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REVIEW ARTICLE

Endothelial cell autophagy in homeostasis and cancer

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Autophagy, the major lysosomal pathway for the degradation and recycling of cytoplasmic materials, is increasingly recognized as a major player in endothelial cell (EC) biology and vascular pathology. Particularly in solid tumors, tumor microenvironmental stress such as hypoxia, nutrient deprivation, inflammatory mediators, and metabolic aberrations stimulates autophagy in tumor-associated blood vessels. Increased autophagy in ECs may serve as a mechanism to alleviate stress and restrict exacerbated inflammatory responses. However, increased autophagy in tumor-associated ECs can re-model metabolic pathways and affect the trafficking and surface availability of key mediators and regulators of the interplay between EC and immune cells. In line with this, heightened EC autophagy is involved in pathological angiogenesis, inflammatory, and immune responses. Here, we review major cellular and molecular mechanisms regulated by autophagy in ECs under physiological conditions and discuss recent evidence implicating EC autophagy in tumor angiogenesis and immunosurveillance.

Keywords: autophagy; cancer; endothelial cells; immunosurveillance; tumor vasculature

The role of autophagy in noncancer cells within the tumor microenvironment (TME) and its effects on tumor progression is increasingly being recognized. However, both the global aspects and the precise mechanisms through which autophagy regulates stromal and host responses influencing cancer progression and therapeutic outcome remain poorly elucidated at present. We recently reviewed the effects of endothelial cell (EC) autophagy on tumor angiogenesis and tumor vascular structure. Here, we instead focus on how autophagy shapes the interface between ECs and the immune system and discuss potential mechanisms which may regulate this interaction.

Abbreviations
AMPK, AMP-activated protein kinase; ATG, autophagy-related genes; ATP, adenosine triphosphate; BECN1, Beclin1; cGAMP, cyclic guanosine monophosphate–adenosine monophosphate; cGAS, cGAMP synthase; CQ, chloroquine; CXCL, chemokine CXC motif ligand; DC, dendritic cells; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; ERGIC, endoplasmic reticulum–Golgi intermediate compartment; FA, fatty acids; FAO, fatty acid oxidation; FOXO1, forkhead box protein O1; HIF1α, hypoxia-inducible factor 1α; ICAM-1, intracellular adhesion molecule 1; IDO, indoleamine 2, 3-dioxygenase; IFN, interferon; IL, interleukin; IRF3, interferon regulatory factor 3; LAP, LC3-associated phagocytosis; MAM, mitochondria-associated membrane; MAVS, mitochondrial antiviral signaling protein; MDSC, myeloid-derived suppressor cells; mTOR, mechanistic target of rapamycin; NO, nitric oxide; NOTCH1, notch receptor 1; RIG-I, retinoic acid-inducible gene I; ROS, reactive oxygen species; SIRT1, NAD^+-dependent histone deacetylase sirtuin 1; STING, stimulator of interferon genes; TEC, tumor endothelial cells; TFEB, transcription factor EB; TME, tumor microenvironment; Treg, regulatory T cells; ULK1, unc-51 like autophagy activating kinase 1; UVRAG, UV radiation resistance-associated gene protein; VCAM-1, vascular cell adhesion molecule 1; VEGF(R), vascular endothelial growth factor (receptor); vWF, von Willebrand factor; WPB, Weibel-Palade bodies; WT, wild-type.

A brief outlook on autophagy pathways

Macroautophagy (hereafter called autophagy) is a constitutive catabolic process for bulk or selective encapsulated lysosomal degradation and recycling of obsolete or damaged cytoplasmic cargo including organelles. During nutrient deprivation or in response to various intrinsic and extrinsic stress signals, autophagy is stimulated. This sustains cell survival and its metabolic needs, by mitigating cellular damage and by increasing macromolecule recycling [1]. Hence, autophagy fulfills cell's vital requirement for quality control and stress adaptation, making it crucial for maintaining cellular, tissue, and organism homeostasis. Beyond these key cell-autonomous roles, autophagy exerts cell nonautonomous functions by regulating unconventional secretion, inflammatory, and immune responses [2]. Not surprisingly, therefore, dysregulation of autophagy is a common trait of numerous pathological states including metabolic diseases, neurodegeneration, and cancer, among others [3].

At the molecular level, autophagy entails the formation of double membrane-bound structures called autophagosomes, which are destined for cargo delivery to the lysosomes. Formation of autophagosome distinguishes this catabolic process (macroautophagy) from other types of autophagy. These include microautophagy, in which cargo is directly internalized by the lysosomes/late endosomes and chaperone-mediated autophagy, which guides aberrant proteins for LAMP2A-mediated lysosomal degradation through recognition of their KFERQ-like motif by the cytosolic heat-shock cognate 70 (Hsc70) chaperone [4]. The autophagic machinery consists of a conserved set of autophagy-related genes (ATG) hierarchically organized to coordinate the initial phase of autophagosome formation, its elongation, trafficking, and fusion with the lysosomes (for a complete view of molecular autophagy, readers are referred to [1,5]). These events are controlled by mechanistic target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) signaling pathways, which sense changes in nutrient availability, or by other forms of induced physiological or metabolic stress. Nutrient starvation leads to decreased mTOR activity and subsequent dephosphorylation of transcription factor EB (TFEB), the master regulator of lysosomal biogenesis [6]. TFEB then translocates from the cytoplasm to the nucleus and leads to transcription of several autophagy and lysosomal genes [7]. Two major complexes, namely the unc-51 like autophagy activating kinase 1 (ULK1) complex (consisting of ULK1, RB1CC1/FIP200, ATG13, and ATG101) and the class III PI 3-kinase complex I [Beclin1 (BECN1), PIK3C3/VPS34, PIK3R4/VPS15, ATG14, NRBF2, and UV radiation resistance-associated gene protein (UVRAG)] along with the ATG9 trafficking system (ATG2A or ATG2B, WDR45/WIPI4 and the transmembrane protein ATG9A) tightly regulate the formation of the initial sequestering structure, termed the phagophore. Subsequently, membrane expansion and phagophore elongation are carried out by two ubiquitin-like (Ubl) conjugation systems composed of the ATG12-ATG5 complex requiring the action of E1 and E2 enzymes ATG7 and ATG10, which subsequently bind to ATG16L1 and ATG8-family proteins (including the LC3 and GABARAP subfamilies). Conjugation of the ATG8/LC3 family proteins to the phagophore membrane resident lipid phosphatidylethanolamine (known as LC3 lipidation) drives further expansion of phagophore, which eventually encloses a portion of the cytoplasm and closes to form the autophagosome. The exact source of the phagophore membrane is yet to be identified, but different subcellular compartments including the ER, ER–Golgi intermediate, the Golgi, mitochondria, and the plasma membrane can seemingly serve as suppliers. The last step of the autophagy pathway involves the fusion of the autophagosome with the lysosome, ultimately leading to the degradation of the cargo through lysosomal hydrolases and the subsequent release of various metabolites through autolysosomal membrane-associated permeases [5] (Fig. 1).

