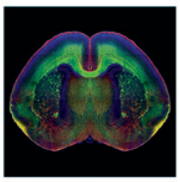
Autophagy



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REVIEW

Autophagy in natural and therapy-driven anticancer immunosurveillance

Federico Pietrocola[a,b,c,d,e], José Manuel Bravo-San Pedro[a,b,c,d,e], Lorenzo Galluzzi[c,f,g,†], and Guido Kroemer[a,b,c,d,e,h,i,†]

[a]Equipe 11 labellisée par la Ligue contre le Cancer, Centre de Recherche des Cordeliers, Paris, France; [b]INSERM, U1138, Paris, France; [c]Université Paris Descartes/Paris V, Sorbonne Paris Cité, Paris, France; [d]Université Pierre et Marie Curie/Paris VI, Paris, France; [e]Metabolomics and Cell Biology Platforms, Gustave Roussy Comprehensive Cancer Institute, Villejuif, France; [f]Department of Radiation Oncology, Weill Cornell Medical College, New York, NY, USA; [g]Sandra and Edward Meyer Cancer Center, New York, NY, USA; [h]Pôle de Biologie, Hopitâl Européen George Pompidou, AP-HP, Paris, France; [i]Department of Women's and Children's Health, Karolinska University Hospital, Stockholm, Sweden

ABSTRACT

Autophagy is primordial for the maintenance of metabolic and genetic homeostasis in all eukaryotic organisms. Owing to its cell-intrinsic effects, autophagy robustly inhibits malignant transformation, yet can support the progression of established neoplasms as well as their resistance to conventional treatments. The notion that autophagy inhibition sensitizes neoplastic cells to chemotherapy and radiation therapy rivals with the capacity of autophagy to contribute to natural and therapy-driven anticancer immunosurveillance via a multitude of mechanisms. Indeed, autophagy ensures an optimal release of immunostimulatory signals by dying cancer cells and hence boosts their capacity to initiate an immune response. Moreover, autophagy is important for the activity of several components of the immune system involved in tumor recognition and elimination, including antigen-presenting cells and $CD8^+$ cytotoxic T lymphocytes. In this review, we discuss how cancer cells disable autophagy to bypass immune control and how strategies aiming to enhance autophagy can be envisaged to improve the efficacy of immunogenic cancer therapies.

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Introduction

Oncogenesis has long been conceived as a merely cell-intrinsic process resulting from the accumulation of (epi)genetic defects that underlie the acquisition of a malignant phenotype.[1] From this perspective, the causal nexus between the cell-intrinsic functions of macroautophagy (herein referred to as autophagy) and the multistep process through which a normal cell first becomes neoplastic (malignant transformation) and then forms an ever more aggressive and clinically manifest cancer (tumor progression; exhaustively reviewed in ref. 2) readily comes into sight. As a catabolic process consisting of the sequestration and the lysosomal breakdown of cytoplasmic material, autophagy contributes to the maintenance of metabolic homeostasis (by supplying substrates for bioenergetic metabolism from disposable cellular components and/or energy stores in conditions of nutrient deprivation)[3] and genomic stability (through the turnover of potentially genotoxic (dam)aged organelles and protein aggregates)[4] in every cell lineage. Thus, the transient loss of autophagic functions (favoring malignant transformation) followed by the restoration of autophagic proficiency (supporting the viability of established neoplasms in harsh environmental conditions) perfectly fits in the multistep model classically adopted to describe oncogenesis.[2,5]

The demonstration that immunodeficient mice are more susceptible to spontaneous and chemical carcinogenesis than their immunocompetent counterparts has unveiled the notion that cancer is not (only) an (epi)genetic disease of individual cells, but (also) an immunological disorder.[6] According to the immunosurveillance model, the clinical manifestation of cancer invariably results from the escape of neoplastic cells from recognition by the innate or adaptive arm of the immune system.[7] Tumors elude immunosurveillance in a dual manner: (1) as cancer cell variants with limited immunogenicity are selected by the immune system (immunoediting), and/or (2) as neoplastic cells actively suppress tumor-targeting immune responses (immunosubversion).[8,9] Therefore, the (re)activation of adaptive immune responses, usually mediated by $CD8^+$ cytotoxic T lymphocytes (CTLs) that recognize tumor-specific (neo)-antigens, is of the utmost importance for controlling tumor progression.

