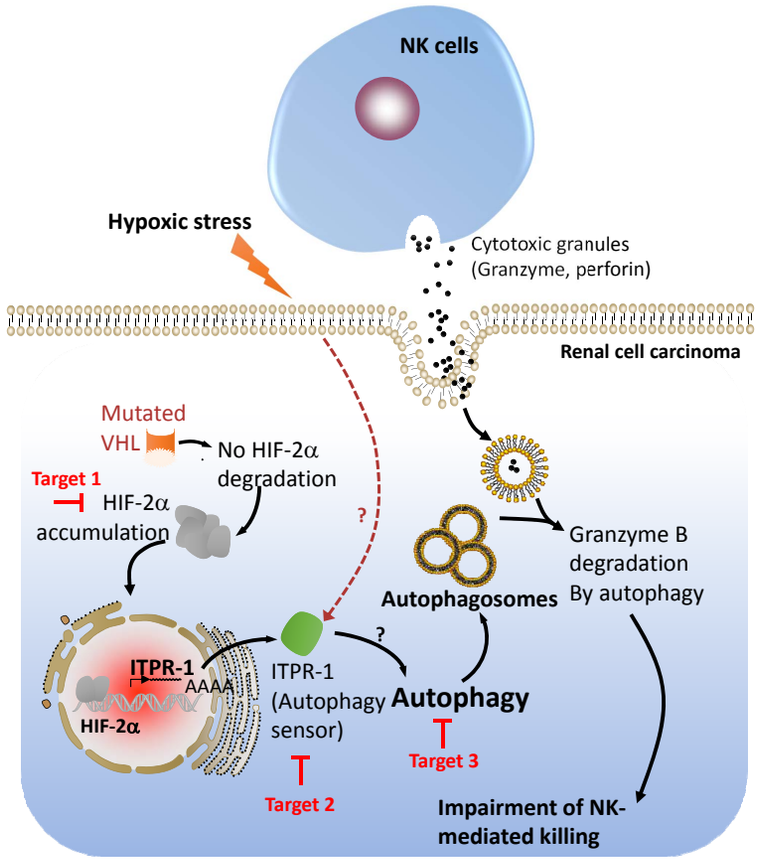

Figure 3. Targeting the autophagy sensor ITPR1 in renal cell carcinoma improves NK-mediated tumor cell killing. The expression of mutated VHL in renal cell carcinoma leads to the accumulation of HIF-2α. Accumulated HIF-2α translocates to the nucleus and induces the transcription of its target gene ITPR1. ITPR1 plays a key role in sensing a yet undefined signal derived from NK cells to activate autophagy by a mechanism that is not fully understood. The activation of autophagy in renal carcinoma cells leads to the degradation of NK-derived granzyme B and ultimately impairs NK-mediated tumor cell killing. At least 3 targets in this pathway (indicated in the figure as Targets 1, 2, and 3) may improve NK-mediated killing in renal cell carcinoma.

level and activity. In light of our *in vitro* observations, the relevance of HIF-2α/ITPR1/autophagy pathway on NK-dependent antitumor immune response using Renca murine RCC was investigated. The authors demonstrated first that NK cells control *in vivo* Renca tumor development, by showing that the depletion of host NK cells significantly increased tumor growth. Furthermore, they observed a significant decrease of tumor volume in mice engrafted with ITPR1-defective Renca cells as compared to control cells. This decrease might be due to the improvement of NK-mediated antitumor immune response. Consistent with this hypothesis, the regression of ITPR1 defective tumors was no longer observed in NK-depleted mice. Taken together, these results suggest that inhibiting ITPR1/autophagy in tumors improves their elimination by NK cells *in vivo*. While several studies claim that autophagy inhibitors could improve anticancer therapies, other reports indicate that the use of autophagy inhibitors may also have negative effect in the context of cancer immunotherapy. This study highlights that targeting the autophagy sensor ITPR1 could be an alternative strategy to improve NK-mediated antitumor immune response in renal carcinoma [46, 47].