Autophagy can be stimulated by various intrinsic and extrinsic stressors and can be nonselective or selective. Nonselective autophagy leads to the bulk degradation of a part of the cytoplasm and has no cargo selectivity. In selective autophagy, a number of signaling molecules (often with redundant functions) responding to specific stress signals operate as adaptors or receptors to link a specific cargo to the autophagy machinery and deliver it for degradation. Among the different forms of selective autophagy, the pathway for clearance of damaged mitochondria, called mitophagy, is probably the best studied, owing to the key role of mitochondria in cellular fitness, metabolism, and inflammation [8–10]. A variety of genetic and pharmacological studies have shown that disposal of damaged mitochondria by mitophagy is central for the ability of autophagy to dampen inflammation driven either by intrinsic or by extrinsic stressors [11]. PARKIN-mediated ubiquitination of mitochondria followed by p62-mediated mitophagy prevents mitochondria from releasing reactive oxygen species (ROS), cardiolipin, and mitochondrial DNA, which all act as molecular mediators of the NLRP3

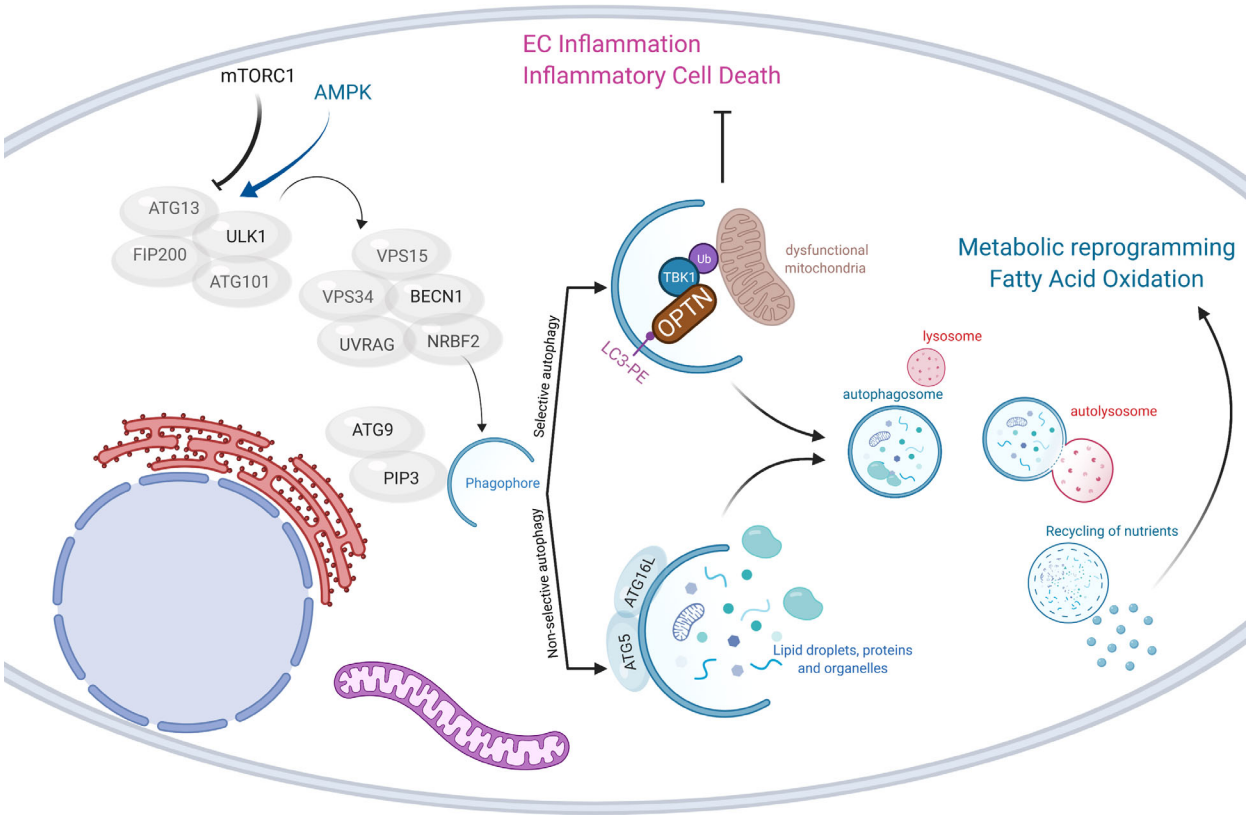

Fig. 1. In healthy EC, autophagy is a quality control mechanism that maintains intracellular homeostasis. Different ATG proteins are involved in the multistep autophagy cascade. Superfluous, obsolete, or damaged proteins and even whole organelles (e.g., damaged mitochondria that are otherwise a source of cell-damaging ROS) are engulfed into a membrane-coated vesicle that ultimately fuses with the lysosome. Selection of the cargo can either happen in bulk or by more selective mechanisms like in mitophagy, where specific adaptor molecules such as TBK1 and OPTN are used to tether (ubiquitinated) cargo-specific surface proteins to LC3-PE which is embedded in the phagophore. After fusion, degradation of the cargo takes place to their fundamental building blocks (e.g., amino acids, fatty acids, etc.) to be metabolically recycled (e.g., beta oxidation) or save the EC from inflammatory cell death caused by mitochondrial ROS.