Conventional anticancer agents often fail to mediate therapeutic effects in immunocompromised mice, suggesting that their mode of action involves immunological mechanisms.[10] Growing evidence indicates indeed that multiple chemotherapeutics as well as radiation therapy can stimulate anticancer immune responses, either as they directly act on the immune system ("off-target" immunostimulation), and/or as they exacerbate the immunogenicity of malignant cells ("on-target" immunostimulation).[10] Immunogenicity is a function of antigenicity, i.e., the ability of cancer cells to be targeted by CTLs upon recognition of tumor-associated epitopes (exposed on the cell surface in association with MHC class I molecules) and

CONTACT Lorenzo Galluzzi deadoc@vodafone.it Weill Cornell Medical College, Stich Radiation Oncology, 525 East 68th Street, Box # 169, New York, NY 10065, USA; Guido Kroemer kroemer@orange.fr Centre de Recherche des Cordeliers, Equipe 11, 15 rue de l'Ecole de Médecine, 75006 Paris, France.
[†]share senior authorship

adjuvanticity, i.e., the provision of immunostimulatory signals in response to sublethal or lethal stress.[11] Different classes of chemotherapeutics including (but not limited to) anthracyclines (doxorubicin, epirubicin, mitoxantrone), platinum-based compounds (oxaliplatin) and alkylating agents (cyclophosphamide), as well as some forms of radiation therapy, photodynamic therapy, and oncolytic virotherapy, succeed in restraining tumor growth as they trigger immunogenic cell death (ICD), a specific form of cellular demise that culminates in the activation of an adaptive tumor-targeting immune response.[12] The ability of some anticancer agents to kill malignant cells in 'the right way' converts them into prototypical antigen-donor cells (ADCs) while enhancing the proficiency of antigen-presenting cells (APCs), hence building the foundations for a robust CTL-driven adaptive immune response.[12]

The cancer cell-extrinsic effects of autophagy cooperate with their cancer cell-intrinsic counterparts for the inhibition of malignant transformation. As an example, autophagic responses in myeloid cells have profound anti-inflammatory functions (related to the inhibition of inflammasome activation), and hence prevent the establishment of chronic inflammatory foci that support transformation.[13] Conversely, the cancer cell-extrinsic functions of autophagy rival with their cancer cell-intrinsic counterparts in the context of anticancer immunosurveillance. Indeed, autophagy not only supports the adjuvanticity of malignant cells, but also stimulates the activity of various components of the immune system involved in the recognition and elimination of malignant cells including APCs and CTLs.[14] The purpose of this review is to discuss the cell-extrinsic effects of autophagic responses within malignant cells in the context of natural and therapy-driven anticancer immunosurveillance.

Autophagy in natural immunosurveillance

The elimination of newly transformed cells by the immune system constitutes one of the first barriers against tumor development. Consequently, malignant cells have to develop a wide spectrum of strategies to suppress the immune response that would normally lead to their recognition and elimination.[7] Thus, the qualitative and quantitative nature of the immunological tumor infiltrate changes considerably depending on growth stage. Initially, adaptive immune responses are enabled owing to the presence of dendritic cells (DCs) endowed with the capacity to engulf cancer cell debris and present tumor-derived epitopes on MHC class II and class I molecules to $CD4^+$ and $CD8^+$ T cells, respectively.[15] In many instances, cancer cells progressively modify the tumor microenvironment upon the recruitment (and/or local differentiation) of myeloid-derived suppressor cells (MDSCs) and $CD4^+$IL2RA/$CD25^+$ $FOXP3^+$ regulatory T (T_{REG}) cells, the latter playing a major role in the inhibition of CTL activity.[16]

In a murine model of $KRAS^{G12D}$-driven pulmonary carcinogenesis, the ablation of the essential autophagy gene *Atg5* (autophagy related 5) ensuing the intranasal delivery of an adenovirus encoding the Cre recombinase accelerates the manifestation of malignant lesions as it increases the frequency of tumor-infiltrating T_{REG} cells.[17] Manipulations designed to deplete (i.e., the injection of an antibody specific for IL2RA/CD25 [interleukin 2 receptor, α chain]) or functionally inhibit T_{REG} cells (i.e., the administration of an antibody targeting IZUMO1R/FOLR4/R4 [IZUMO1 receptor, JUNO])[18] retard the development of autophagy-deficient tumors, underscoring the concept that a proficient autophagic program in malignant cells facilitates their immunological control.[17] The subversion of immunosurveillance upon autophagy inhibition affects the capacity of cancer cells to release immunostimulatory signals commonly referred to as damage-associated molecular patterns (DAMPs), which are sensed by specific pattern recognition receptors (PRRs) expressed by immune cells.[19] Most likely, DAMP release also occurs during early oncogenesis as cancer cells suffer (and sometimes succumb to) oncogenic stress. In the context of non-small cell lung cancer (NSCLC), the autophagy-dependent secretion of immunostimulatory ATP (see below)[20] is counteracted by the KRAS (KRAS proto-oncogene, GTPase)-driven overexpression of ENTPD1/CD39 (ectonucleoside triphosphate diphosphohydrolase 1), an ecto-enzyme that initiates the conversion of ATP into immunosuppressive adenosine, which also involves NT5E/CD73 (5′-nucleotidase, ecto).[21] While ATP binds to purinergic receptors such as P2RY2 (purinergic receptor P2Y, G-protein coupled 2) on immature DCs to favor their recruitment, adenosine works as a chemoattractant for T_{REG} cells through its action on ADORA2A (adenosine A2a receptor) and ADORA2B (adenosine A2b receptor).[21] Hence, when autophagy is inhibited, malignant cells preferentially recruit T_{REG} cells over DCs, thus generating an immunosuppressive tumor microenvironment.

