The Role of Autophagy at the Nano/Bio Interface - Underlying Mechanisms and Therapeutic Potential in Cancer

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Graphical Abstract

Abstract

Autophagy plays an essential role in maintaining cellular homeostasis by responding to certain stressed conditions such as nutrient deprivation, organelle damage, pathogen infection, and exposure to certain nanomaterials. Through deliberate tuning of the physicochemical properties, the fate of nanomaterial-treated cells is subjected to various cellular toxicity, stress responses, and immune responses, some of which involve various autophagic mechanisms. Beginning from the molecular basis of the autophagy machinery, we briefly introduce the major understanding of autophagy in inflammation, immunity, cancer metabolism, and therapy. Different working mechanisms will be discussed to classify the impact of physicochemical characteristics on autophagy induction or inhibition by engineered nanomaterials. From the perspective of autophagy-targeting cancer therapeutics, we will further delineate the advanced nano-formulations for improved drug delivery to impact autophagy in the setting of cancer diseases and designing co-delivery nanomedicine that targets autophagy along with another major cancer pathway to achieve *in vivo* synergy. Moreover, cancer immunotherapy, aiming at immune cells or checkpoints, is also integrated with autophagy-regulatory components using multiple

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nano-platforms as an emerging strategy for cancer treatment. Considering the recent breakthroughs of nanotechnology targeting autophagy in cancer cells, antigen-presenting cells, or other cell types within tumor microenvironment by precisely designed nanomedicine may provide additional solutions for cancer treatment through autophagy-dependent metabolic regulation or immune pathways. However, one should closely monitor the extent of autophagy alternation and side effects from certain nanoparticles to avoid severe toxic responses.

Keywords:

Autophagy, engineered nanomaterials, cancer therapy, immune response, nanomedicine

Purpose and Rationale

Breaking the word “autophagy” into two pieces, “auto” refers to “self”, and “phagy” is an alternative way to say “eat”. Observed initially in the glucagon-perfused rat liver, scientists reported this interesting phenomenon by demonstrating the degradation of mitochondria and other organelles within lysosomes [1]. While an essential catabolic process for maintaining cellular homeostasis and attenuating cell stresses, autophagy and its regulatory mechanisms are not thoroughly investigated yet [2, 3]. This profound process also intensively involves many pathological conditions, which is best described in cancer. The process of autophagy has been conceptually divided into three major categories, *i.e.*, macro-autophagy, micro-autophagy, and chaperone-mediated autophagy; among these mechanisms, macro-autophagy is the primary and the best-characterized form. While we will briefly discuss these mechanistically distinct macro-autophagy processes in section 1, suffice to mention here that more than 30 autophagy-related genes (ATG) have been discovered in various organisms, including mammalian cells [4-6]. Noteworthy, this sophisticated cell machinery becomes the basis for understanding the impact of external materials, which could be an insult or therapeutically beneficial to the cell type of interest.

With the rapid emerging of engineered nanomaterials (ENMs), ample evidence has revealed the significant impact of ENMs on autophagy [7], which could exert a hazardous response or become useful therapeutics. For the former, a variety of ENMs have been demonstrated to regulate autophagy since ENMs are frequently sequestered in the autophagosomal and/or lysosomal compartments [8]. For the latter, early studies have demonstrated the possibility when using nano-enabled approaches to target autophagy in

various disease scenarios, such as cancer, overcoming drug resistance, infection diseases, and certain rare diseases (*i.e.*, Huntington's disease [9]). In this mini review, we will begin from the fundamental biology of autophagy to illustrate its critical roles in tumor biology, inflammation, and immunity. This will be followed by a discussion about how the physicochemical properties of ENMs could impact autophagy. Finally, from the therapeutic perspective, we will focus on nano-enabled approaches to target autophagy for direct cancer treatment and the creative design of nano-platforms for cancer immunotherapy. Before we conclude, we also share our perspectives on this interesting topic.

Summary of relevant literature

Autophagy in cancer

Macroautophagy is mediated by autophagosome that delivers engulfed cargos to the lysosomes for degradation. The autophagosome formation requires the hierarchically ordered activities of ATG proteins recruited at the phagophore assembly site (PAS) [10]. A classical macroautophagy process includes the following: (i) activation of the ULK1 kinase complex to initiate the phagophore formation; (ii) nucleation through class III phosphatidylinositol-3-OH kinase (PI(3)K) complex (Vps34/Beclin 1/Vps15/ATG14L); (iii) membrane elongation and completion through ATG12/ATG7/ATG5/ATG16 and microtubule-associated protein 1A/1B–light chain 3 (LC3)/ATG8 ubiquitin-like conjugation systems; and (iv) maturation by fusing with lysosomes [11, 12]. Recently, several ATG proteins have been discovered to function in a non-canonical autophagic manner [13-16]. These ATG proteins utilize a portion of the canonical autophagy machinery and bypass some steps to create a “short-path” for enhanced degradation. For example, LC3-associated phagocytosis (LAP) is a process that

is independent of the ULK1 complex but requires PI(3)K complex and ubiquitin-like conjugation systems. The degradation is mediated by the fusion between LC3-positive phagosomes with lysosomes [13]. We recently identified a Beclin 2-mediated non-canonical degradation pathway that bypasses Beclin 1 and

the ATG5/7/16L conjugation system, during which Beclin 2-associated ATG9A⁺ vesicle could direct fuse with the pre-autophagosomal structure for target protein disposal [15, 16]. The differences among canonical and non-canonical autophagic processes are illustrated in Fig. 1.