inflammasome. Activation of the inflammasome leads to CASPASE-1 activation and consequent processing and release of potent pro-inflammatory cytokines such as interleukin-1b (IL-1b) and IL-18 [12]. Autophagy can also directly control inflammation since several components of the inflammasome undergo autophagic degradation [13]. Xenophagy, the lysosomal pathway for the removal of invading pathogens (bacteria and viruses), is stimulated by the recognition of pathogen-associated molecular patterns *via* pattern recognition receptors such as the Toll-like receptor and NOD-like receptor to degrade pathogens and attenuate pathogen-induced inflammation. Interestingly, xenophagy and mitophagy share molecular features: Indeed, both intracellular bacteria and mitochondria are recognized by autophagy receptors such as p62, NBR1, and NDP52 to promote their autophagic clearance [14]. Given that mitochondria derive from bacterial endosymbionts, xenophagy and mitophagy could highlight a primordial and evolutionarily conserved function of autophagy that mitigates inflammation triggered by intrinsic or extrinsic stressors in multicellular organisms [15].

Autophagy pathways also set a threshold for the antiviral immune responses elicited by the two principal sensors for cytosolic DNA and RNA, cGAMP synthase (cGAS), and retinoic acid-inducible gene I (RIG-I), respectively. Upon activation by viral DNA, cGAS-mediated production of cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) leads to the activation of stimulator of interferon genes (STING). Conversely, after sensing viral RNA, RIG-I binds to and activates mitochondrial antiviral signaling protein (MAVS) complex located at the outer membrane of the mitochondria. Both pathways converge in the activation of interferon regulatory factor 3 (IRF3)

and NF-kB driven production of type I interferon (IFN) and other cytokines, which elicit host resistance to viral infections and activate key components of the innate and adaptive immune system [16]. Both the activation of STING and MAVS pathway stimulates autophagy [17–19]. In a recent study, upon translocation of STING to endoplasmic reticulum–Golgi intermediate compartment (ERGIC) and the Golgi, STING containing ERGIC was shown to be a membrane source for LC3 lipidation and ATG5-driven autophagy, which facilitated clearance of DNA and viruses in the cytosol [20]. Interestingly, STING from the anemone *Nematostella vectensis* induces autophagy in HeLa cells but no IFN response, which suggests that autophagy may be a primordial function of the cGAS-STING signaling pathway [20]. Both the antimicrobial and the inflammatory arms of the cGAS-STING pathway cross-talk at mitochondria level, since STING and NLPR3 interact with MAVS within a specific microdomain of ER–mitochondria apposition, called mitochondria-associated membrane (MAM) [21]. Importantly, MAM also coordinates mitochondrial morphology and autophagosome formation [22]. The tight compartmentalization of pro-inflammatory and innate immunity complexes with the autophagy machinery at the MAM highlights the crucial role of autophagy in sensing inflammatory and innate immune responses against intracellular pathogens. However, excessive activation of STING- and MAVS-mediated antiviral responses can elicit severe inflammatory diseases (for an extensive review, see [23]), implicating the need of a rheostat mechanism to prevent their excessive activation.

Other forms of selective autophagy, including ER phagy (the removal of portions of the ER membrane) and lipophagy (the specific degradation of lipid droplets) with increasing implications in physiological and pathological conditions, are also emerging. Moreover, different *ATG* genes are increasingly recognized to have 'moonlighting' functions implicated in broader intracellular vesicular trafficking processes that are independent of their degradative functions. These include, but are not limited to, exocytosis, conventional, and unconventional (i.e., independent of the ER-to-Golgi anterograde system) secretion [2]. LC3-associated phagocytosis (LAP) is a recently recognized form of ULK1- and ubiquitin-independent phagocytosis engaged against several pathogens, which targets extracellular entities for lysosomal degradation [24,25]. LAP utilizes several *bona fide* autophagy proteins including UVRAG, BECN1, VPS34, ATG5, ATG7, ATG12, and members of the LC3 family [2] and it is thought to have developed as part of the host–pathogen co-evolution [26].

In conclusion, the functions of autophagy in cellular homeostasis (Fig. 2) are complex and the impact of the specific degradation of organelles or cytoplasmic materials on the cell's fate has far larger implications than originally thought. It is also likely that components of the autophagy machinery may be differentially recruited to support selective degradation of proteins [27,28]. This would depend not only on the type of stressor to which the cell is exposed, but also on the specialized function, differentiation, or metabolic state of the targeted cell, as discussed in the following sections.

Autophagy in the vascular endothelium

Endothelial cells are the main cellular constituents of the vascular system in vertebrates. Maintaining a functional EC status is therefore essential for vascular homeostasis. EC lining the vasculature forms the main interface between blood circulating nutrients, oxygen, signaling molecules, and all tissues of the body. The vasculature is also the main conduit for leukocytes and various inflammatory cells, thus playing an essential role in wound healing, immune responses, and resolution of inflammation. Consequently, defects in EC functionality caused by pathological insults or aging processes are causative of a variety of diseased states including, among others, atherosclerosis, metabolic diseases, uncontrolled inflammation, and cancer. Although the role of autophagy in EC has not been completely unraveled, accumulating evidence implicates this catabolic process to vascular development, homeostasis, and a variety of (age-related) vascular pathologies [3]. In line with this, activation of autophagy by caloric restriction exerts vasculoprotective functions and delays vascular aging [29,30]. Here, we will briefly discuss known and novel connections linking EC autophagy to redox homeostasis, metabolism, and secretion (a more detailed discussion can be found in recent reviews [31,32]).

