Blocking Hypoxia-Induced Autophagy in Tumors Restores Cytotoxic T-Cell Activity and Promotes Regression

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Abstract

The relationship between hypoxic stress, autophagy, and specific cell-mediated cytotoxicity remains unknown. This study shows that hypoxia-induced resistance of lung tumor to cytolytic T lymphocyte (CTL)-mediated lysis is associated with autophagy induction in target cells. In turn, this correlates with STAT3 phosphorylation on tyrosine 705 residue (pSTAT3) and HIF-1α accumulation. Inhibition of autophagy by siRNA targeting of either beclin1 or Atg5 resulted in impairment of pSTAT3 and restoration of hypoxic tumor cell susceptibility to CTL-mediated lysis. Furthermore, inhibition of pSTAT3 in hypoxic Atg5 or beclin1-targeted tumor cells was found to be associated with the inhibition Src kinase (pSrc). Autophagy-induced pSTAT3 and pSrc regulation seemed to involve the ubiquitin proteasome system and p62/SQSTM1. In vivo experiments using B16-F10 melanoma tumor cells indicated that depletion of beclin1 resulted in an inhibition of B16-F10 tumor growth and increased tumor apoptosis. Moreover, in vivo inhibition of autophagy by hydroxychloroquine in B16-F10 tumor-bearing mice and mice vaccinated with tyrosinase-related protein-2 peptide dramatically increased tumor growth inhibition. Collectively, this hypothesis establishes a novel functional link between hypoxia-induced autophagy and the regulation of antigen-specific T-cell lysis and points to a major role of autophagy in the control of in vivo tumor growth. Cancer Res; 71(18); 5976–86. ©2011 AACR.

Introduction

Tumor growth and spread depend as much on the host response as on the biologic characteristics of the tumor itself and on the influence of the tumor microenvironment. Hypoxic microenvironment plays a role in shifting the normal balance toward malignancy. Evidence indicates that cancer cells which remain viable in hypoxia often possess an increased survival potential and an aggressive growth (1). A better understanding of the hypoxic tumor context could improve the prospect of developing effective cancer immunotherapy.

Cytolytic T lymphocytes (CTL) are important effector cells during tumor rejection (2). Currently, most cancer immunotherapy approaches involve the generation of CTLs against tumor-associated antigens (TAA) through vaccination strategies that induce or optimize TAA-specific immune responses. However, tumor rejection does not always follow successful induction of tumor-specific immune responses (3). Numerous studies have shown a paradoxical coexistence of cancer cells with TAA-specific T cells in an immune-competent host. Moreover, tumor cells themselves play a crucial role in controlling the antitumor immune response (4) allowing them to maintain their functional disorder and evade destruction by CTLs. Clearly, a strong and sustained CTL response is insufficient for successful elimination of tumors. Tumor growth is influenced by tumor cell recognition by specific CTLs and tumor susceptibility to T cell-mediated target cell death. In addition to chemo- and radioresistance, tumor cells also develop resistance to CTL-mediated cytotoxicity (resistance to perforin, granzyme-B, or defect in death receptor expression or signaling) leading to tumor cell survival and proliferation (5). In addition, resistance of tumor cells to cell-mediated cytotoxicity involves several strategies including cross-talk with the tumor microenvironment (6). Therefore, a better understanding of how the tumor microenvironment contributes to the resistance of tumors to killer cells is critically important for improved immune intervention. In this regard, hypoxic areas within tumors results from morphologically and functionally inappropriate neovascularization leading to the expression of genes involved in proliferation, glycolysis, and angiogenesis (7). Tumor cells evade adaptive immunity under hypoxia (8), and the adaptation to hypoxia results in an aggressive and metastatic cancer phenotype associated with resistance to therapy (9).
with a poor treatment outcome (9). In addition, hypoxia favors the emergence of tumor phenotypes that are resistant to therapy approaches (10). Signaling pathways involving hypoxia-inducible factor HIF-1α (11) and hypoxia-dependent activation of STAT3 (12) have been well elucidated (13). These hypoxia-dependent signaling pathways contribute to malignant transformation (14) and progression by transactivating host target genes involved in tumor proliferation, survival, self-renewal, invasion, and angiogenesis (15).

Autophagy, initially described as a mechanism involved in cell homeostasis and protection (16), has been also proposed to mediate tumor progression and promotion of cancer cell death (17). Moreover, autophagy has been involved in regulating adaptive immune responses and tolerance induction (18). Autophagy is also associated with tumor cell resistance to different apoptotic inducers (19, 20) and can modulate different stages of cancer development. Recently, a relationship between tumor hypoxia and autophagy has been described (21) to promote cell survival (22).