Fig. 1 Scheme illustration of canonical macroautophagy, Beclin 2-mediated non-canonical autophagic degradation, and LC3-associated phagocytosis pathways.

Notably, autophagy dysregulation has been frequently found in tumor cells and perpetuated inflammation. Autophagy serves as a catabolic process to break down proteins and damaged organelles into amino acids to support the nutrition need for sustained tumor growth. Therefore, a group of small-molecule compounds targeting different steps in autophagy has been applied, repurposed, and tested in clinical trials for cancer treatment, such as hydroxychloroquine (HCQ). The commonly used activators and inhibitors for autophagy manipulation process are illustrated in Fig. 2. On the contrary, several ATG proteins have been found to play tumor-inhibitory roles through directly suppressing the oncogenes or tumor-associated inflammation. For example, Beclin 1 is a haploinsufficient tumor-suppressor gene inhibiting anti-apoptotic Bcl-2 family proteins *via* its BH3 domain by restraining tumorigenesis [17]. Heterozygous deletion of *Beclin 1* in mice increases the incidence of spontaneous tumors [18, 19]. Therefore, strategies using Beclin inducer such as Tamoxifen to increase the synthesis of Beclin 1, became a well-recognized anti-tumor drug for breast cancer treatment [20]. By contrast, Beclin 2, a homolog protein of Beclin 1, although sharing a ~57% sequence identity with Beclin 1 and a predicted BH3 domain, suppresses tumorigenesis by inhibiting the inflammatory responses and MAPK pathways [14, 15]. Autophagy balances inflammation in innate immunity and appears to generate multiple effects on immunity. Innate immune signaling pathways, including type I interferon, NF- κ B, and inflammasomes, are activated through recognition of pathogen-associated molecular

patterns (PAMPs) and damage-associated molecular patterns (DAMPs) by innate immune receptors such as DNA sensors, toll-like receptors, and NOD-like receptors (NLRs) [21-24].

Fig. 2 Autophagy regulation using inhibitors and activators.

While the innate immune response is critical in sensing malignant cells (such as DAMPs released from damaged cancer cells) and supporting an effective adaptive immune response, excessive inflammatory responses may promote cancer development [25-27]. The recognition that autophagy has an anti-inflammatory function, stems from the observation that the production of interleukin 1 beta (IL-1 β) and IL-18 is increased in the absence of functional ATG16L1 in a mouse model of Crohn's disease [28]. An inflammasome complex, consist of pro-caspase 1, the adaptor protein ASC, and a cytoplasmic sensor protein, could respond to PAMPs and DAMPs by inducing the proteolytic processing and secretion of IL-1 β and IL-18 [29]. Several convergent reports show that autophagy has a negative role in inflammasome activation by removing intracellular DAMPs, degrading inflammasome components, and the control of biogenesis and secretion of IL-1 β production [1-5, 30-34]. Autophagy deficiency leads to an accumulation of depolarized mitochondria,

leading to the leakage of mitochondrial DNA (for AIM2 recognition) and reactive oxygen species (ROS) production (which activates the NLRP3 sensor) [33]. We recently identified that Beclin 2 could mediate the degradation of multiple inflammasome sensors by delivering them to autophagosomes through ATG9A-associated vesicles [16]. Loss of Beclin 2 could induce excessive inflammasome activation and IL-1 β production to exacerbate the alum-induced peritonitis in a mouse model [16]. We also showed that Beclin 2 could target essential kinases to control NF- κ B and ERK1/2 signaling to inhibit the inflammation-associated spontaneous lymphoma development [15]. Autophagy could also suppress type I interferon signaling in response to viral infection or gut microbiota [35, 36]. Autophagy-deficient mice could produce more microbiota-dependent IFN-I production to protect against infection and chemical injury of the intestine [36]. SARS-CoV-2 M protein could induce mitophagy (mitochondria elimination through autophagy) to inhibit the type I IFN response

while blocking autophagy using inhibitors (3-MA or chloroquine) inhibited viral replication [35]. As an unconventional form of autophagy, LAP could also reduce pro-inflammatory responses and type I interferon signaling pathways in tumor-associated macrophages (TAM) to promote tumor immune tolerance for tumor progression [13]. Therefore, targeting specific ATG proteins or autophagic pathways either through canonical or non-canonical form may alter innate immune signaling and affect tumor initiation and progression.