Autophagy as a guardian of EC homeostasis

In adult organisms, the endothelium is in a quiescent state, but it can become activated and proliferative in response to several physiological and pathological stressors. During wound healing, angiogenesis (e.g., the formation of new blood vessels from pre-existing ones) is essential for tissue restoration and to alleviate

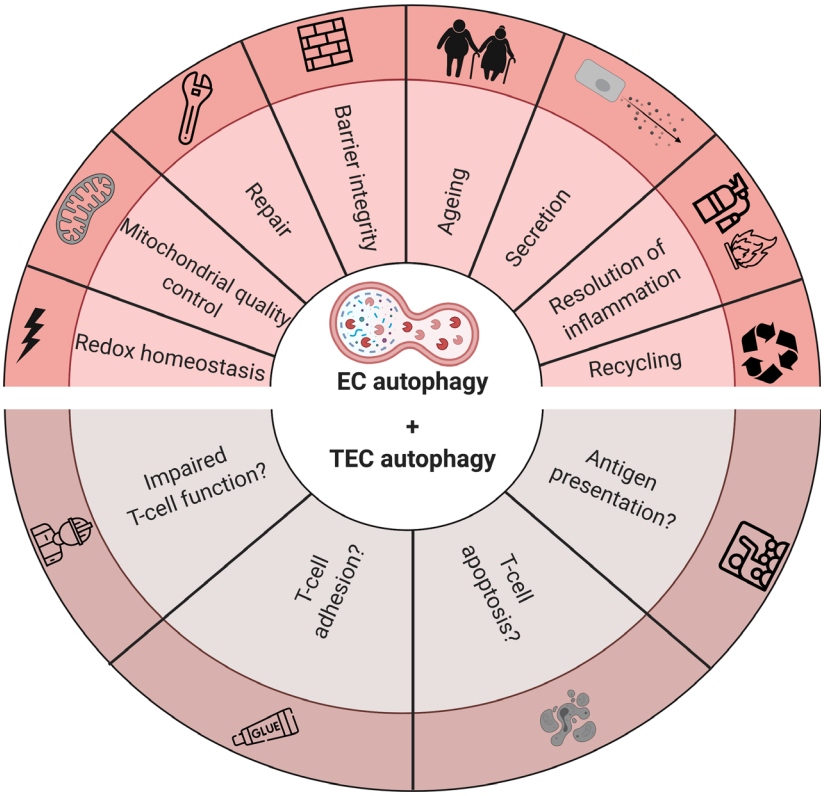

Fig. 2. Autophagy in EC has vasculoprotective functions and supports the major hallmarks of healthy EC, by preserving redox homeostasis, mitochondria quality control, repair, and adequate responses to inflammation, among others. As such, EC autophagy plays important roles at the tissue and organism levels. However, as discussed in this review and still speculative, autophagy in tumor EC (TEC) may be corrupted by the hostile TME and metabolic stress. In this case, TEC autophagy may contribute to—among other functions—maintain an immunosuppressive TME by interfacing with immune cells, through downregulation of adhesion molecules, impairment of T-cell survival and functionality.

tissue inflammation by supplying oxygen, nutrients and by regulating the trafficking of immune cells through the expression of adhesion molecules and homing factors/cytokines [33]. EC possess a high degree of plasticity, which allows them to rapidly respond to various pro-angiogenic cues and to return in a quiescent status after restoration of tissue homeostasis. Return to a quiescent state is essential after resolution of wound healing. If EC activation continues in an uncontrolled manner, EC can perpetuate to a state of chronic inflammation. The efficiency of this plasticity/adaptation mechanism in mice declines with the aging, which is indeed accompanied by lower expression of several autophagy genes [34].

Autophagy in EC is emerging as a crucial mechanism to keep EC quiescence in-check and to control EC permeability [35]. This is largely due to its ability to regulate the cellular redox tone, which is central to proper EC function and permeability and is required for the maintenance of their quiescent phenotype [36]. While a basal level of oxidative stress is thought to maintain proper EC functions, increased levels of ROS stimulate angiogenesis or may lead to cell death [37]. Under physiological conditions, ROS induced by laminar shear stress stimulates EC autophagy through flow-induced activity of NAD^+-dependent histone deacetylase sirtuin 1 (SIRT1) and TFEB [6,38]. SIRT1 can stimulate autophagy by direct deacetylation of both pro-autophagic proteins and of the forkhead box protein O1 (FOXO1), triggering transcriptional upregulation of a subset of autophagy genes in EC [39]. Apart from its transcriptional effects, FOXO1 regulates autophagy by binding to ATG7 in the cytoplasm and restricting EC migration and angiogenesis [40]. Moreover, shear stress-induced SIRT1/FOXO1 signaling maintains quiescence by repression of c-Myc [39]. Interestingly, SIRT1 itself is susceptible to autophagic degradation, a process that contributes to the loss of SIRT1 during aging [41]. Whether this bidirectional control between SIRT1/FOXO1 activity and autophagy is involved in the overall reduced autophagic capacity in the aged endothelium is unknown but is an interesting connection to be explored in future studies.

Not only do ROS induce autophagy, but this catabolic process in turn maintains low/homeostatic redox levels, thereby controlling vascular permeability and barrier integrity [36]. Genetic or pharmacological manipulation of autophagy in EC elevates ROS and increases EC permeability *in vitro*, which can be partially rescued by antioxidants [36]. Moreover, regulation of the redox tone is implicated to signaling and bioavailability of nitric oxide (NO), a molecule which

plays a vital role in preserving EC functions [42]. Oxidative stress in EC induces expression of endothelial nitric oxide synthase (eNOS), which causes uncoupling of eNOS from a NO-producing enzyme to a superoxide anion-producing enzyme, further promoting EC dysfunction [43]. Whether the decreased autophagic flux observed in the aged endothelium [44] contributes to a ROS-mediated change in the activity of eNOS and impaired bioavailability of NO is an interesting connection which remains to be explored. However, several studies in animal models indicate that vascular ROS (especially mitochondria-generated ROS) are involved in the development of hypertension [45]. Moreover, EC autophagy plays a crucial role in stabilizing atherosclerotic plaques [46] and EC-specific *ATG7*-deficient mice retain higher LDL levels with an increased atherosclerotic burden compared to wild-type (WT) mice [47]. TFEB was identified as a vasculoprotective therapeutic target by reducing EC inflammation and atherosclerosis formation *via* the canonical autophagy pathway but also by autophagy-independent mechanisms [6]. Altogether, these studies support the critical importance of autophagy in redox control and as a crucial atheroprotective mechanism.