Immunohistochemical analyses of human breast carcinoma lesions revealed that the presence of cytoplasmic MAP1LC3B/LC3 (microtubule associated protein 1 light chain 3 β) puncta linked to reduced levels of SQSTM1/p62 (sequestosome 1), which together are indicative of a functional autophagic response, correlated with an improved ratio of CTLs over T_{REG} cells.[22] Similarly, in a rodent model of non-alcoholic fatty liver disease (NAFLD)-driven hepatocellular carcinoma, in which autophagy is disabled by the accumulation of toxic lipid droplets in hepatocytes, tumor progression was associated with the depletion of tumor-infiltrating $CD4^+$ T lymphocytes.[23] Altogether, preclinical and clinical evidence suggests that, at least in some cancer types, the autophagic proficiency of malignant cells modulates tumor infiltration by myeloid and lymphoid cells to support the establishment of an immunostimulatory tumor microenvironment.

Of note, autophagy may not only boost the adjuvanticity of cancer cells but also exacerbate their antigenicity.[24] As a matter of fact, the immunopeptidome of autophagy-competent cells substantially diverges from that of their autophagy-incompetent cells. Owing to the block in protein translation that characterizes the initiation of autophagic responses, as well as to the role of autophagy in miRNA homeostasis, autophagic cells are indeed characterized by a unique repertoire of novel MHC class I epitopes.[14] Such peptides can be either presented on the surface of cancer cells or processed and cross-presented by DCs (upon loading on MHC class I molecules) through a cascade of events that is facilitated by autophagy.[24]

Autophagy also mediates immunostimulatory functions as it supports the survival and function of APCs and CTLs. Besides its effects on the processing of exogenous MHC class II epitopes

(reviewed in ref. 25), autophagy is involved in antigen cross-presentation by DCs, although the underlying mechanisms remain to be precisely elucidated.[19] Moreover, the autophagy-dependent adaptation of mature (vs. immature) T cells to new metabolic requirements (which is prominently based on extensive mitochondrial rewiring) accounts for the extrathymic survival of T lymphocytes, especially $CD8^+$ memory T cells.[26] In summary, the crosstalk between the cell-intrinsic and cell-extrinsic functions of autophagy in all the players of the adaptive immune response to malignant cells dictates natural anticancer immunosurveillance.

Autophagy in cancer therapy

Malignant cells exploit homeostasis-supporting functions of autophagy to the same extent as their non-transformed counterparts. Robust cytoprotective autophagic responses account indeed for the metabolic adaptation of cancer cells to a hypoxic and nutrient-depleted environment, as well as for the survival of disseminated and metastasis-prone neoplastic cells.[3] Moreover, the activation of autophagy has been associated with resistance to different therapeutic treatments including chemotherapy and radiation therapy.[27] Altogether, these findings delineate a decisive role for autophagy in tumor progression and resistance to treatment. Throughout the past decade, this assumption inspired a considerable amount of work dedicated to the development of autophagy-inhibitory strategies that would mediate antineoplastic effects per se or would synergize with conventional anticancer treatments. Nonetheless, the actual efficacy of these strategies in restraining tumor growth remains controversial, due to several problematic issues.