Autophagy in disease-related immune response

Another essential role of autophagy is the involvement in antigen presentation. Regulation of autophagy could potentially affect the antigen-presentation through major histocompatibility complex (MHC) class I and class II molecules to impact the subsequent adaptive immune responses. Recent studies have shown that autophagy can deliver cytoplasmic materials or pathogens to the lysosomes for MHC class II antigen presentation [37-39]. Starvation-induced autophagy could enhance the MHC class II presentation of peptides from intracellular sources. On average, the highest peptides presentation level was raised by 131% after 24 hours of autophagy induction [37]. In Epstein Barr virus-transformed lymphoblastoid cells, viral antigens accumulate in autophagosomes when lysosomal acidification is blocked, indicating a role of autophagy in delivering viral antigen to lysosomes.

Additionally, blocking autophagy in Epstein Barr virus-transformed B cells leads to reduced recognition by antigen-specific CD4⁺ T cells, further indicating the critical role of autophagy in viral antigen presentation by MHC class II molecule [37, 38]. Dendritic cells (DCs) deficient in ATG5 appear to have compromised antigen processing and presenting by MHC class II molecules, leading to an impaired CD4⁺ T cell response after herpes simplex virus infection [40]. These studies indicated that DCs require functional autophagic machinery to process and present antigens onto MHC class II molecules efficiently. Other evidence also indicated a role of autophagy in the cross-presentation of exogenous antigen via MHC class I. In a macrophage cell line, autophagy

inhibitor blocked MHC class I antigen presentation and the antigen-specific CD8⁺ T cell responses against HSV-1 infection. A similar finding was also evident in tumor antigen presentation that inhibition of autophagy almost completely abolished cross-presentation, while induction of autophagy enhanced the cross-presentation of tumor antigens [41, 42]. These reports have led to an expanded view of antigen presentation in which autophagy can play an active role.

Although the regulatory mechanisms of ATGs in cancer have not yet been fully clarified and the functional pathways vary, the role of ATG proteins in metabolism, innate immune signaling, and immunity made them potential therapeutic targets for cancer therapy [11, 43, 44]. Therefore, regulating autophagy protein or machinery at the nano/bio interface could potentially create novel therapeutics against malignancy. Furthermore, precision nanomedicine by rational design that targets specific ATG proteins or certain autophagic steps may provide better solutions for complicated scenarios, especially for tumor therapy or tumor microenvironment (TME) regulation.

Assessment and monitoring of autophagy

To correctly monitor autophagy, multiple techniques should be applied depending on each experimental setting. According to the latest guideline published by Daniel J. Klionsky et al., briefly, autophagy could be assessed through the observation of autophagosomes under transmission electron microscopy, formation of GFP-ATG8 puncta under fluorescent microscope, detection and quantification of autophagic substrate proteins such as p62 or ATG8 family proteins turnover by western blotting with and without lysosome inhibitors [45]. Another method of evaluating autophagy flux is to measure the decomposition rate of a given protein through autophagy. Autophagy can be blocked at a given point, and then the accumulation of organelles, organelle markers, cargo markers, or the entire cargo at the clogged point over time is recorded. One can also track the decrease rate of the protein markers of autophagic degradation over time after completely inhibiting the protein synthesis. Under certain conditions, researchers

also need to determine if they need to assess the early or late autophagic compartments (phagophores, autophagosomes, autolysosomes). No single detection method can be entirely satisfactory to every situation, especially when assessing the nanoparticle-related autophagic process [45].

The roles of intrinsic physicochemical properties of engineered nanomaterials on autophagy

An increasing number of ENMs have been reported to modulate the process of autophagy. After ENMs are taken up and trapped within the endomembrane system, autophagy and lysosomal dysfunction are frequently found as an emerging mechanism of ENMs toxicity. Reported examples include metals, metal oxides, rare earth oxide (REO) nanoparticles, carbon-based materials, polymeric nanoparticles, quantum dots, and Stober silica. Exposure of cobalt and chromium (CoCr) nanoparticles to the BeWo trophoblast barrier could alter the autophagic flux and induce a significant production of IL-6. Interestingly, indirect CoCr nanoparticle exposure by transferring conditioned media from the BeWo trophoblast culture could induce DNA damage in neuron cells and astrocytes, and such effect depends on autophagy in the BeWo barrier. As a result, inhibiting autophagy in the BeWo barrier significantly reduced the level of DNA damage in astrocytes. These results collectively suggest a critical role of autophagy dysfunction in initiating CoCr nanoparticle-induced DNA damage [46-50].

By contrast, another study reported that extravillous trophoblast (EVT) cells might utilize autophagy to maintain cellular homeostasis after phagocytosing platinum nanoparticles (nPt). Herein autophagy functions as a protective mechanism to reduce nPt cytotoxicity in human trophoblast cells [46-50]. Regarding the autophagy alternation, various mechanisms are involved, including oxidative stress, direct or indirect ubiquitination of nanomaterials, and inhibition of mTOR signaling, etc [8, 46, 51-55]. While many nanoparticles were found to be engulfed to trigger the formation of autophagosomes [47, 56, 57], other reports suggest that nanoparticles may suppress autophagy flux by lysosome