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3.3. Autophagy activation during epithelial-to-mesenchymal transition and tumor cell plasticity

Epithelial-to-mesenchymal transition (EMT) has become one of the most exciting fields in cancer biology. While its role in cancer cell invasion, metastasis and drug resistance is well established [48, 49], the molecular basis of EMT-induced immune escape remains unknown. EMT is a fundamental process in embryogenesis [50] that allows immobilized epithelial cells to migrate as single cells to localize in different organs. Mechanisms driving EMT in development have also been co-opted by carcinoma cells to promote cell plasticity, invasion, and metastasis [10]. Most carcinoma cells exhibit a spectrum of EMT phenotypes or "epithelial cell plasticity," which is directly linked to histological grading and thus contributes to prognosis, stemness, immune suppression, and development of resistant cell variants [51–53]. Epithelial cells are characterized by a well-defined apico-basal polarity involved in the establishment of junctions between cells [54, 55]. The adhesive receptor E-cadherin is a critical component of adherens junctions, and it is often downregulated during tumor progression. Adherens junctions are thus most likely a major structure implicated in the control of epithelial cell plasticity [56]. Upon exposure to EMT inducers, polarized normal or transformed epithelial cells undergo morphological transition by launching a complex program of transcriptional, translational and posttranslational mechanisms.

So far, the relationship between autophagy and EMT in tumors is not well elucidated and studies addressing this issue in the context of tumor immune response are emerging. Thus, the first evidence showing that the acquisition of an EMT phenotype in breast cancer cells is associated with the induction of autophagy and the escape from T-cell-mediated lysis has been published recently [57] (Figure 4). Indeed, using the breast MCF-7-derived tumor cells that have undergone EMT following overexpression of wild-type SNAI1/SNAIL or the constitutively activated (SNAI1-6SA) protein, or by the acquisition of TNF/TNF-α resistance (2101 cells), the authors showed that EMT transcription factors are not the only way to induce an enhanced phenotypic plasticity resulting in breast cancer cell resistance to CTLs. They also showed that the acquisition of resistance to TNF leads to the induction of EMT and the subsequent resistance to antigen-specific killer cells. It is worth noting that the acquisition of resistance to TNF and the high EMT score of TNF-resistant (2101) cells suggest the existence of a level of complexity in the EMT process in which multiple molecules act together to mediate EMT, rather than the master regulators acting on their own.

Consistent with the role of autophagy as a cell protective mechanism, the authors further investigated whether the activation of the EMT program in tumor cells is associated with the induction of autophagy. The results showed that expression of SNAI1 in breast cancer cells induces an epithelial dedifferentiation program that coincides with a drastic change in cell morphology and the activation of autophagy flux. Interestingly, they found that BECN1 is upregulated in mesenchymal cells compared to epithelial cells.

Although the molecular mechanism by which the EMT program affects the expression of BECN1 remained to be addressed, several lines of evidence indicate that this may be related to SNAI1- or EMT-dependent repression of miRNA(s) involved in modulation of BECN1 expression. Indeed, it has been reported that MIR30A inhibits the expression of BECN1, and

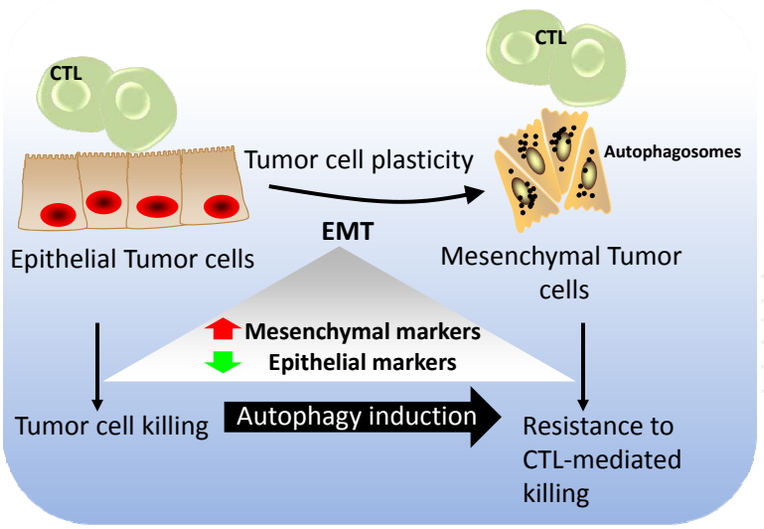

Figure 4. The acquisition of an EMT phenotype of tumor cells through loss of epithelial and gain of mesenchymal markers confers resistance to CTL-mediated lysis through autophagy induction.