This study aims to examine the involvement of hypoxia-induced autophagy in the regulation of tumor cell lysis by specific CTLs. We show that hypoxia-induced autophagy promotes tumor cell resistance to specific lysis by a mechanism dependent on STAT3 phosphorylation. We also show that autophagy and the proteasome pathway cooperate to regulate STAT3 activation in hypoxic tumor cells. Simultaneous stimulation of immune system and inhibition of autophagy in vivo significantly inhibited tumor growth. These data uncover a key role of autophagy in the regulation of antitumor immunity.

Materials and Methods

Tumor cells and CTLs culture

IGR-Heu lung carcinoma cell line and Heu171 cell clone were derived and maintained in culture as described (23). B16-F10 melanoma cell line was purchased from American Type Culture Collection. Cells were transfected with LC3 cDNA fused with GFP (provided by Dr. Mizushima, Tokyo, Japan). To detect ubiquitination, cells were transfected with Hemagglutinin (HA)-tagged ubiquitin vector (pMT123), provided by Dr. Matthias Treier (EMBL, Germany) using Lipofectamine 2000 (Invitrogen). Unless otherwise indicated the induction of HIF-1α in cells was done in a hypoxia workstation (Invivo2 400, Ruskinn), as previously described (23).

Reagents and antibodies

Reagents include protease inhibitors and anti-HA (12CA5; Roche); bicinchoninic acid protein assay reagent (Pierce); CHAPs, E64d, pepstatin, PP2, 3-methyladenine (3-MA), cobalt chloride (CoCl₂), and hydroxychloroquine (HCQ) sulfate (Sigma); and bortezomib (Velcade; Millennium Pharmaceuticals). Antibodies include the following: mouse anti-HIF-1α and p62 (BD-Transduction Laboratories); rabbit anti-LC3B, -pSTAT3, -Src, -pSrc, -Bcl-1, -Atg5, and mouse anti-STAT3 and -p62 (BD-Transduction Laboratories); rabbit anti-LC3B, cals). Antibodies include the following: mouse anti-HIF-1α (Sigma); and bortezomib (Velcade; Millennium Pharmaceuticals). Reagents and antibodies were derived and maintained in culture as described (23). B16-F10 melanoma tumor cells are available upon request.

Immunohistochemical analysis

B16-F10–engrafted tumors were fixed in 4% PFA, and paraffin sections were stained with hematoxylin-eosin-saffron. Apoptotic cells were quantified using in situ cell death detection kit (Roche) based on the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method as previously described (24). Hypoxic tumor areas were detected after injection of mice with pimonidazole (60 mg/kg i.p. via tail vein)

Cytotoxicity assay

Cytotoxic activity of CTL clone (Heu171) was measured by a conventional 4-hour Cr51 release assay and percent-specific cytotoxicity was calculated as described (23).

RNA interference and generation of short hairpin RNA

Atg5 and Beclin tumor cells

Cells were transfected by electroporation with 50 nmol/L siRNA of human and mouse BECN1 (beclin1), p62 or Atg5 (Qiagen), or Luciferase (Invitrogen) as previously described (23). Atg5 short hairpin RNA (shRNA) expressing cells were generated by infection with commercial human APG5 shRNA lentiviral particles (Santa Cruz Biotechnology) according to manufacturer’s protocol. Details for the generation of shRNA Beclin B16-F10 cells are available upon request.

Western blot and immunoprecipitation

Western blotting was done as previously reported (23). Immunoprecipitation was done on 1 mg of total protein using Dynabeads protein G (Invitrogen) according to the manufacture’s protocol.

Live cell imaging and confocal microscopy

Time-lapse video microscopy was done using Axiovert 200 M microscope (Carl Zeiss MicroImaging). Cells were cultured with 150 μmol/L CoCl₂ to mimic hypoxia condition over a period of 24 hours. Frames were taken with ×40 oil objective lens in intervals of 1 frame/3 minutes. Cells were analyzed with a Zeiss laser scanning confocal microscope LSM-510 Meta (Carl Zeiss).

In vivo experiments

C57BL/6 mice (Charles River Laboratories) were housed at the Institut Gustave Roussy animal facility and treated in accordance with institutional animal guidelines. Six to 7-week-old mice (n = 10 per group) were inoculated s.c. with 3 × 10⁶ B16-F10 cells. Tumor volume was measured using a caliper every other day and estimated as follows: Volume (cm³) = (width)² × length × 0.5.