First, most pharmacological blockers of autophagy are rather nonspecific as they target PIK3C3/Vps34 (phosphatidylinositol 3-kinase catalytic subunit type 3), such as wortmannin and 3-methyladenine, or affect lysosomal acidification, such as chloroquine and its derivatives.[28] While the efficacy of these drugs, alone or in combination with a plethora of chemotherapeutics or with radiation therapy, has been extensively corroborated in vitro or in patient-derived cancer cell lines implanted into immunodeficient mice,[27] the link between their anticancer activity and autophagy remains questionable. Thus, chloroquine sensitizes mouse cancer cells to the antineoplastic effects of the DNA-damaging agent cisplatin irrespective of the expression of ATG12 (autophagy-related 12).[29] The knockout of *ATG7* (autophagy-related 7) fails to affect the antiproliferative activity of chloroquine in multiple cancer cell lines expressing mutant KRAS.[30] Finally, the ability of chloroquine to reduce the growth of melanoma cell xenografts is tied to anti-angiogenic effects rather than to bona fide autophagy inhibition.[31]

Second, most studies suggesting that autophagy inhibition improves the efficacy of cancer therapy, including those in which autophagy was genetically blocked with short-hairpin RNAs (shRNA) targeting essential components of the autophagic machinery, were performed in immunodeficient mice, which obviously excludes any crosstalk between autophagy and the immune system from the working hypothesis.[32]

Third, several groups used models of genetically driven cancers in which the overexpression of an oncogene was coupled to tissue-specific ablation of floxed *Atg5* or *Atg7*. In the context of $KRAS^{G12D}$- and $BRAF^{V600E}$-driven pulmonary and pancreatic oncogenesis,[17,33,34] autophagy-incompetent adenomas fail to evolve into adenocarcinomas, confirming the involvement of proficient autophagic responses in tumor progression. Although these findings were obtained in immunocompetent hosts, the evidence that autophagy-mediated repression of tumor growth relies on the presence of a functional TRP53/p53 (tumor protein p53) system[17,35] complicates data interpretation. Moreover, it is uncertain whether a total and irreversible inhibition of autophagy (as obtained by the deletion of *Atg5* or *Atg7*) truly reflects the effects of pharmacological autophagy inhibitors (which would be expected to be partial and reversible). Indeed, combined treatment with chloroquine or hydroxychloroquine fails to yield any significant improvement in the efficacy of multiple anticancer agents in clinical studies.[32] Instead, it has been proposed that such treatments might initiate a vicious cycle facilitating malignant transformation in other tissues.[2,13]

In summary, although much emphasis has been laid on autophagy inhibition as a potential strategy to reduce the fitness of malignant cells, whether this kind of therapeutic intervention provides actual benefits to cancer patients remains to be formally demonstrated.

Autophagy in immunogenic chemo- and radiotherapy

As mentioned above, some chemotherapeutic agents can trigger ICD. Such agents lead to a spatiotemporally coordinated emission of chemotactic and phagocytic signals by neoplastic cells, which can consequently be approached and phagocytosed by DCs. This event provides a major contribution to the establishment of a tumor microenvironment prone to CTL infiltration.[2] Mechanistically, the release of DAMPs from cells succumbing to ICD is characterized by (1) endoplasmic reticulum stress-dependent exposure of CALR (calreticulin) on the cell surface, knowing that CALR is necessary for the engulfment of dying cells by DC precursors expressing LRP1/CD91 (LDL receptor related protein 1); (2) pre-mortem release of ATP, which relies on an autophagy-dependent process involving the CASP3 (caspase 3)-mediated proteolytic activation of PANX1 (pannexin 1) channels, knowing that ATP is an obligatory chemoattractant for tumor infiltration by myeloid cells (via P2RY2)[20] and triggers the inflammasome-dependent secretion of IL1B (interleukin 1 β) that is required for optimal CTL recruitment (via P2RX7 [purinergic receptor P2 X 7]);[36] (3) pre-mortem secretion of type I interferon and autocrine stimulation of type I IFN receptors for the generation of chemokines such as CXCL10 (chemokine [C-X-C motif] ligand 10), which is also required for tumor infiltration by CTLs;[37] (4) post-mortem release of the non-histone nuclear protein HMGB1 (high mobility group box 1), which stimulates the maturation of APCs and optimal antigen presentation;[12] as well as (5) release of ANXA1 (annexin A1), which interacts with FPR1 (formyl peptide receptor 1) on maturing DCs to ensure their recruitment into close proximity of dying cancer cells.[38] All these functional aspects represent an indispensable condition for chemotherapy-induced ICD to be

perceived as immunogenic, and constitute a unique tool for the identification of novel ICD inducers.[39]