destabilization, during which the unique physicochemical property plays a dominant role [51, 54, 58]. The primary mechanism reported for autophagy induction is the ability of ENMs to generate abundant ROS as a “stress” signal to trigger autophagy [47]. Reactive oxygen and nitrogen species are essential signaling molecules at basal levels; however, both could damage mitochondria at high levels. So far, autophagy is the primary mechanism for responding to danger signals from damaged mitochondrial, such as mitochondrial DNA (mtDNA), to execute mitochondrial turnover. With a high surface area to volume ratio, Nanoparticles are therefore potent ROS inducers to trigger autophagic process. By contrast, the inhibition of autophagic flux is primarily caused by ENM-induced lysosome dysfunction through impairing the autophagosome maturation process [47, 51, 54, 56-58]. Nanoparticles, primarily taken up via endocytosis, will end up in lysosomes for degradation. Due to the inability to degrade inert nanoparticles such as gold nanoparticles, the accumulated nanoparticles may destabilize the lysosome. Therefore, among a substantial number of studies describing the ENM-induced autophagy, inorganic hard ENMs (such as gold nanoparticles) have more likelihood to affect autophagy over soft materials (such as polymers). On top of that, the surface modification and size were the dominant factors that affect the autophagic process since both significantly affect the ROS production and lysosome integrity and function.

CdSe/ZnS quantum dots (QDs) could provoke autophagosome accumulation in a ROS-dependent manner [30]. Further studies compared the cytotoxicity and intracellular processing of QDs of different sizes in human bone marrow mesenchymal stem cells and found that smaller QDs could regulate autophagy and induce autophagic cell death more extensively than larger QDs [55]. Similarly, zinc oxide (ZnO) nanoparticles could induce ROS-dependent mitochondrial damage, the formation of autophagosomes, and autophagic cell death. Such an effect can be rescued by ROS scavenger treatment [59]. Interestingly, autophagy induction by ENMs may have cell-type specificity. Iron oxide nanoparticle-induced autophagy is correlated

with ROS production and mitochondrial damage in cancer cells (A549) but not normal cells (IMR-90) [52]. Studies have also shown that nanoparticles, such as Au nanoparticles (Au NPs) and silica nanoparticles, may induce autophagy in a dispersion-dependent manner, and the degree of autophagy was regulated by adjusting the dispersion status of nanoparticles [56]. Another possibility while seeing the accumulation of autophagosomes is not autophagic induction but the impairment of lysosome function. As a leading example, Ma et al. demonstrated a size-dependent lysosomal alkalization and blockage of autophagosome maturation, owing to the size-dependent uptake and accumulation of Au NPs in lysosomes in normal rat kidney cells. Larger Au NPs (50 nm) that were taken up more efficiently compared to their smaller counterparts (10 and 25 nm) impaired the autophagy flux more potently [58]. Another finding showed that silica nanoparticles inhibited autophagic degradation via a similar lysosomal impairment mechanism [54]. We recently identified an endocytosis-independent autophagy impairment by ultrasmall Au NPs. We examined the oxidative stress of BMDCs after exposure to 200 µg/mL Au 4.5, 13, 30, and 70 nanoparticles for 6 hours. Our data showed that 4.5 nm-sized Au NPs (Au 4.5) were localized in cytoplasm and produced a higher level of ROS than larger-sized counterparts.

Interestingly, Au4.5 could target LC3, a central autophagic machinery protein, for proteasome-mediated degradation. The direct cell penetration ability of Au4.5 causes ubiquitination of LC3 proteasome-mediated degradation to inhibit the autophagy machinery without inducing significant cytotoxicity [51]. Next to size, some studies have uncovered the surface charge-dependent autophagy regulation by ENMs. Positively charged nanoparticles, in general, have higher uptake efficiency and membrane permeabilization ability, thus leading to a higher capability to cause lysosome damage. Therefore, cationic nanoparticles induced more autophagosome accumulation than negatively charged ones even though both are of similar diameters [58]. Comparatively, PEG-functionalization will likely hinder the nano/bio interface and thus impede cellular uptake-dependent autophagy. While formulating a nanoparticle into a fine-tuned

size and surface charge may alter the cellular processing, surface modification of peptide RE-1 (ACTARSPWICG), RE-1 peptide variants, or RGD could also regulate the autophagy-inducing activity [49]. By contrast, to avoid nanoparticle-induced lysosome accumulation or autophagy dysfunction, a popular idea is to design “smart” elements for lysosome escape. The polymeric nanoparticles with pH-responsive elements could efficiently escape from the endo-lysosome compartments to enhance the cytoplasmic delivery of drugs, proteins, peptides, antisense oligonucleotides, or siRNA [60-62]. For example, using equimolar amounts of pH-sensitive 2-propyl acrylic acid (PAA) and DMAEMA to formulate neutral charged smart polymer nanoparticles could cause pH-dependent membrane damaging activity for efficient siRNA delivery. In another study, a unique pH/redox double sensitive cationic monomolecular NP containing imidazole residues was developed for cytoplasmic siRNA delivery. The efficient endosomal/lysosomal escape of NPs-siRNA complex was empowered through osmotic expansion and endosomal/lysosomal membrane destruction [60-62].