TRP-2 180–188 peptide vaccination and HCQ treatment

Lyophilized TRP-2(180-188) peptide (SYVDFFVWL; Proteogenix) was diluted in dimethyl sulfoxide and stored at −20°C. Synthetic CpG ODN 1826 (TCCATGACGTTCCTGACGTT; Oligofectory) was dissolved in sterile PBS. TRP-2(180-188) peptide vaccination was done by s.c. injection of 50 μg CpG ODN and 50 μg TRP-2(180-188) emulsified in 100 μL of IFA on the left side of back. The vaccination was boosted at day 4 and day 12. Daily HCQ treatment [30 mg/kg administered intraperitoneally (i.p.)] was started at day 8 for 10 days.
hypoxyprobe-1 (Chemicon) 60 minutes before killing. Frozen, acetone-fixed sections were stained using anti-cleaved LC3B (Abgent), anti-pimonidazole (Chemicon) and Mouse CD31 (BD biosciences) antibodies.

Statistics

Data were analyzed with GraphPad Prism. Student t test was used for single comparisons. Data were considered statistically significant when \( P < 0.005 \).

Results

Hypoxic stress-induced impairment of tumor susceptibility to CTL-mediated lysis is associated with the induction of autophagy

We have previously reported (23) that hypoxic stress-induced HIF-1\( \alpha \) is associated with a decrease in CTL-mediated tumor cell lysis (Fig. 1A) and correlated with an increase in pSTAT3 (Fig. 1B). To investigate whether hypoxia-dependent impairment of target cell susceptibility to CTL-mediated lysis is associated with autophagy activation, we analyzed the expression of p62/SQSTM1 and the lipidated form of microtubule-associated protein light chain 3 (LC3-II). Figure 1C shows a time-dependent decrease in p62/SQSTM1 expression in hypoxic cells which correlated with an accumulation of LC3-II only in hypoxic cells treated with lysosomal protease inhibitors E64 and pepstatin (Fig. 1D) reflecting the activation of autophagy under hypoxia. This was further confirmed by time-lapse video microscopy (Supplementary Data S1A and B) using GFP-LC3 expressing IGR-Heu cells (Supplementary Data S2). Representative images from time-lapse experiments showed a time-dependent increase of autophagosomes per cell under hypoxia (Fig. 1E, top and bottom). The molecular mechanism...
involved in the activation of autophagy was next investigated. Results shown in Supplementary Data S3A reveal a lower amount of Bcl-2 coimmunoprecipitated with Beclin1 in hypoxic as compared with normoxic cells suggesting that the activation of autophagy involves a disruption of the autophagy inhibitory complex of Beclin 1/Bcl-2. BNIP3 and BNIP3L seem to be involved in displacing Beclin1 from Bcl-2 under hypoxia (21) because the expression of BNIP3 and BNIP3L was increased in hypoxic as compared with normoxic cells (Supplementary Data S3B and C). Silencing BNIP3 and BNIP3L inhibited autophagosome formation in hypoxic IGR-Heu cells (Supplementary Data S3D). Collectively, our results show that hypoxic stress–induced impairment of tumor susceptibility to CTL-mediated lysis is associated with the induction of autophagy in IGR-Heu cells by a mechanism involving BNIP3/BNIP3L.

Targeting autophagy restores IGR-Heu tumor cell susceptibility to CTL-mediated lysis

To investigate whether the inhibition of hypoxia-induced autophagy is able to restore tumor cell susceptibility to CTL-mediated lysis, we targeted autophagy genes Atg5 and Beclin1 by siRNA (Fig. 2A). As shown in Fig. 2B, there was a remarkable reversal of hypoxia-induced inhibition of CTL-mediated killing in autophagy defective cells. Interestingly, CTL-induced killing of autophagy defective cells was observed at all effector–target (E:T) ratios tested, compared with control cells. These data suggest that hypoxia-induced autophagy plays an important role in the acquisition of tumor cell resistance to T cell receptor–dependent killing.
Hypoxia-induced pSTAT3 is regulated by autophagy in IGR-Heu cells

Because hypoxia-dependent impairment of IGR-Heu cell susceptibility to CTL-mediated lysis correlated with HIF-1α and pSTAT3 induction (23), we investigated whether the autophagic process operates upstream of hypoxia-dependent induction of pSTAT3. Targeting Beclin1 or Atg5 under hypoxia abrogates pSTAT3 expression without affecting STAT3 expression (Fig. 3A). Similar results were obtained using autophagy inhibitors 3-MA and HCQ, which block the early and late events of autophagy, respectively (Fig. 3B). Figure 3C shows a strong accumulation of autophagosomes in hypoxic cells treated with HCQ, thus showing the effectiveness of the HCQ concentration used in blocking the autophagic flux. These results show that hypoxia-induced autophagy regulates the induction of pSTAT3.