As a modifier of cellular adjuvanticity, autophagy is actively involved in the release of ICD-associated DAMPs. Thus, autophagy-deficient cells fail to expose phosphatidylserine (a prominent phagocytic signal) on the plasma membrane as they die, as a consequence of pre-mortem intracellular ATP depletion.[40] Along similar lines, an intact autophagic machinery is required for the routing of ATP from lysosomes to the extracellular milieu in the course of ICD.[20] Through this mechanism, autophagy favors the generation of an extracellular ATP gradient that engages purinergic P2RY2 and P2RX7 receptors on myeloid cells, including DCs and their precursors.[21] In an immunocompetent syngeneic background, the efficacy of mitoxantrone- or oxaliplatin-based chemotherapy against mouse MCA205 fibrosarcomas and mouse CT26 colorectal carcinomas was reduced by the inhibition of autophagy in malignant cells with *Atg5*- or *Atg7*-targeting shRNAs.[41] The limited response of autophagy-deficient tumors to chemotherapy with ICD inducers can be reproduced: (1) in tumor-bearing athymic *nu/nu* mice (which are constitutively immunodeficient, because they lack T lymphocytes); (2) in wild-type mice that are depleted from CTLs by means of a CD8-targeting antibody; (3) in wild-type mice bearing ENTPD1-overexpressing tumors. Conversely, the co-administration of the ENTPD1 inhibitor ARL67156 rescues the response of autophagy-deficient tumors to mitoxantrone and oxaliplatin by increasing pericellular ATP levels.[41] Consistently, in a murine model of $BRAF^{V600E}$-driven melanoma in which delivery of the Cre recombinase into melanocytes engenders oncogene activation and loss of the oncosuppressor *Pten*, the systemic administration of mitoxantrone mediates optimal therapeutic effects only in autophagy-proficient tumors. Indeed, mitoxantrone loses its efficacy when *Atg7* is ablated in melanoma cells as well as when $CD4^+$ T lymphocytes are depleted by means of specific antibodies.[42]

Similarly, the irradiation of CT26 colorectal carcinomas delays tumor progression only if malignant cells are implanted in immunocompetent settings and if tumors can mount an effective autophagic response. Thus, while the stable downregulation of *Atg5* by a specific shRNA sensitizes CT26 cells to radiation therapy when tumors are established in athymic *nu/nu* mice, this maneuver causes relative radioresistance in immunocompetent syngeneic C57BL/6 mice. Also in this model, the administration of ARL67156 restores the response of autophagy-deficient neoplasms to treatment as it promotes tumor infiltration by CTLs.[43] Altogether, these findings delineate a landscape in which autophagy-dependent ATP release in response to immunogenic cancer treatment is required for the induction of a therapeutically relevant tumor-targeting immune response. In further support of this notion, it has recently been shown that *Atg5*- and *Atg12*-targeting shRNAs fail to alter $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cell infiltration in B16 melanomas established in immunocompetent syngeneic mice when chemotherapy is unable to mediate therapeutic effects.[44] Thus, autophagy-dependent danger signaling may not compensate for the intrinsically low immunogenicity of some tumors or the inefficacy of some therapeutic regimens.

Autophagy stimulation in cancer therapy

It is increasingly accepted that the nature and intensity of the stress caused by treatment in cancer cells determine its immunological consequences. As a possible scenario, the activation of adaptive autophagic responses in healthy malignant cells might be immunologically neutral or even tolerogenic. In contrast, autophagy occurring in the context of failing adaptation to stress, i.e., as a preface to cell death, would render cancer cells immunogenic. Thus, autophagy may finely tune the threshold of immunostimulation underlying a proper immune response. In an immunocompetent murine model of $HRAS^{G12V}$-driven glioma, the selective loss of *Trp53* mediated by the orthotopic delivery of the Cre recombinase via a lentiviral vector accelerates tumor progression. Noteworthy, the combinatorial administration of the antidepressant imipramine and the anticoagulant tiprimine *de facto* induces autophagy-dependent cell death in glioma cells and retards tumor progression.[45] Interestingly, these 2 agents increase the cellular levels of cyclic adenosine monophosphate (cAMP) while inhibiting P2Y12 (purinergic receptor P2Y, G-protein coupled 12) receptors on cancer cells. We surmise that these events could favor ATP release and activate TMEM173/STING (transmembrane protein 173), a cAMP-dependent protein that stimulates the expression of type I IFN-related genes, hence mimicking anthracyclines in their capacity to elicit viral mimicry in cancer cells.[37]