So far, it is inconclusive what is the “most effective” size or modification to affect autophagy since many parameters contribute to the induction and alternation of the autophagy process. For example, autophagy may act as a protective mechanism against ENMs invading by responding to ROS stress signal [53], but excessive ROS production could overwhelm the cells to induce autophagic cell death [59]. Furthermore, while smaller-sized nanoparticles can induce ROS due to the substantially higher surface-over-volume ratio than the larger counterparts, cellular uptake was postulated more efficiently for 40-60 nm-sized NPs. Therefore, systemically characterize the autophagy alternation ability of NPs with different sizes, surface charges, and modifications are warranted. This data collection and understanding are crucial for the design of safe and effective nanoparticles for medical uses.

Design of nanocarriers to impact autophagy in cancer

While profound and highly attractive as a therapy, one of the key challenges in using

autophagy targeting drugs is the inadequate tumor access and non-specific biodistribution *in vivo*. Take the weak-base autophagy blockers chloroquine (CQ) and HCQ, for instance, the protonation effect under the acidic tumor TME leads to the formation of cell membrane-impermeable molecules, preventing the efficient use of these payloads as free drugs. Moreover, for synergistic tumor inhibition, it has been popular to design drug pairs to target autophagy in addition to a major cancer pathway, which demands synchronized pharmacokinetics (PK) and optimal drug ratio at the cancer site.

While the role of autophagy is complex and sometimes contradictory, it is generally believed that chemotherapeutic agent-induced stress response can induce autophagy, which becomes a major mechanism to promote cancer cell survival. This has become a popular approach, some of which are being tested clinically, to improve the killing effect of chemotherapy drugs or restore drug sensitivity by introducing autophagy inhibitor plus chemo. Various pharmacological inhibitors were tested in the literature, including PI3K inhibitors such as 3-methyladenine and wortmannin and lysosomal neutralization reagents such as CQ, HCQ, and bafilomycin A1 [41]. Similar advancement has been made in the content of nanomedicine, which has led to exciting outcomes at the intact animal level. Want et al. demonstrated the possibility of making an HCQ liposome, which also carries a TH-RGD targeting peptide [63]. Using this delivery platform, the authors showed a significant increase in intratumoral drug concentration for more than 10-fold and major toxicity reduction in a B16F10 melanoma mouse model. When combining with chemotherapeutics, such as doxorubicin (DOX) or liposomal DOX, the best-performing treatment by co-administration of DOX and HCQ liposomes gave >60 days median survival outcome, compared to the control treatment using free drug pair (median survival of 16 days) [63]. The same group also showed the successful synthesis of paclitaxel/HCQ co-delivery liposome, leading to enhanced potency inhibiting primary tumor growth and reducing lung metastasis in the

B16F10 model *in vivo* [64]. Interestingly, the authors' data strongly indicate the immunomodulatory effects of their formulation, such as the CXCR4/CXCL12 axis, which exemplified a new direction of autophagy-mediated nano-immunotherapy for cancer management [64].

Noteworthy oncology findings suggested that both autophagy inhibition and autophagy induction are therapeutically helpful as the latter may impact the anti-apoptosis pathways. Literature suggested that autophagy induction by mTOR or Bcl-2 inhibitors may synergize with chemo options, evidenced in a long list of cancer types, such as glioma, breast cancer, renal cancer, prostate cancer, and mesothelioma [4, 65, 66]. Therefore, these creative combinations were also formulated and tested using various nanoparticles (see Table 1 at the end of the article). In addition to design nanocarriers using autophagy drugs plus chemo agents, we developed a ratiometrically designed nanocarrier targeting autophagy pathway plus a cell-cycle pathway cyclin-dependent kinase (CDK)4/6 for efficient pancreatic cancer treatment (Fig. 3) [67]. We optimized the synergistic drug ratios when combining HCQ plus palbociclib (PAL) in a panel of cultured pancreatic cancer cells, demonstrating PAL:HCQ of 5:1 exerted across-the-board drug synergy in multiple cell lines (Fig. 3). When ratiometrically co-packaged into a mesoporous silica nanoparticle (MSNP)-based carrier, the leading formulation could yield a synchronized PK profile and secured drug ratio, which are key factors for optimal drug synergy in subcutaneous (s.c.) and orthotopic PANC1 models. Nano-enabled ratiometric co-delivery outperformed a list of control treatments, including free drug mixture. We continued to show that repetitive administrations of this drug combination formulation paradoxically activated an anti-apoptosis pathway. This has promoted the introduction of a small molecule pan-Bcl-2 inhibitor, ABT-737, to potentiate the effectiveness of our particles, yielding a long-lasting anti-cancer outcome (Fig. 3).