Mitochondria are the powerhouse for ATP production and generation of mitochondrial ROS enhances the inflammatory traits of EC [48]. Thus, removal of damaged mitochondria through mitophagy may contribute to the intrinsic beneficial effect of EC-associated autophagy on redox homeostasis, energy balance, and inflammatory responses. The PARKIN/PINK1 pathway for mitophagy is activated in response to metabolic stress in EC and prevents mitochondrial dysfunction and metabolic stress-induced endothelial injury [49]. However, the functional link between mitochondria clearance in EC and the control inflammatory responses remains poorly explored. Interestingly, DRP1-driven mitochondrial fission and consequent increase in ROS production in EC is required for the induction of the NF-kB pathway, vascular cell adhesion molecule 1 (VCAM-1), and leukocyte adhesion in response to TNF and other pro-inflammatory factors both *in vitro* and *in vivo* [50]. Given that mitochondrial dynamics and autophagy are two intimately linked processes [51], accumulation of fragmented and ROS producing mitochondria could ultimately stimulate mitophagy as a mechanism to facilitate resolution of the EC inflammatory responses. In line with this, liver-specific deletion of *ATG5* or *ATG7* in EC in mice impairs the ability to handle oxidative stress and results in the upregulation of pro-inflammatory genes in liver EC, which then favors the development of liver fibrosis and nonalcoholic steatohepatitis [52]. However, downregulation of *ATG7* due to thrombin exposure in cultured EC attenuates actin stress fiber generation, which is required for NF-kB activation and expression of inflammatory genes [53]. This suggests that depending on the inflammatory mediator and its downstream signaling pathway, specific autophagy pathways may either repress or amplify inflammatory responses in EC.

Recent *in vivo* studies underscore that EC metabolism is a key regulatory trait of EC specification, function, and angiogenesis [54]. Glycolysis in quiescent EC is elevated in response to pro-angiogenic stimuli and fosters endothelial migration and proliferation [55]. Yet, mitochondrial fatty acid oxidation (FAO) is the prevalent metabolic pathway supporting the tricarboxylic acid cycle for redox homeostasis and vasculoprotection in quiescent EC [56], while sustaining nucleotide synthesis in the highly proliferative EC [57]. Interestingly, during aging in mice, reduced SIRT1 activity in EC is accompanied by impaired fatty acids (FA) uptake, impaired FAO, mitochondrial dysfunction, and oxidative stress [58]. The functional link between SIRT1 and autophagy in EC [38] and the emergence of lipophagy as a source of FA through the degradation of lipid droplets in several cell types [59] suggests that lipophagy (co)regulates EC redox tone and the quiescent EC phenotype by supplying FA for FAO. This is an interesting hypothesis warranting further investigations.

Furthermore, being the most important lysosomal degradation route and given the noncanonical roles attributed to several *ATG* in the regulation of endocytic pathways and secretion, autophagy in EC may control both the composition of the surface proteome and of their secretome [2]. Mice with endothelial-specific conditional deletion of *ATG7* or *ATG5* are viable, with no obvious defects in vessel structure or capillary density. However, these mice display a deficit in the secretion of von Willebrand factor (vWF), an important mediator in the coagulation cascade, and prolonged bleeding time after epinephrine stimulation [60]. Along with bioactive molecules such as P-selectin, IL-8, angiopoietin 2, and endothelin 1, vWF is contained in endothelial secretory granules called Weibel–Palade bodies (WPB) which require acidic pH to be packaged and secreted [61]. In the absence of ATG5 or ATG7 (but not of Beclin1), WPB displays a more alkaline pH thereby reducing the ability of vWF to be included into functional WPB and thus to be secreted. Although chloroquine (CQ) phenocopies the *in vivo* effects of the genetic loss of *ATG5 or ATG7* in EC, thus strengthening the relevance of an acidic pH for the trafficking and release of vWF by EC, the precise

molecular mechanism underpinning this autophagy-regulated process remains unknown. However, other studies indicate that altering lysosomal pH and thus autophagosomal degradation by CQ, does not always recapitulate the effects of the genetic deletion of autophagy genes in EC [32]. EC-specific knockout of *ATG5* does not affect Notch receptor 1 (NOTCH1) signaling, which is a critical regulator of EC quiescence [62]. On the other hand, CQ leads to the activation of NOTCH1 signaling through the endocytic route, resulting in NOTCH1 intracellular domain-mediated EC quiescence and vessel normalization in tumor-bearing mice (Fig. 3A) [62]. Hence, various autophagy pathways operate in EC with the main purpose to protect EC functions, to maintain EC redox and metabolic homeostasis, and to coordinate EC responses to inflammatory cues.

Heightened autophagy is a hallmark of the TME

Autophagy is a malleable pathway and it is now clear that it co-evolves with the TME. In growing tumors, autophagy is elevated as a result of the hypoxic, pro-inflammatory, and nutrient-deprived microenvironment [63]. At the initial stages of tumor development, autophagy exerts relevant quality control mechanisms in cancer cells and mitigates tumor formation through the downregulation of tumor-promoting inflammation, thereby favoring immunosurveillance. In rapidly growing tumors, autophagy is hijacked by oncogenes to support the increased metabolic needs of cancer cells [64]. Eventually, cancer cell-autonomous autophagy begins to operate as a machinery involved in trafficking and exporting pro-inflammatory/pro-angiogenic cytokines or chemotactic/pro-invasive molecules such as extracellular ATP [65], thus educating the TME.

Interestingly, not only tumor autophagy but also host autophagy is emerging as a key regulator of the TME and cancer development. In host cells, autophagy can support tumor growth by providing essential nutrients in circulation or within the TME [66–68]. In addition to supplying nutrients, host autophagy counteracts key immunosurveillance mechanisms within the TME [69]. The functional role of autophagy in the tumor-associated vasculature, especially in response to stressful conditions like hypoxia and uncontrolled angiogenesis in the TME, remains an open question. To fulfill the increased demand for oxygen and nutrients, cancer cells initiate vascular endothelial growth factor (VEGF)-mediated angiogenesis resulting in heightened proliferation of tumor ECs TEC [31]. These rapidly dividing TEC form a functionally abnormal vasculature with a weak structural integrity (patchy endothelium and discontinuous basement membrane) and insufficient number of supporting mural cells (pericytes and smooth muscle cells) [70]. Structural and functional abnormalities of the vessel wall result in the disruption of blood flow and inadequate perfusion of oxygen/nutrient-rich blood. Disruptions in blood flow and oxygen availability foster hypoxia, nutrient deprivation, acidity, and inflammation, ultimately promoting tumor growth and dissemination of cancer cells [71].