that the transcription factors SNAI1 and ZEB1 bind to E-boxes in the MIR34A/B/C promoters, thereby repressing MIR34A and MIR34B/C expression. While much remains to be learned mechanistically, this result extended the role of SNAI1 as a regulator of autophagy and paved the way to an interesting topic of research. Although targeting BECN1 in mesenchymal cells is sufficient to restore CTL-mediated tumor cell lysis, it has no effect on cell morphology and the expression of EMT markers. This finding suggests that autophagy is a downstream target of the EMT program in breast cancer cells [57, 58]

4. Autophagy as a target for improving anticancer therapies

Intrinsic resistance mechanisms evolved by cancer cells are a key limitation to improve response rates and survival of patients treated with anticancer therapies. It is now clearly established that autophagy activation in cancer cells under stress conditions allows resistance to chemotherapy [59], radiotherapy [60, 61], and immunotherapy [17, 44, 62, 63]. On the basis of these observations, it is not surprising that autophagy has emerged as a potential therapeutic target, and research efforts have intensified to develop autophagy inhibitors that could be used in combination with anticancer therapies.

Pharmacological inhibitors of autophagy identified so far can be classified in two main groups depending on which stage of the autophagy process is targeted. Sequestration inhibitors such as 3-methyladenine (3-MA), LY294002, and wortmannin act at the early stage of the autophagy pathway by inhibiting the class III phosphatidylinositol-3 kinase (PI3K). Recently, a potent small molecule inhibitor of autophagy, called spautin-1, was identified which causes the degradation of the class III PI3K complex by targeting BECN1 [64]. Most of other inhibitory compounds act as later stage. Microtubule poisons such as vinca alkaloids, taxanes, nocoda-

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zole, and colchicine cause blockade of autophagosome and lysosome fusion. Inhibitors of lysosomal enzymes (e.g., leupeptin, pepstatin A, and E-64d) or compounds that elevate lysosomal pH (e.g., bafilomycin A1, chloroquine) impair autophagy through the inhibition of cargo degradation by lysosomal hydrolases (reviewed in [28]). Chloroquine (CQ) and its derivative hydroxychloroquine (HCQ) have long been used as antimalarial and antirheumatic drug, and they were the only autophagy inhibitors approved by the US Food and Drug Administration. As single agent, CQ has shown anticancer activity in lymphoma [65], pancreatic [66], and breast cancers [67]. In addition, the use of CQ or HCQ in combination with conventional therapies has provided convincing results in preclinical models [68]. Indeed, autophagy blockade enhances anticancer effects of apoptosis-inducing agents [69] and Src family kinase inhibitors [70]. Moreover, this strategy has shown promising results on patient's survival in the first phase III clinical trial using CQ as adjuvant treatment to conventional anticancer therapy for glioblastoma [71]. Currently, more than 30 clinical trials are registered with the National Cancer Institute to evaluate the effects of autophagy inhibition in a variety of human cancers (http://clinicaltrials.gov). The Table 1 summarize the clinical trials involving CQ or HCQ in combinational treatment of refractory malignancies.

Cancer type	Drug intervention	Phase status	Clinical trial ID	Title of the clinical trial
Pancreatic cancer	HCQ +Gemcitabine +Abraxane	I/II Active	NCT01506973	A Phase I/II/Pharmacodynamic Study of Hydroxychloroquine in Combination with Gemcitabine/ Abraxane to Inhibit Autophagy in Pancreatic Cancer
Breast cancer	HCQ	II Active	NCT01292408	Autophagy Inhibition Using Hydrochloroquine in Breast Cancer Patients
NSCL cancer	HCQ +Paclitaxel +Carboplatin +Bevacizumab	II Active	NCT01649947	Modulation of Autophagy in Patients with Advanced/Recurrent Non-small Cell Lung Cancer - Phase II
Renal cancer	HCQ+RAD001	I/II Active	NCT01510119	Autophagy Inhibition to Augment mTOR Inhibition: A Phase I/II Trial of RAD001 and Hydroxychloroquine in Patients with Previously Treated Renal Cell Carcinoma
SCLC	CQ +Chemotherapy +Radiotherapy	I Active	NCT00969306	Chloroquine as an Anti-Autophagy Drug in Stage IV Small Cell Lung Cancer (SCLC) Patients
Colorectal cancer	HCQ+FOLFOX +Bevacizumab	I/II Active	NCT01206530	FOLFOX/Bevacizumab/ Hydroxychloroquine (HCQ) in Colorectal Cancer