Figure 4. Autophagy and ubiquitin proteasome system cooperate to regulate pSTAT3 in hypoxic IGR-Heu cells. A, expression of pSrc by immunoblot in normoxic (N) and hypoxic cells transfected with Luciferase (Luc), Beclin1 (BECN1) or Atg5 siRNA. B, expression of pSrc and pSTAT3 by immunoblot in normoxic (N) and hypoxic cells untreated (−) or treated with Src kinase inhibitor (PP2, 10 μmol/L). C, immunoblot analysis of ubiquitinated protein in hypoxic cells untreated (−) or treated (+) with 3MA or Bz. D, expression of pSrc and pSTAT3 in normoxic (N) and hypoxic cells untreated (−) or treated (+) with Bz. Hypoxic cells were transfected with Luc, BECN1, or Atg5 siRNA. E, immunoblot analysis of pSrc and pSTAT3 polyubiquitination. Left, immunoblot analysis of the expression of HA-ubiquitin in control (C) untransfected and transfected cells cultured under normoxia (N) or hypoxia (H). Middle and right panels represent immunoprecipitation experiments using anti-HA antibody followed by immunoblot using anti-pSrc (middle) or anti-pSTAT3 (right).
Impairment of pSTAT3 in autophagy defective cells is related to Src kinase activity and involves the ubiquitin proteasome system

We next investigated the involvement of the receptor-associated–specific Janus kinases (JAK) and Src in the phosphorylation of STAT3 to get more insights into the mechanisms associated with hypoxia-induced pSTAT3 and to elucidate the role of autophagy in this process. Our data show that inhibition of autophagy in hypoxic cells does not affect the phosphorylation of JAK2 (data not shown) but dramatically decreased the tyrosine-416 phosphorylation of Src (pSrc). It is noteworthy that pSrc expression is highly increased under hypoxia (Fig. 4A). Figure 4B shows that inhibition of Src activity in hypoxic cells by PP2 significantly inhibits hypoxia-induced pSTAT3. Together our data show that targeting autophagy inhibits hypoxia-induced pSrc.

Analysis of the ubiquitination profile of proteins in autophagy-defective cells cultured under hypoxia shows that high-molecular-weight proteins (up to 40 KDa) are highly ubiquitinated (Fig. 4C). The ubiquitination level is decreased in autophagy-defective cells and dramatically reaccumulated in cells treated with the proteasome inhibitor bortezomib, highlighting the role of the ubiquitin proteasome system (UPS) in the clearance of pSTAT3 and/or pSrc in autophagy defective cells under hypoxia. This was supported by our data (Fig. 4D) showing that inhibition of UPS by bortezomib in autophagy-defective cells completely restores the expression of pSTAT3 and partially that of pSrc.

Because UPS-dependent protein clearance implies their ubiquitination, we analyzed the ubiquitination profile of pSTAT3 and pSrc in autophagy defective cells expressing HA-tag ubiquitin. As shown in Fig. 4E left panel, no HA-ubiquitin was detected in untransfected cells, but equal and high expression level of HA-ubiquitin was detected in transfected cells cultured under normoxia and hypoxia. Immunoprecipitation results (Fig. 4E, middle) clearly show the absence of pSrc ubiquitination. However, a strong high-molecular-weight smear was detected with anti-pSTAT3 antibody in hypoxic cells most likely corresponding to a polyubiquitinated form of pSTAT3 (Fig. 4E, right). Together, these results indicate that autophagy and UPS cooperate to regulate pSTAT3 in hypoxic tumor cells.