Role of autophagy in the tumor-associated vasculature

Observations suggest that the autophagic machinery from healthy EC and TEC can respond differently to hypoxia (one of the key features of TME) [72]. Unlike normal EC and similar to cancer cells, increased autophagy in EC imparts resilience to hypoxia-induced cell death by increased activity of hypoxia-inducible factor 1α (HIF1α) and BCL2 interacting protein 3 (BNIP3) [72]. After *in vitro* exposure to hypoxia, quiescent human umbilical vein-derived EC shows impaired autophagic flux and downregulation of *BECN1*, while bone marrow primary multiple myeloma EC maintains the expression of autophagy markers and show resistance to apoptosis after hypoxia. In contrast, as compared to WT mice, hemizygous deletion of autophagy essential *BECN1* increases pro-angiogenic features and the aggressive growth pattern of subcutaneously injected B16-F10 melanoma cells under hypoxia [73]. Lung-derived EC from these *BECN1* hemizygous mice displays enhanced proliferation, migration, tube formation, angiogenic capacity, and hypoxia-induced stress response (upregulation of *HIF1α* expression), suggesting that genetic loss of autophagy enhances hypoxia-stimulated angiogenesis, in these settings. These contradicting results may relate to *in vitro* vs *in vivo* conditions, where TME-derived factors may play a major role in determining TEC fate. However, given the relevant homeostatic role of autophagy in EC, one may wonder whether heightened TEC autophagy is an attempt to reinstate TEC quiescence or rather that the process is hijacked in TEC to promote tumor dissemination and an immunosuppressive TME. Autophagy in TECs has been associated with two arms of EC function—immune sensing and vessel normalization, both capable of modulating immune cell recruitment in tumors. Before discussing details about each, first we describe a snapshot of the effects of hypoxic TME on dialogue between ECs and immune cells.