Cancer type	Drug intervention	Phase status	Clinical trial ID	Title of the clinical trial
Solid tumors	HCQ+Sorafenib	I Active	NCT01634893	Oral Hydroxychloroquine Plus Oral Sorafenib to Treat Patients with Refractory or Relapsed Solid Tumors
Solid tumors	CQ+Carboplatin +Gemcitabine	I Active	NCT02071537	Chloroquine in Combination with Carboplatin/Gemcitabine in Advanced Solid Tumors
Solid tumors	HCQ +Temsirolimus	I Active	NCT00909831	Hydroxychloroquine and Temsirolimus in Treating Patients with Metastatic Solid Tumors that Have not Responded to Treatment
Chronic myeloid leukemia	HCQ +Imatinib mesylate	II Active	NCT01227135	Imatinib Mesylate with or without Hydroxychloroquine in Treating Patients with Chronic Myeloid Leukemia
Pancreatic cancer	HCQ +Gemcitabine +Nab-Paclitaxel	II Active	NCT01978184	Randomized Phase II Trial of Pre-operative Gemcitabine and Nab Paclitacel with or without Hydroxychloroquine
Melanoma, prostate or kidney cancers	HCQ+MK2206	I Active	NCT01480154	Akt Inhibitor MK2206 and Hydroxychloroquine in Treating Patients with Advanced Solid Tumors, Melanoma, Prostate or Kidney Cancer
Multiple myeloma	HCQ +Cyclophosphamide +Dexamethasone +Sirolimus	I Active	NCT01689987	Hydroxychloroquine, Cyclophosphamide, Dexamethasone, and Sirolimus in Treating Patients with Relapsed or Refractory Multiple Myeloma
Solid tumors	HCQ+Vorinostat	I Active	NCT01023737	Hydroxychloroquine + Vorinostat in Advanced Solid Tumors
Pancreatic cancer	HCQ +Radiotherapy	II Active	NCT01494155	Short Course Radiation Therapy with Proton or Photon Beam Capecitabine and Hydroxychloroquine for Resectable Pancreatic Cancer
Glioma	HCQ +Radiotherapy	II Active	NCT01602588	A Randomised Trial Investigating the Additional Benefit of Hydroxychloroquine (HCQ) to Short Course Radiotherapy (SCRT) in Patients Aged 70 Years and Older with High Grade Gliomas (HGG)

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Cancer type	Drug intervention	Phase status	Clinical trial ID	Title of the clinical trial
Soft tissue sarcoma	HCQ+Sirolimus	II Active	NCT01842594	A Phase II Trial of Combined Hydroxychloroquine and Sirolimus in Soft Tissue Sarcoma
Melanoma	HCQ +Vemurafenib	I Active	NCT01897116	A Phase I Trial of Vemurafenib and Hydroxychloroquine in Patients with Advanced BRAF Mutant Melanoma
Renal cancer	HCQ+Aldesleukin	I/II Active	NCT01550367	Study of Hydroxychloroquine and Aldesleukin in Renal Cell Carcinoma Patients (RCC)
Colorectal cancer	HCQ+Vorinostat	II Approved	NCT02316340	Vorinostat Plus Hydroxychloroquine Versus Regorafenib in Colorectal Cancer
Advanced cancer	HCQ+Vorinostat or Sirolimus	I Active	NCT01266057	Sirolimus or Vorinostat and Hydroxychloroquine in Advanced Cancer
Prostate cancer	HCQ +Navitoclax +Abiraterone acetate	II Active	NCT01828476	Navitoclax and Abiraterone Acetate with or without Hydroxychloroquine in Treating Patients with Progressive Metastatic Castrate Refractory Prostate Cancer
Melanoma	HCQ +Dabrafenib +Trametinib	I/II Active	NCT02257424	The BAMM Trial: BRAF, Autophagy and MEK Inhibition in Metastatic Melanoma: A Phase I/2 Trial of Dabrafenib, Trametinib and Hydroxychloroquine in Patients with Advanced BRAF Mutant Melanoma
Solid tumors	HCQ +Temozolomide	I Active	NCT00714181	Hydroxychloroquine and Temozolomide in Treating Patients with Metastatic or Unresectable Solid Tumors
Multiple myeloma	HCQ+Bortezomib	I/II Active	NCT00568880	Hydroxychloroquine and Bortezomib in Treating Patients with Relapsed or Refractory Multiple Myeloma
Pancreatic cancer	HCQ +Gemcitabine	I/II Closed	NCT01128296	Study of Pre-surgery Gemcitabine + Hydroxychloroquine (GcHc) in Stage IIb or III Adenocarcinoma of the Pancreas
Colorectal cancer	HCQ +Capecitabine +Oxaliplatin +Bevacizumab	II Closed		Hydroxychloroquine, Capecitabine, Oxaliplatin, and Bevacizumab in Treating Patients with Metastatic Colorectal Cancer