Culturing malignant cells in nutrient deprived-medium or fasting mice for 2 d (with *ad libitum* access to water) are strong triggers of autophagy. Nutrient starvation (by itself or combined with anticancer agents) restrains tumor progression unless malignant cells have activating mutations in PIK3CA (phosphatidylinositol 3-kinase, catalytic, α polypeptide), which operates downstream of IGF1R (insulin-like growth factor 1 receptor) to suppress autophagy.[46] Similarly, the reduction in circulating IGF1 (insulin-like growth factor 1) levels normally associated with fasting cycles of 48 h synergizes with immunogenic chemotherapies (including doxorubicin, cyclophosphamide, mitoxantrone and oxaliplatin) in limiting the growth of mouse 4T1 breast carcinomas, B16 melanomas and/or MCA205 fibrosarcomas evolving in immunocompetent syngeneic mice.[47] The ability of fasting to synergize with chemotherapy could be replicated by IGF1R inhibitors, yet is obliterated: (1) if tumors are inoculated into athymic *nu/nu* mice;[48] (2) upon systemic administration of excess IGF1;[47] (3) when non-immunogenic chemotherapeutics such as cisplatin are used;[49] (4) if MCA205 cells are genetically manipulated to deplete ATG5 and ATG7.[50] These results, along with the evidence that starvation enhances extracellular ATP secretion,[51] support the idea that fasting causes an autophagy-dependent immunostimulatory effect.

One starvation cycle of 48 h causes a drastic, yet reversible, weight loss of approximately 20% in mice.[47,50] Alternative strategies have been elaborated to induce autophagy without a major weight loss. Fasting-mimicking diet (FMD) cycles induce a relatively mild weight loss as compared with complete nutrient starvation, while causing a similar drop in circulating IGF1 levels. Combining such a FMD and doxorubicin significantly delays the progression of 4T1 breast carcinoma established in

immunocompetent (but not immunodeficient) mice.[48] Similarly, alternate day fasting (resulting in 30% reduction in calorie intake as compared with standard dietary regimens on normal chow) synergizes with radiation therapy in limiting the progression of mouse 4T1 and 67NR triple-negative breast cancer cells orthotopically injected into immunocompetent BALB/c mice.[52]

The beneficial impact of fasting on the efficacy of immunogenic chemotherapy can be reproduced by caloric restriction mimetics (CRMs). CRMs reduce, as cellular starvation does, the level of cytoplasmic protein acetylation in multiple distinct cell types including cancer cells, hence robustly inducing autophagy.[53] This effect can be obtained through: (1) inhibition of cytosolic acetyl-CoA synthesis by ACLY (ATP citrate lyase), with hydroxycitrate (HC) or SB204990; (2) inhibition of EP300 (E1A binding protein p300) acetyltransferase activity, with spermidine or C646; and/or (3) activation of SIRT1 (sirtuin 1)-dependent deacetylation, with resveratrol.[53] Interestingly, CRMs elicit perturbations in the metabolome and in trophic signals (namely a drop in circulating IGF1) that are largely convergent with those induced by fasting, though without provoking any significant weight loss. The systemic administration of CRMs enhances the efficacy of mitoxantrone- and oxaliplatin-based chemotherapy against MCA205 fibrosarcomas evolving in immunocompetent mice. However, this combinatorial treatment has no effects when neoplasms are established in immunocompromised mice (athymic *nu/nu* mice or mice in which $CD8^+$ lymphocytes have been depleted by a specific antibody). Moreover, CRMs fail to improve the efficacy of chemotherapeutic agents that do not induce ICD (such as cisplatin).[50] The administration of HC improves the efficacy of mitoxantrone in various tumor models including transplantable CT26 colorectal cancers, transplantable TC1 lung cancers, as well as breast carcinomas driven by hormones (i.e., medroxyprogesterone acetate) and carcinogens (i.e., 7,12-dimethylbenz[a]anthracene). Moreover, the administration of HC as a standalone agent significantly reduces the number of malignant lesions in a model of $KRAS^{G12D}$-driven NSCLC. The immunostimulatory activity of HC relies on its capacity to increase the autophagy-dependent secretion of ATP driven by mitoxantrone (as monitored in vivo, in CT26 carcinomas engineered to express an ATP-detecting luciferase variant on the cell surface).[50] Moreover, MCA205 fibrosarcomas overexpressing ENTPD1or an *Atg5*-targeting shRNA fail to respond to mitoxantrone plus HC. Along the same line, HC does not reduce the number of $KRAS^{G12D}$-driven pulmonary lesions if *Atg5* is concomitantly deleted or ENTPD1 concomitantly overexpressed by malignant cells.[50]

The immunological infiltrate of wild-type MCA205 fibrosarcomas evolving in immunocompetent syngeneic hosts, but not that of ATG5-depleted or ENTPD1-overexpressing