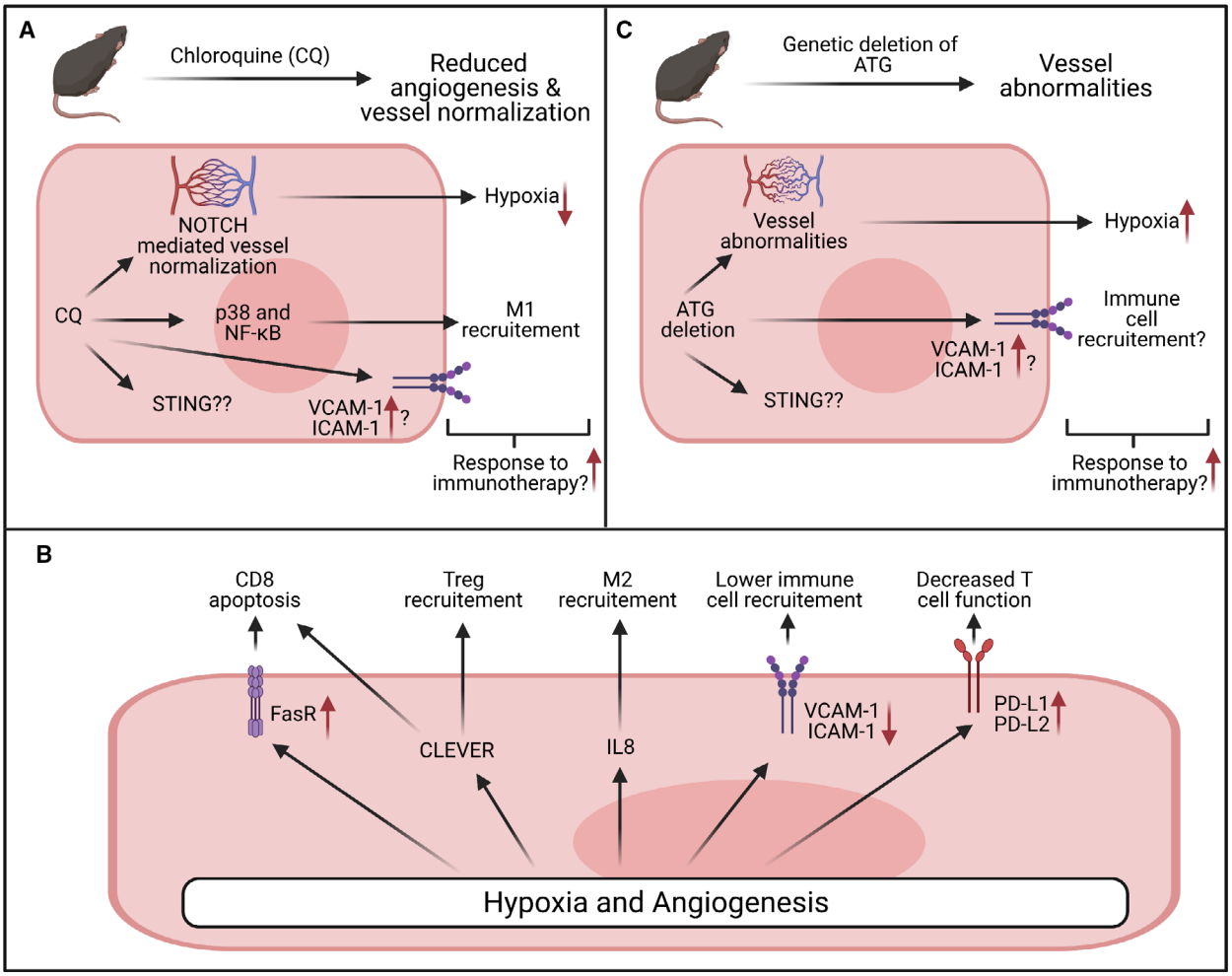

Fig. 3. (A) Chloroquine (CQ), when administrated in tumor-bearing mice, displays autophagy-independent effects in TEC, which are mediated by the activation of the anti-angiogenic NOTCH1 signaling. A major effect of CQ-induced vessel normalization is decreased intratumoral hypoxia, which may promote antitumor immunity. Another mechanism through which CQ may contribute to ameliorate antitumor immunity is by resetting tumor-associated macrophage polarization toward an M1 phenotype, through the activation of p38 and NF-kB pathways by the release of lysosomal Ca^{2+}. It remains speculative what the effects of CQ are on key mediators of the EC::T-cell interface, such as STING or TEC adhesion molecules, and their consequences for anticancer immunotherapy. (B) TEC have an important interfacing function toward circulating immune cells and thereby control their adhesion, activation, and survival *via* various mechanisms. In general, in response to hypoxia and angiogenesis in the TME, this interface facilitates immunosuppressive effects. (C) Genetic deletion of essential autophagy genes (ATG) favors a pro-angiogenic TEC phenotype which may impact tumor hypoxia. The effects of TEC-specific ablation on antitumor immunity remain largely unexplored. Loss of ATG enhances the pro-inflammatory EC status causing an upregulation of ICAM-1 and VCAM-1. However, both the global effects and precise mechanisms through which autophagy affect key mediators in the EC:: T-cell interface remain largely unknown.

Hypoxic TME interferes with EC-mediated immunosurveillance

During inflammation, pro-inflammatory cytokines such as TNFα, IFNγ, and IL activate EC and prepare them for infiltration of immune cells. The infiltration and activation cascade involve upregulation of adhesion proteins including VCAM-1, intracellular adhesion molecule 1 (ICAM-1), and E-selectin on the surface of EC, chemotactic signaling by chemokine CXC motif ligands (CXCL9, CXCL10, and CXCL11), diapedesis and activation *in situ*. Moreover, being semi-professional antigen-presenting cells, EC expresses major histocompatibility complex (MHC) class I and II on their surface and various co-stimulatory (e.g., CD40, LFA-3, OX40L) and co-inhibitory [e.g., PD-L1, PD-L2, indoleamine 2,3-dioxygenase (IDO), CD276] molecules, whose expression can be altered in the TME

(Fig. 3B) [74]. Altogether this highlights the variety of effects that the tumor vasculature can elicit to regulate extravasation, survival, and activation of effector T cells. As discussed previously, tumor vasculature is ill-defined, hypoxic, and structurally abnormal. Tumor vessels together with consistent VEGF signaling negatively influence immune cell infiltration, maturation, and function and create an immunosuppressive microenvironment [75] and cause immune tolerance [76] by molecular pathways which are only partially understood.

Hypoxic TEC increases surface expression of Fas ligand, which binds to the Fas receptor on T cells and initiates apoptotic cell death [77]. CD8+ T cells, which comprises cytotoxic T cell have higher propensity of FasL-induced cell death as compared to regulatory T cells (Treg) [78], thus favoring the presence of Treg in the TME. Hypoxic TEC not only preferentially wipe out CD8+ T cells, but they also increase recruitment of immunosuppressive Treg by upregulating stabilin-1 (CLEVER) protein expression [79]. In addition, hypoxia-induced VEGF-A, HIF1α, and IL-8 signaling in TEC recruits immature myeloid cells into tumors, which form either myeloid-derived suppressor cells (MDSC) or immunosuppressive M2 macrophages [80]. MDSC suppresses immune function by secreting TGF-β [81] which supports an immunosuppressive microenvironment by either maintaining dendritic cells in their immature state (hence, poorer antigen presentation) or by promoting differentiation and proliferation of Treg [82]. Moreover, PGE2 secreted from MDSC in the absence of a pro-inflammatory environment promotes development of Treg, induces immunosuppressive chemokine production, and inhibits the transendothelial migration of T cells by improving the barrier function of EC [83,84]. Angiogenic signals by VEGF-A stimulation also lead to EC anergy [85] due to which TEC fails to upregulate surface expression of VCAM-1 and ICAM-1 in response to the pro-inflammatory cues from the inflamed tissues [86], resulting in poorer lymphocyte adhesion.

Ordeals of CD8+ T cells do not end upon infiltration into tumors. Hypoxia-induced HIF1α can also inhibit the effector function of CD8+ T cells by increasing expression of inhibitory checkpoint molecules PDL1 and PD-L2 on EC [87]. On the other hand, VEGF-A signaling stimulates inhibitory receptors including Tim3, PD1, and CTLA-4 on intratumoral T cells [88]. Effects of hypoxia on immune function extend beyond HIF1α induction in EC. Hypoxic TME forces TEC metabolism to rely on anaerobic glycolysis and the excreted lactic acid [12] can metabolically reprogram infiltrating T cells [89,90]. In addition to lactate, TEC-derived NO, sphingosine-1-phosphate, and IDO also modulate T-cell function [91]. In the following sections, we discuss some recent literature proposing a link between tumor vasculature-associated autophagy and the regulation of immune responses.

TEC autophagy: a role in immune sensing mechanisms?

STING is an important innate immune sensor responsible for antitumor adaptive T-cell responses at local and systemic levels. In response to tumor cell-derived DNA, both professional [dendritic cells (DC)] and nonprofessional antigen-presenting cells such as EC activate type 1 IFN response and prime CD8+ T cells [92–94]. In analogy with the inflammasome, autophagy mediates a negative feedback mechanism which prevents excessive STING and MAVS pathway activation and restricts exacerbated innate immune responses. Certain pro-autophagic proteins like ATG12–ATG5 directly bind to RIG-I and MAVS (important for activation of STING pathway) and induce their removal by autophagy [95,96]. Others, like ATG9A, negatively influence trafficking of ER-associated STING and inhibit activation of the IRF3 kinase: TANK-binding kinase 1 [97]. Dissociation of ATG1/ULK1 from its repressor protein AMPK leads to ATG1/ULK1-mediated phosphorylation and consequent inactivation of STING protein, while p62 prevents prolonged innate immune response by favoring the degradation of ubiquitinated STING protein [98]. STING pathway and its role in antitumor immunity have been recently recognized. Existing evidence suggests that TEC may represent the earliest source of STING-induced type 1 IFN such as IFN-β [93]. Upon intratumoral injection of cGAMP in B16-F10 tumors, the earliest IFN-β-expressing cells co-stained with EC markers (CD31 and VEGFR2) but do not express any immune cell (B cells, T cells, DCs, NK cells, monocytes/macrophages, and neutrophils) lineage markers [92]. In addition, cells co-stained with IFN-β and EC markers are absent in STING-deficient mice while induction of IFN-β is unaltered in CD11c-depleted mice, reinforcing the importance of STING signaling in EC. Recently, the EC STING pathway was demonstrated to have vasculature normalizing effects by upregulating type 1 IFN, increased expression of vascular stabilizing genes (including *CDH5*, *ANGPT1*, and *PDGFRb*), and higher tumor T-cell infiltration [92]. However, the effects of genetic deletion of autophagy genes specifically in EC or TEC on the STING pathway and thereby on the immune contexture remain an open question and will

need further investigation, especially in context of TME.