Cancer type	Drug intervention	Phase status	Clinical trial ID	Title of the clinical trial
Solid tumors	HCQ +Sunitinib malate	I Closed	NCT00813423	Sunitinib Malate and Hydroxychloroquine in Treating Patients with Advanced Solid Tumors that Have not Responded to Chemotherapy
Multiple myeloma	Rapamycin or HCQ +Cyclophosphamide +Dexamethasone	NS Closed	NCT01396200	Cyclophosphamide and Pulse Dexamethasone with Rapamycin or Hydroxychloroquine
Glioblastoma	HCQ +Radiotherapy +Temozolomide	I/II Closed	NCT00486603	Hydroxychloroquine, Radiation Therapy, and Temozolomide in Treating Patients with Newly Diagnosed Glioblastoma Multiforme

Table 1. Examples of clinical trials involving autophagy inhibitors in combination with anticancer therapies (http://cancer.gov/clinicaltrials). CQ: chloroquine; HCQ: hydroxychloroquine; NSCL: non-small cell lung; SCLC: small cell lung cancer; NS: not specified.

Despite the encouraging preclinical results supporting the use of autophagy blockers in combination with chemotherapy, more attention should be paid to evaluate the impact of such inhibitors on tumor cell microenvironment. Indeed, recent evidence has emphasized that the cross-talk between cancer cells, and their microenvironment is crucial in determining efficient anticancer immune responses [72, 73]. It is now clear that a potent antitumor immune response is an important prognostic factor for cancer patient overall survival [74], suggesting that simultaneously blocking autophagy in tumor cells and boosting the immune system may be of critical importance to achieve successful anticancer treatment. We recently demonstrated that increased autophagy in tumor cell suppressed the antitumor immune response and that autophagy blockade enhances CTL- and NK-mediated tumor cell killing once they have been activated to lyse tumor cells [17, 63]. Given the limited successes encountered by many immunotherapeutic approaches, these data imply that strategies based on adoptive transfer of T cells, dendritic cell (DC) vaccines, or administration of antibodies or recombinant cytokines such as IL-2, could only be effective if the blockade of autophagy is effective in tumor cells [68]. Indeed, Liang *et al.* showed that the combination of high dose of Interleukin-2 (IL-2) with CQ promotes long-term survival, decreased toxicity, and enhanced immune cell proliferation and infiltration in advanced murine metastatic liver tumor model [75]. This group has now initiated a clinical protocol to evaluate the combinational administration of IL-2 and HCQ in patients with advanced renal cell cancer.

While experimental and preclinical studies were mainly focused on the rational to use autophagy inhibitors in cancer therapy, pharmacological approaches aiming to upregulate autophagy have recently received considerable attention. Accumulating evidence highlights that autophagy plays a crucial role in increasing the immunogenicity of tumor cell and actively participates in tumor-associated antigen processing and presentation [76]. Indeed, cancer cell-associated autophagy contributes to immunogenic cell death (ICD) through the release/

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exposure of immunostimulatory danger signals that stimulate the antitumor immune response. Such signaling molecules include secreted ATP, surface-exposed calreticulin, and high mobility group box 1 (HMGB1) release [77, 78]. This important role of autophagy in eliciting ICD was reported in a recent study showing how autophagy-competent cells, but not autophagy-deficient cells, enable to release ATP and recruit dendritic cells and T lymphocytes into the tumor bed in mice [79]. Recently, the same group has confirmed that chemotherapy-induced autophagy in cancer cells determines the outcome of melanoma therapy. Systemic treatment with the anthracycline mitoxantrone reduced the growth of autophagy-competent melanomas but not autophagy-deficient tumors. This growth-inhibitory activity of mitoxantrone observed on autophagy-competent melanomas was shown to be mediated through CD4+ and CD8+ T lymphocytes, suggesting that autophagy is required to trigger a potent anticancer response [80].