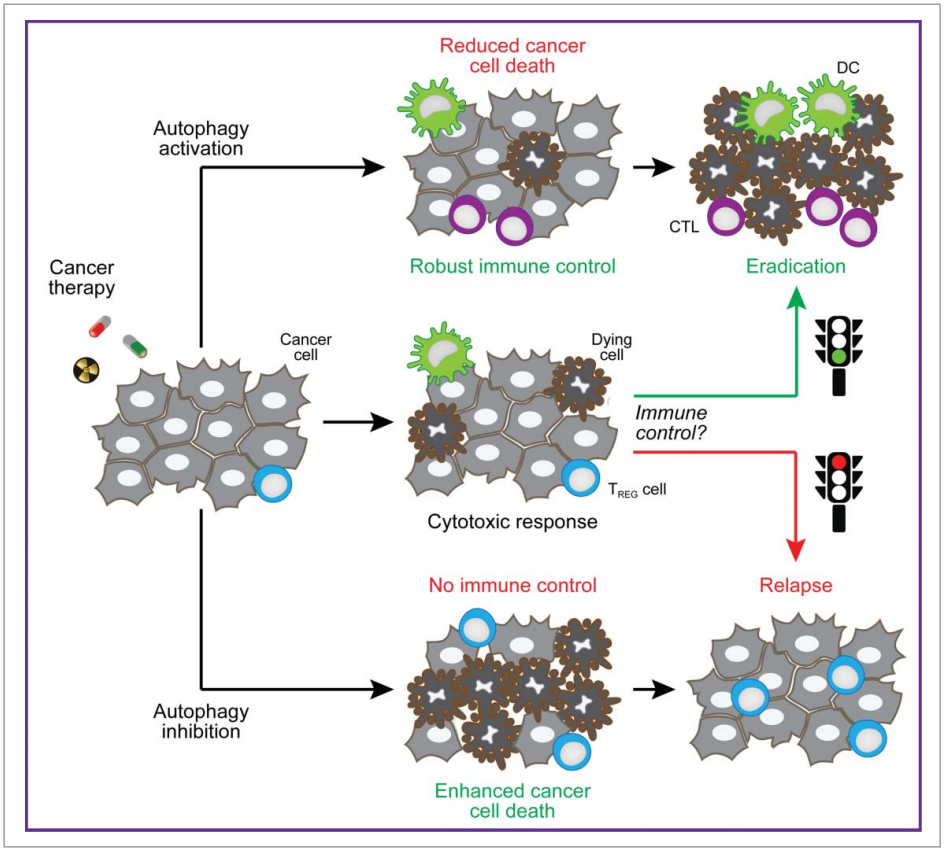

Figure 1. Autophagy modulation in cancer therapy. Since the cancer cell-intrinsic effects of autophagy contribute to tumor progression and resistance to treatment, it has been postulated that autophagy inhibitors may synergize with chemotherapy or radiation therapy to promote disease eradication. However, although inhibiting autophagy may increase the amount of cancer cells succumbing to chemotherapy or radiation therapy, it also prevents the activation of a therapeutically relevant tumor-targeting immune response, hence favoring relapse. Conversely, activating autophagy may limit the amount of malignant cells dying in response to treatment, yet support the elicitation of an anticancer immune response that eradicates disease. CTL, $CD8^+$ cytotoxic T lymphocyte; DC, dendritic cell; T_{REG}, $CD4^+IL2RA/CD25^+FOXP3^+$ regulatory T.

tumors, is characterized by activated and proliferating $ICOS^+$ $MKI67^+$ CTLs and by a reduced number of T_{REG} cells.[50] In this setting, autophagy induction contributes to antitumor immunosurveillance by increasing ATP (over adenosine) levels in the tumor microenvironment, eventually leading to T_{REG} cell depletion.[21] The positive effect of HC on the efficacy of mitoxantrone can indeed be reproduced by: (1) systemic injection of a IZUMO1R-specific antibody to inhibit T_{REG} cell function, (2) diphtheria-toxin mediated depletion of T_{REG} cells in DEREG mice, which have been engineered to express the diphtheria toxin receptor under the control of the *Foxp3* promoter, (3) administration of an antibody specific for NT5E, which limits the extracellular formation of adenosine.[50] Moreover, the treatment of immunocompetent mice bearing 4T1 breast carcinoma with a FMD plus doxorubicin specifically reverts the overexpression of HMOX1 (heme oxygenase 1) by malignant cells, which is a strategy used by tumors to modify the microenvironment to their own benefit.[48] This maneuver, which induces autophagy per se,[54] specifically reduces tumor infiltration by T_{REG} cells.[48]