Fig. 3 Use of ratiometrically designed nanocarrier targeting CDK4/6 and autophagy pathways for effective pancreatic cancer treatment. (A) Use of CompuSyn software to determine Combination index (CI) in a panel of pancreatic cancer cell lines receiving PAL/HQ mixtures at various combination molar ratios. CI < 1 indicated a synergistic effect and CI ≤ 0.5 indicated a strong level of synergy. A drug ratio of 5:1 led to an across-the-board synergy in all the tested cell lines. (B) Ratiometric design of PAL/HQ co-delivery nanoparticle based on using lipid-coated mesoporous silica (silicasome) nanoplateforms. (C) CryoEM visualization of ratiometric co-delivery silicasome. (D) Use of GFP-RFP-LC3B dual reporter assay to determine the blockade on autophagosome-lysosome fusion. This experiment showed that both HCQ-only and co-delivery liposomes prevented autophagosome-lysosome fusion. Scale bar: 20 μm. (E) Evaluation of PK profile and PAL/HQ ratio in plasma. Mice received free drug or ratiometric co-packaged PAL/HQ nanoparticle through intravenous administration. The PAL/HQ ratio in plasma was calculated and plotted versus time. (F) Efficacy study. Upper panel: PANC-1 tumor-bearing mice received ratiometric PAL/HQ co-delivery nanoparticles through intravenous administration. Various controls were included, including a free drug mix. Lower panel: In a separate efficacy experiment, the introduction of an anti-apoptosis inhibitor, ABT-737, could further potentiate the effectiveness of PAL/HQ co-delivery nanoparticles, leading to a long-lasting anti-cancer outcome in vivo. Figure adapted from Ji Y et al. Nat Commun. 2020 [67].

Design of autophagy-regulatory nanomedicine for immunotherapy

Owing to the critical role of autophagy in antigen-presentation, a nanoparticle-based delivery system that carries antigens along with autophagy inducers showed great potential to enhance T cell activity (see Table 1). DCs are one of the most professional antigen-presenting cells (APCs) for the activation of naïve T cells to drive antigen-specific immunity against cancer. Therefore, promoting the autophagy in DCs may induce potent antigen-specific immunity after vaccination [27, 42]. Aluminum microparticle (also known as Alum) is the only licensed particulate adjuvant in the US. Alum is well known as a potent adjuvant for antibody responses, but its ability to prime cytotoxic T lymphocytes is limited [68]. By formulating aluminum into nanosized, α-Al₂O₃ nanoparticles could trigger autophagy and deliver antigens to autophagosomes for antigen

presentation. Ovalbumin (OVA), as a model antigen, was conjugated to α-Al₂O₃ nanoparticles, TiO₂ nanoparticles, and α-Fe₂O₃ nanoparticles of similar sizes [57].

Interestingly, α-Al₂O₃ nanoparticles exhibited more potent T cell priming and antigen-cross presentation ability than other particle adjuvants, suggesting a chemical composition-dependent adjuvant effect. Consequently, immunization of mice with tumor antigen-conjugated α-Al₂O₃ nanoparticles resulted in significant tumor regression [57]. Furthermore, conjugating autophagy inducers can achieve manipulation of autophagy to nanoparticles, such as linking a Beclin 1 peptide (NH₂-CGTNVFNATFHIWHSGQFGT-COOH) to the backbone of the polymer. The polymeric nanoparticles that upregulate autophagy in DCs thus leading to a high-efficiency antigen presentation of OVA and OVA-specific T cells activation [69].

In addition to adjuvating antigen-specific T cell responses through the antigen-presentation mechanism, nanoparticles may also improve the antibody responses by modulating the inflammasome activity through autophagy. Our recent study indicated a size-dependent autophagy suppression by Au nanoparticles. Ultrasmall-sized nanoparticles (Au4.5) suppressed autophagy by target LC3 for proteasome-mediated degradation. Being a negative regulator of the inflammasome, suppression of autophagy induced excessive NLRP3 accumulation for enhanced inflammasome activity [51]. Another

comparative analysis was conducted in macrophages receiving ENMs with different chemical compositions, such as multiwall carbon nanotubes (MWCNT) and rare earth oxide (RES) nanoparticles. Li et al. demonstrated that MWCNTs and REO nanoparticles are potent inducers of NLRP3 inflammasome assembly. In the case of MWCNTs, NLRP3 assembly and IL-1 β production were accompanied by intact autophagy homeostatic regulation. Thus, the treated cells were capable of rapidly removing activated NLRP3 complexes.

Fig. 4. Au nanoparticles exhibited potent NLRP3-dependent adjuvant activity and enhanced antibody production through autophagy inhibition mechanism. (A) Optical images of Au nanoparticle suspension and TEM analysis of Au nanoparticles. (B) ELISA test for the induction of IL-1 β production in supernatants of bone marrow-derived DCs primed with LPS (100 ng/ml, 3 h) alone or followed by 6 h of different sized Au nanoparticles (Au4.5, Au13, Au30, or Au70) treatment at increasing doses (50, 100, and 200 μ g/ml), or 1 h ATP (5 mM) treatment. (C, D) Immunoblot analysis of cell lysates (C) and ELISA for IL-1 β in supernatants (D) of DCs primed with LPS (100 ng/ml, 3 h), followed by treatment with 200 μ g/ml Au4.5 for different times. (E-G) Scheme illustration of mice receiving OVA or OVA plus adjuvant immunization. 6-8 weeks old female C57BL/6 wild-type or NLRP3-deficient mice were subcutaneously injected with PBS, OVA alone, OVA adjuvanted by Au nanoparticles (2 mg), or OVA adjuvanted by alum (2 mg) on day 0 and day 7. Serum samples were collected on days 14, 28, and 42 for OVA-specific IgG (H+L) production antibody titer analysis (n=5) (F, G). *p < 0.05, **p < 0.01. Figure adapted from Zhu M et al. ACS Nano. 2020 [51].