Furthermore, it has been described that autophagosomes are essential carriers for cross-presentation of tumor-associated antigens [81]. Li *et al.* have demonstrated that the induction of autophagy in tumor cells, following exposure to alpha-tocopheryloxyacetic acid (alpha-TEA), generates double membrane-bound autophagosomes containing antigens that enhance the cross-priming of CD8+ T lymphocytes. Moreover, the inhibition of autophagy, with 3-MA or by specific silencing of Atg12, partially blocks T-cell activation. The authors showed that vaccination with DC pulsed with autophagosome-enriched fraction, derived from tumor cells treated *in vitro* with alpha-TEA, decreased lung metastasis and increased survival of tumor-bearing mice [82]. Therefore, an autophagy inducer, such alpha-TEA, might be exploited as adjuvant therapy to improve efficacy of immune modulator of T-cell response (anti-CTLA-4 antibody). Moreover, the same group also reported that vaccination with autophagosome-enriched of defective ribosomal products (called DRibbles) or DRibbles loaded onto DC is a potent inducer of the antitumor response in murine cancer models when associated with IFN-γ and Toll-like receptor agonist [83]. Based on these findings, a clinical study was initiated to investigate the efficacy of DRibbles vaccine in patients with non-small cell lung cancer. Recently, Amaravadi *et al.* highlighted that cancer patients are suffering from a "systemic autophagic syndrome," meaning that autophagy is activated in tumor cells while suppressed in immune effectors [68]. Taken together, these observations emphasize that future therapeutic approaches may combine *ex vivo* autophagy induction in immune cells and systemic autophagy inhibition to improve efficacy of immunotherapies. As antigen processing and delivery to major histocompatibility complex (MHC) Class I and II molecules into APC is mediated through autophagy cargos, efficient DC vaccines may require their isolation from patients, followed by *ex vivo* activation with tumor-associated antigens, and reintroduction of the matured DC that would facilitate priming of CD8+ T cells.

5. Conclusion

The ability of cancer cells to evade immune surveillance and resist immunotherapy raises fundamental questions about how tumor cells survive in the presence of a competent immune system. To address this issue, studies have primarily focused on the mechanisms by which

tumor cells avoid recognition by the immune system without considering the impact of the tumor microenvironment. Thus, despite intense investigation, the relatively modest gains provided by immunotherapy can be in part attributed to the activation of mechanisms suppressing the antitumor immunity. It is now clearly established that the majority of these mechanisms are likely evolved in the local tumor microenvironment. In line with this, it may be more accurate to consider cancer, which was initially thought to be a disease of cells, then of genes and then of genomes, as a disease of the microenvironment. While remarkable and fairly rapid progresses have been made over the past two decades regarding the role of the microenvironment in cancer biology and treatment, our understanding of its actual contribution in tumor resistance to immune cell attack is still fragmented.

Emerging data indicate that, by inducing autophagy, hypoxia in the tumor microenvironment plays key role in mediating tolerance to immune cell attack. Therefore, an understanding of how autophagy plays such a role may allow better understanding of tumor adaptation and evolution, and ultimately lead to improve the efficacy of therapies.

Despite recent advances in our understanding about the role of autophagy in cancer, the emergence of consensual strategy implying autophagy modulators in anticancer therapy is still challenging. Indeed, harnessing autophagy for therapeutic purposes will require careful consideration on whether, when and how autophagy is induced as a prosurvival mechanism, or is recruited to promote cancer cell killing. To date, most of the studies have focused on the impact of autophagy on tumor cells themselves but it should be more accurate to consider autophagy in the context of the tumor microenvironment. It has been increasingly clear that autophagy may influence the cross-talk between cancer and immune cells, leading to either immunoevasion or immunostimulation. Further knowledge on the impact of autophagy in tumor cells as well as in the tumor microenvironment is necessary to tailor therapies that selectively block suppressive mechanisms that impede antitumor response while promoting the antitumor immunity.

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