In our opinion, the depletion of T_{REG} cells from the tumor bed caused by fasting or fasting-mimicking strategies is more likely to be associated with the inhibition of T_{REG} cell recruitment by cancer cells than with a direct effect on T_{REG} cell function or differentiation. Indeed, fasting and CRMs inhibit MTOR (mechanistic target of rapamycin [serine/threonine kinase]) complex 1 (MTORC1), which is necessary for T_{REG} cell differentiation.[55,56] However, HC does not affect the differentiation of naïve $CD4^+$ T lymphocytes into T_{REG} cells in vitro (our unpublished data). These observations suggest that pre-mortem autophagic responses in cancer cells (rather than in T_{REG} cells or their precursors) account for reduced T_{REG} cell infiltration in tumors exposed to fasting or fasting-mimicking strategies. In support of this hypothesis, the anti-inflammatory effects associated with fasting correlate with increased infiltration of target organs by T_{REG} cells. Irrespective of these considerations, the administration of IGF1 (which inhibits HC-induced autophagy) abrogates the anticancer effects of mitoxantrone plus HC, as IGF1 reverses therapy-induced T_{REG} cell depletion. Interestingly, IGF1 supplementation also promotes the expansion of T_{REG} cells,[57] and the involvement of this pathway (possibly dissociated from MTORC1 induction) in the response of malignant lesions to treatment cannot be ruled out.

Finally, CRMs and fasting can promote therapeutic responses by supporting several immunological functions. In mice bearing CT26 carcinomas, spermidine administration reportedly favors tumor infiltration by myeloid cell precursors, hence delaying disease progression.[58] Moreover, spermidine can increase (in an autophagy-dependent manner) the formation of memory $CD8^+$ T cells.[59] Similarly, a FMD has been found to revert immunosenescence (the decline in immunological functions generally associated with aging) and to increase the production of common lymphoid progenitors, which might contribute to tumor infiltration by CTLs.[48] In conclusion, non-immunosuppressive autophagy inducers including fasting and CRMs exacerbate the capacity of immunogenic cancer therapies to elicit a tumor-targeting immune response.

Concluding Remarks

The downregulation of autophagy may constitute a stratagem for cancer cells to evade immune recognition, progress and become clinically manifest. Once tumors are established, the inhibition of autophagic responses within neoplastic cells may promote resistance to ICD-inducing treatments, including some chemotherapeutics and radiation therapy, as a consequence of decreased DAMP emission and limited tumor infiltration by APCs. In this context, the pharmacological stimulation of autophagy can enhance the therapeutic activity of ICD inducers by boosting adaptive tumor-targeting immune responses. Cancer cells can also escape immunosurveillance by directly inhibiting T-cell functions.[60] Indeed, tumor-infiltrating T lymphocytes often express exhaustion markers including CTLA4 (cytotoxic T-lymphocyte-associated protein 4) and PDCD1/PD-1 (programmed cell death 1), hence becoming sensitive to inhibition by molecules of the B7 family, which bind CTLA4 and are mainly expressed by tumor-infiltrating APCs, and CD274/PD-L1 (CD274 antigen) or PDCD1LG2/PD-L2 (programmed cell death 1 ligand 2), which bind PDCD1 and are mainly expressed by neoplastic cells.[60] Clinically approved immunotherapeutics based on the inhibition of CTLA4 and PDCD1 by specific antibodies (so-called checkpoint blockers) restore CTL responses and reinstate immunosurveillance. Despite a proven clinical efficacy in the treatment of various cancers (including metastatic melanoma, NSCLC and renal cell carcinoma), a significant fraction of patients fails to respond to checkpoint blockers as a consequence of: (1) limited tumor antigenicity, (2) an immunosuppressive tumor microenvironment characterized by limited CTL infiltration, or (3) reduced antigen presentation.[61] Since the activation of autophagy participates in the normalization of the tumor microenvironment and promotes CTL infiltration, the co-administration of autophagy stimulators, ICD inducers and checkpoint blockers might significantly improve therapeutic outcome. At this stage, we must further evaluate the possibility of combining autophagy inducers with immunotherapies in preclinical models, so that we can propose optimized therapeutic schedules for clinical evaluation.

Abbreviations

ADC	antigen-donor cell
APC	antigen-presenting cell
cAMP	cyclic adenosine monophosphate
CRM	caloric restriction mimetic
CTL	cytotoxic T lymphocyte
DAMP	damage-associated molecular pattern
DC	dendritic cell
FMD	fasting-mimicking diet
HC	hydroxycitrate
ICD	immunogenic cell death
MDSC	myeloid-derived suppressor cell
MTORC1	mechanistic target of rapamycin (serine/threonine kinase) complex 1
NAFLD	non-alcoholic fatty liver disease
NSCLC	non-small cell lung cancer
PRR	pattern recognition receptor

shRNA	short-hairpin RNA
T_{REG}	regulatory T

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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