This differs from REO ENMs mediated lysosomal injury severity-wise, which damages this organelle to the extent that it disrupts autophagosome fusion and removal of the activated NLRP3 complexes; this leads to exaggerated IL- β production [51]. Importantly, NLRP3 inflammasome activation has been implicated for T cell-mediated cancer immunotherapy [70] and adjuvant activity for antibody production [71, 72]. Furthermore, the nanoparticles that activated NLRP3 inflammasomes have been demonstrated to markedly enhance OVA-specific antibody production in an NLRP3-dependent manner (Fig. 4) [51]. Therefore, tuning autophagy by nanoparticles with selective properties could enable the development of novel cancer vaccine adjuvants.

Immune checkpoint blockade therapy aims to take the “brake” off the immune system to activate the anti-tumor immunity fully. The levels of programmed cell death-1 (PD-1) with

its ligand (PD-L1), two major immune checkpoints, have been reported to be upregulated by autophagy inhibitors in different cancers such as melanoma, ovarian cancer, and gastric cancer [73]. Liu et al. developed a lipid bilayer-coated MSNP, also known as a silicasome, for chemo-immunotherapy [74]. Irinotecan, a weak-basic chemo-drug, neutralizes the acidic lysosomal pH, leading to autophagy inhibition and PD-L1 expression in pancreatic ductal adenocarcinoma (PDAC) cells. Therefore, checkpoint blockade therapy, combined with silicasome-empowered irinotecan delivery, synergistically enhanced the anti-tumor effect in an orthotopic PDAC mouse model [74]. Similarly, legumain-responsive Au nanoparticles were loaded with DOX and autophagy inhibitor HCQ [75]. Co-administration of the nanoparticle with anti-PD-L1 antibody enhanced the anti-glioma effect and efficiently prevent glioma recurrence [75].

Conclusions and perspective

Although the correlation between autophagy and cancer is complicated and sometimes contradictory, the critical role of ATG protein in metabolism, innate immune signals, and immunity makes it a potential therapeutic target for cancer. Considering autophagy’s essential role in maintaining cellular homeostasis, it may impact various aspects during cancer development and progression.. Alternatively, autophagy may suppress or facilitate cancer growth through different mechanisms, as illustrated in Fig. 5.

Fig. 5 Competing outcomes of autophagy inhibition/promotion in cancer development. Abbreviations: TAM, tumor-associated macrophage; LAP, LC3-associated phagocytosis; DC, dendritic cells.

in tumor development, cell-type-specific nano-formulations for targeted therapy are needed to manipulate autophagy in cancer cells, cancer-associated macrophages APCs, or other cells within TME. For example, to deliver cancer antigens for cancer immunotherapy, autophagy inducers could be co-delivered by nanoparticles to promote autophagy-dependent antigen-presentation for a potent anti-tumor immune response. By contrast, autophagy inhibitors could be delivered specifically to cancer cells by nanoparticles to prohibit tumor nutrition supply when aiming at cancer metabolism. In addition, with the advent of nanotechnology, it has become popular to consider constructing co-delivery nanocarriers or designing nano-enabled combination therapy to implement “autophagy interference” + “X”. The candidates for “X” could be a nuclear acid-based active pharmaceutical ingredient (API) or their combinations, which *per se* largely require nano-formulation. The apparent advantage of such an approach is the “synergy” by implementing synchronized PKs, multi-target combinations, and improved safety profile of the combination. These become the major strengths to augment the likelihood of a successful tumor therapy in vivo. The third interesting aspect is to systemically dissect the critical roles of nanomaterial properties in autophagy regulation. However, when attempting to do this, one should closely monitor the extent of autophagy alternation to avoid unexpected toxic responses due to engineered nanomaterials' diverse physical and chemical properties. This may become the basis of designing drug-free nano-formulations to replace the classical pharmacological compounds that target autophagy.

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Conflict of interest

The authors declare no conflicts of interest. For signed statements, please contact the journal office: editor@precisionnanomedicine.com

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Supporting File:

Table 1 The use of autophagy-regulatory nanoparticles for cancer therapy

Target	Cancer model	Treatment	Antitumor effect	Key observation of autophagy	Ref
Tumor cells	SK-Hep-1 hepatoma xenograft model	Carboxy-functional iron oxide nanoparticle (Fe ₂ O ₃ @DM SA)	SK-Hep-1 tumor cells with NPs co-implanting model: ~33% tumor inhibition compared to PBS; SK-Hep-1 subcutaneous tumor model: ~42% tumor inhibition compared to PBS	LC3-II and p62 protein↑ Formation of autophagosomes (LC3B, p62, Beclin 1, ATG2A, ATG16L1) mRNA↑ Autophagic cell death↑	[76]
	MCF-7 cancer cells xenograft tumor model	Bec1 (an autophagy-inducing peptide)-modified pH-sensitive polymers	~40% tumor inhibition compared to PBS	LC3-II/LC3-I protein↑ p62 protein↑ LC3-II protein↑	[77]
	A549 lung cancer xenograft model	Au NPs (5 nm in diameter) attached onto the amino-functionalized MSNs (denoted as GCMSNs) loaded with camptothecin (CPT)	Significantly inhibited tumor growth by high CPT-loaded GCMSNs treatment (p < 0.05)	LC3-I, LC3-II protein↑ ATG5 protein↑ LC3 protein↑	[78]
	143B osteosarcoma xenograft model	Fullerene C60 nanocrystals (s.c.) + chemical inhibitor KN-93 (s.c.)	~25% tumor inhibition compared to PBS	Autophagic flux↓ (autophagosome accumulation and impairment of autophagic degradation) LC3-II protein↑ p62 protein↑ Cathepsin B and cathepsin D↓	[79]
	A549 lung cancer xenograft model	Pt(IV)-peptide-bis(pyrene), cRGD NPs encapsulated	A549 tumor nude mice model: 86.04% tumor inhibition compared to PBS;	1. siBec1@PPN induced autophagy inhibition 2. A549 tumor cells: Beclin 1 and LC3-II ↓	[80]
	Pt-resistant A549 lung cancer xenograft model	Beclin 1 siRNA (siBec1@PPN)	Pt-resistant A549 tumor-bearing	3. Pt-resistant A549 tumor cells: Beclin 1 and LC3-II ↓ (non-	

		athymic nude mice: 83.98% tumor inhibition compared to PBS	treated group higher than normal A549 tumor cells)	
C6 glioma xenograft model; C6 glioma rechallenge model	Legumain- responsive Au NPs (D&H- A-A&C) encapsulated DOX and HCQ+ anti- PD-L1 antibody	C6 glioma model: 56 days median survival time compared to control ~28 days; C6 glioma rechallenge model: 60.9 days median survival time compared to control ~18 days	1. LC3-II/LC3-I ratio ↑ 2. Autophagosomes ↑ observed <i>via</i> transmission electron microscope (TEM)	[81]
KB (s.c.) tumor model; orthotopic 4T1 breast cancer model;	3- bromopyruvat e (3BP, an autophagy promoter and hypoxia ameliorator) integrated into photosensitize r chlorin e6 (Ce6)- encapsulated nanoparticles (CD-Ce6-3BP NPs)	1. KB model: complete inhibition; low systemic toxicity; 2. Orthotopic 4T1 model: complete inhibition; reduced metastatic tumor nodules in lung	LC3-II/LC3-I protein ratio ↑ p62 protein ↓ LC3 puncta dots ↑ Autophagosomes↑	[82]
4T1 breast cancer model	Au nanocage encapsulated pentacarbonyl	Complete inhibition	1. LC3 protein↑ 2. Autophagosomes ↑	[83]
Panc-1 s.c. and orthotopic xenograft model	Lipid-coated MSNP co- encapsulate CDK4/6 inhibitor PAL and HCQ	Panc-1 s.c. model: ~43% tumor inhibition compared to phosphate buffered saline (PBS); Panc-1 orthotopic models: ~20% tumor inhibition compared to PBS; ~36% tumor inhibition compared to PBS when combined with BCL2 inhibitor ABT-737	1. Autophagosomes ↑ 2. p62 protein↑ 3. ROS ↓ 4. LC3-II/LC3-I ratio ↑	[67]

DCs	CT26 tumor xenograft model;	Honeycomb calcium carbonate (CaCO ₃) nanoparticles (HOCN)+ mitoxantrone (MTX)	10% tumor inhibition compared to saline; secondary tumors: complete inhibition	1. LC3-II ↑ and LC3-I ↓ in DCs 2. Autophagosomes in DCs ↑ 3. Antigen was encapsulated by autophagosomes in DCs ↑; colocation rate of antigen and autophagosome was up to 84% 5. LC3-II ↑ in the lymph nodes	[84]
	CT26 abscopal tumor model				
	B16-F10-OVA xenograft model	Polymer conjugated with Beclin 1 peptide and antigen peptide OVA ₂₅₇₋₂₆₄ (NP-B-OVA)	~12% tumor inhibition compared to saline (NPs by s.c./i.v.)	1. LC3B II ↑, p62 ↓ in DCs 2. Antigen cross-presentation ↑ by inducing autophagy	[85]
	B16-OVA melanoma xenograft model	α-Al ₂ O ₃ -OVA	11% tumor inhibition compared to PBS	1. Nanoparticles co-localized with the autophagosome marker-LC3 2. Autophagosomes containing α-Al ₂ O ₃ nanoparticles was observed by TEM 3. knockdown of Beclin 1 in DCs blocked the cross-presentation of OVA	[86]

NP = nanoparticles; PBS = phosphate buffered saline; TEM = transmission electron microscope.