P62/SQSTM1 is required for autophagy-dependent pSTAT3 degradation

P62/SQSTM1 (p62) is a multifunctional protein acting as an adaptor between ubiquitylated protein aggregates and autophagy (25). p62 degradation by autophagy represents another way for cancer cells to survive under hypoxia (26). We targeted p62 in autophagy-competent and -defective cells and analyzed the expression of pSTAT3 and pSrc. Figure 5A shows that targeting p62 in autophagy-defective cells induces a reaccumulation of pSTAT3 and pSrc under hypoxia. siRNA p62 alone did not affect the expression of pSTAT3 and pSrc in autophagy-competent cells (Fig. 5A). These results suggest that p62 is required for autophagy-dependent pSTAT3 degradation and could play a role as a cargo for pSTAT3 or pSrc degradation. Immunoprecipitation experiments on normoxic and hypoxic
cells using anti-p62–specific antibody (Fig. 5B) provided compelling evidence for a direct interaction between p62 and pSTAT3 under hypoxia, suggesting that p62 interacts with pSTAT3 to trigger its degradation. These results are supported by data in Fig. 5C showing that under hypoxia pSTAT3, pSrc, and a fraction of p62 are present in the nuclear compartment.

**Autophagy is activated in B16-F10 hypoxic tumor areas**

To determine whether autophagy was induced in hypoxic zones of tumors, we analyzed the colocalization of LC3 (autophagy marker) in the hypoxic zones (stained with pimonidazole) of the B16-F10 tumors established in C57BL/6 mice. B16-F10 melanoma cells are able to activate autophagy under hypoxia (Supplementary Data S4A and B). Figure 6A (I) shows the presence of hypoxic areas (green), and Fig. 6A (II and III) show a colocalization of LC3 (blue staining in II and red staining in III) in hypoxic areas (green), suggesting that autophagy was strongly induced in hypoxic zones of the tumor. Moreover, Fig. 6A (IV) shows a cytoplasmic expression of LC3 in the entire hypoxic zone with strongest expression seen in extreme hypoxic areas. The impact of Beclin1 silencing on B16-F10–engrafted tumor progression was next evaluated. Figure 6B shows that the inhibition of autophagy in beclin1 silenced B16-F10–engrafted tumors resulted in a significant decrease in tumor growth. TUNEL staining clearly shows the presence of apoptotic foci and an increase of the number of TUNEL-positive nuclei in autophagy-defective tumors as compared with control (Fig. 6C). These data strongly suggest a role for autophagy in mediating hypoxia tolerance.

**In vivo inhibition of autophagy potentiates the antitumor effect of tyrosinase-related protein-2 peptide vaccination**

Vaccination with peptide from the melanocyte differentiation antigen tyrosinase-related protein-2 (TRP2) in combination with oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODN) as adjuvant has been reported to be efficient in the induction of tumor cell–specific CTLs (27).

We hypothesized that autophagy blockade in combination with TRP2 peptide vaccination would result in a potent antitumor effect. B16-F10–engrafted tumors were treated with different doses of HCQ (30 mg/kg) and vaccinated with TRP2 peptide antigen. A significant decrease in tumor

![Figure 6](image-url)
growth was observed as compared with control mice (Fig. 7A). A similar effect on the inhibition of tumor growth with 60 mg/kg HCQ was observed (data not shown). Strikingly, the combination of TRP2 peptide vaccination and HCQ treatment resulted in complete abrogation of tumor growth (Fig. 7A). These results were further confirmed by TUNEL staining, which showed an increase in the number of TUNEL-stained positive nuclei following HCQ treatment and vaccination (Fig. 7B and C). Moreover, a dramatic increase (20-fold) in TUNEL-positive nuclei was observed in tumors after the combined treatment.

Discussion

Hypoxia is a common feature of solid tumors and a major limiting factor in successful cancer treatment (7). Previously, we showed that hypoxia impairs tumor susceptibility to CTL-mediated cell lysis by a mechanism involving cooperation between HIF-1α and pSTAT3 (23). Given the role of autophagy in induction of the adaptive immune system (18) and cellular survival under hypoxia (28), we sought to determine the molecular basis of the hypoxia/autophagy interaction and its interference with tumor cell survival and growth. Here,
we show that hypoxia-induced autophagy acts as a tumor cell resistance mechanism to specific T cell-mediated lysis. Targeting autophagy was sufficient to significantly restore tumor cell susceptibility to CTL-mediated lysis. We show for the first time that autophagy is a protective cell survival mechanism against CTL-mediated lysis under hypoxia.
Autophagy and Hypoxia-Induced Immune Resistance

It has been suggested that the unfolded protein response enhances the capacity of hypoxic tumor cells to activate autophagy by PKR-like endoplasmic reticulum kinase (PERK) and ATF4-dependent upregulation of Atg5 and LC3 mRNA (20). This mechanism seems unlikely to be involved in the induction of autophagy in hypoxic IGR-Heu cells because there is no evidence for the activation of PERK and ATF4 and for the upregulation of Atg5 and LC3 mRNA (data not shown). However, we show (Supplementary Data S1) that hypoxia induces autophagy by BNIP3/BINP3L-dependent displacement of the autophagy-inhibitory complex Beclin1/Bcl-2 (21).