In addition, recent findings show that deletion of autophagy in liver sinusoidal ECs, enhanced features of EC inflammation (higher expression of *VCAM-1, IL6, Ccl2*, and *Ccl5*) and promoted liver fibrosis [52]. These results add another piece of data indicating a role of EC autophagy in suppressing liver inflammation. It would be interesting to test whether this is a general autophagy-regulated response in the context of the tumor vasculature.

TEC autophagy and lysosomal degradation in vessel normalization—prospects for immunotherapy

Tumor vasculature is emerging as a crucial TME element to fully unleash antitumor immune responses. In advanced tumors, vessel remodeling through unproductive angiogenesis generates a barrier to T cells [99] and lower T-cell infiltration caused by hypoxia and other aberrations of the TME reduces the efficiency of immunotherapy [100]. Anti-angiogenic therapies can lead to a transient window of vessel normalization, by re-establishing EC quiescence and improving vessel maturation, while prolonging their effects leads to vessel pruning [75]. Genetic or pharmacological manipulation leading to vessel normalization [75,101] provides the strongest link between TEC and immunotherapy. Vessel normalization improves structural and functional features of blood vessels, resulting in better perfusion (hence less hypoxia), increased drug delivery, and therapy responses that depend on adequate blood supply to the tumor [102].

Seminal studies have shown that outcomes of genetic deletion (systemic and vascular) and pharmacological inhibition of autophagy (systemic) on tumor growth are dependent on several factors. In the context of pharmacological inhibition, different antitumor outcomes seem to be dependent on specificity and duration/dose of the inhibitor [62,103–105]. *In vivo* chloroquine treatment (at approximately 7 days after tumor injection when tumors were 100 mm^3) induced vessel normalization, reduced growth of B16-F10 melanoma tumors, and increased delivery and efficacy of anticancer drug cisplatin. However, these effects of chloroquine on tumor growth were found to be independent of EC autophagy and occurred *via* a NOTCH1-dependent pathway [62]. Similar antitumor effects of chloroquine were observed when the drug administration was performed before tumor cell injection [105]. In this study, authors did not investigate autophagy markers, but the antitumor effects of chloroquine were shown to be mediated by release of calcium, followed by subsequent activation of p38 and NF-κB pathways which lead to polarization of tumor resident macrophages to M1 phenotype [105]. Interestingly, when chloroquine was injected around day 13 out of 18-days post-tumor injection, authors did not observe beneficial effects of chloroquine on tumor growth and tumor resident immune cell population in B16 and 4T1 tumor-bearing mice [104]. Together, these studies suggest that the time of initiation and duration of chloroquine administration may be critical to elicit antitumor effects. One may also question the nonspecificity of chloroquine as an inhibitor of autophagy since it inhibits autophagy by altering lysosomal pH and thus blocking lysosomal recycling and multiple studies show that chloroquine also exerts autophagy-independent effects [62]. Upon deletion of *ATG5* specifically in the EC compartment (*ATG5*-ECKO), mice unexpectedly displayed abnormal vasculature (smaller, less mature and ill perfused vessels), a phenotype which was opposite to that observed after chloroquine administration. Yet, *ATG5*-ECKO mice showed reduced growth of B16-F10 tumors [62]. This implies the existence of yet to discover mechanisms responsible for reduced tumor growth after inhibition of TEC autophagy. Thus, whether and how autophagy proteins in TEC affect antitumor immunity remains an open question (Fig. 3C). Due to lack of EC specificity of the pharmacological inhibitors against autophagy used in studies so far, antitumor or pro-immunotherapy benefits of pharmacologic inhibitors of autophagy on the vascular compartment are incompletely understood. However, thanks to the development of a drug delivery system such as vascular zip coding [106], pharmacological modulation of vascular autophagy has potential. Development of modulators of autophagy that can be attached to a homing peptide RGR (CRGRRST) and delivered directly to the vasculature is an important avenue which should be addressed in future studies.

Conclusions

The field of autophagy has been booming in the past decades and our understanding of how this catabolic pathway regulates cancer and other inflammatory diseases have made significant strides. It has also become clear that autophagy influences several aspects of the EC biology and vascular functions, both in physiological and in cancer-associated conditions. Several *in vivo* studies using transgenic mouse models with specific deficiency of certain *ATG* in ECs have highlighted the essential role of EC autophagy as a quality control

and vasculoprotective mechanism that preserves redox homeostasis in ECs, and regulates angiogenesis and inflammatory responses. However, several questions still remain unanswered. For example, it is unknown which selective autophagy pathway confers metabolic plasticity on ECs and favors the switch between the quiescent and angiogenic phenotype. Even though emerging evidence suggests that many inflammatory signaling pathways activate autophagy and in turn are controlled by it, the molecular mechanisms through which EC intrinsic autophagy limits inflammation and favors the resolution phase of inflammatory responses are still undefined. Likewise, whether and how TEC autophagy regulates the TEC interface with T cells or other immune cells and its impact on immunosurveillance has not been revealed yet (Fig. 3). Given the emerging heterogeneity of TECs and the diversity and functional roles of selective autophagy pathways, it will be important to understand whether autophagy is a hallmark of distinct EC phenotypes. Ultimately, all these studies will tell us whether correction of EC dysfunctions by the development of selective next-generation autophagy modulating drugs that target autophagy in EC could be a useful therapeutic objective to improve therapeutic responses in cancer and other inflammatory diseases.

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Author contributions

JV, MA designed & co-wrote the paper. JB co-wrote the paper. PA designed, co-wrote and supervised the paper.

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