We attempted to investigate the role of autophagy in the regulation of tumor susceptibility to CTL-mediated lysis by blocking autophagy. Although much still remains to be learned mechanistically, our data show that autophagy inhibition compromised the hypoxia-dependent induction of pSTAT3. Consistent with the clearance function of autophagy, it is difficult to reconcile the idea that the autophagy machinery plays a role in a specific stabilization of pSTAT3 and/or pSrc. Indeed, it is now well documented that there is cross-talk between the UPS and autophagy (25, 29). On the basis of our results, we believe that the decreased level of pSTAT3 in hypoxic and autophagy-defective IGR-Heu cells is related to its degradation by UPS. This was supported by our data showing that hypoxia induces a polyubiquitination of pSTAT3 and our results showing that inhibition of UPS by bortezomib accumulates pSTAT3 in hypoxic and autophagy-defective IGR-Heu cells. It is noteworthy that a UPS-dependent degradation of phospho-p53 (30) and phospho-PKC-δ (31) has been previously described. The interplay between autophagy and UPS is mainly mediated by the adaptor protein p62/SQSTM1, which plays a role as a cargo for targeting proteins to proteasomes degradation and autophagy (32). Because p62 is an autophagic substrate, an accumulation of this protein was observed in autophagy-deficient cells. In this regard, it has been recently reported that an excess of p62 inhibits the clearance of ubiquitinated proteins destined for proteasomal degradation by delaying their delivery (32). Our results show discrepancy with this mechanism as we show that the accumulation of p62 in autophagy-defective cells correlates with a decrease, rather than an accumulation, of pSTAT3 in hypoxic IGR-Heu cells, and targeting p62 in autophagy-defective cells reaccumulates pSTAT3. Although additional work needs to be done to clarify this discrepancy, one possible reason is that degradation of p62 by autophagy in IGR-Heu cells under hypoxia could represent a feedback mechanism to restrict further autophagy and excessive destruction of proteins under hypoxia. Such a feedback mechanism has been recently described (26).

Vaccine approaches have proven effective in enhancing antitumor immunity against tumor cells expressing targeted antigens in vitro (33). However, clinical and animal studies have shown little success in the inhibition of tumor growth by vaccines (34). One of the most important factors that could be responsible for this failure is the hypoxia-dependent activation of prosurvival pathways. Inhibition of autophagy has been reported to sensitize tumor cells to cytotoxic treatments including antitumoral agents and irradiation (35). In light of our in vitro observations, we asked whether targeting of hypoxia-induced autophagy could influence in vivo tumor growth. To address this issue, we used the transplantable murine melanoma B16-F10 cell line that expresses different TAA, including TRP2 (36). Our data showed that autophagy is primarily localized to tumor hypoxic regions as previously reported in other tumor models (20). We also show that targeting beclin1 reduced tumor growth, which correlated with an increase in TUNEL staining. In addition, we observed a significant decrease of tumor growth in mice treated with the autophagy inhibitor HCQ, used at a concentration that had no effect on cell proliferation (data not shown). These observations are in agreement with recent reports indicating that inhibition of autophagy by HCQ inhibits in vitro cell growth and in vivo tumor growth via induction of apoptosis (37, 38). Our results are also supported by several studies showing that inhibition of autophagy promotes cancer cell death (39) and potentiates anticancer treatments (19, 40, 41). HCQ has been used in vitro for the inhibition of autophagy in several tumor models including melanoma (42) and is currently being tested in different ongoing phase II studies (43, 44).

Finally, with regard to potential therapeutic treatment, we show that the combination of TRP2 peptide vaccination with HCQ treatment results in a strong inhibition of tumor growth. These observations further establish the significance of hypoxia in inducing autophagy, demonstrate that it is an adverse prognostic factor, and confirm its critical role not only in radio- and chemoresistance but also in immune effector cell resistance. A better understanding of the hypoxic tumor context is likely to improve the prospect of developing effective cancer immunotherapy. Together, our results show that targeting autophagy could have significant therapeutic implications for tumor progression and extend the notion that simultaneously boosting the immune system and targeting of autophagy could enhance the therapeutic efficacy of cancer vaccines and may prove beneficial in cancer immunotherapy.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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