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Autophagy in infection, inflammation, and immunity

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Preface

Autophagy is a fundamental cell biological pathway affecting immunity. Whereas autophagy is an antimicrobial effector of conventional pattern recognition receptors (PRRs), autophagic adaptors termed SLRs represent a new subset of PRRs and provide the mechanistic basis for autophagic elimination of intracellular microbes. Autophagy controls inflammation via regulatory interactions with innate immunity signalling, by removing endogenous inflammasome agonists, and through effects on secretion of immune mediators. Autophagy contributes to antigen presentation, T cell homeostasis, and affects T cell repertoires and polarization including Th17 inflammation. Here, we review the above relationships organized into four principal roles of autophagy in infection, inflammation, and immunity.

Introduction

Autophagy as an immunological process can be organized in four principal manifestations illustrated in Fig. 1: direct elimination of microbes, control of inflammation, antigen presentation and lymphocyte homeostasis, and secretion of immune mediators. Immunological autophagy fits but in some aspects exceeds the scope of autophagy as a cytoplasmic quality and quantity control process that also provides nutrients through cytosol autodigestion at times of starvation¹. The word autophagy (“self-eating”) refers to a collection of diverse processes enabling cells to digest their cytoplasmic constituents in lysosomes. This definition includes macroautophagy, microautophagy, chaperone mediated autophagy and noncanonical autophagy^{2,3}. Several non-autophagic roles, including immunological effects, of individual or subsets of autophagy factors have also been recognized^{4,5}. In this review we cover only the *sensu stricto* autophagy – macroautophagy. We refer to it by name as autophagy and by function as a defined cell biological pathway that depends on specialized Atg factors¹ (Box 1). This pathway is distinct from other cytoplasmic digestive processes, including proteasomal degradation, by its ability to capture and eliminate large targets such as toxic protein aggregates, defunct or disused organelles, and invading microbes.

Autophagy may be the most primordial form of innate immunity against invading microbes. The present view of autophagy as an immune defense has its mechanistic beginnings in the recognition of autophagy as a downstream effector of PKR, a pivotal antiviral protein in mammalian cells^{6,7} and in the reports of autophagic elimination of intracellular streptococci⁸ and *Mycobacterium tuberculosis*⁹. The latter two examples epitomize the most primal immune manifestation of autophagy - direct capture of intracellular microbes for elimination in autophagosomal organelles (Fig. 1a). The appreciation of immunological functions of autophagy has increased in volume and scope¹⁰ since the initial overviews of connections with innate and adaptive immunity¹¹ and today innate immunity manifestations of autophagy are considered as one of its principal roles. One of the tenets of this review is that autophagic capture and elimination of intracellular microbes has been a key contributing driver in the evolution of the autophagy pathway as a whole. In the present day eukaryotes, in particular in mammals, these primordial roots have evolved and become incorporated into multiple layers of innate and adaptive immunity. Here we dissect these relationships organized in four principal categories (Fig. 1) following the escalating complexity of the immunological systems with which autophagy has been integrated.

Autophagy pathway and immune signalling

Autophagy as an Atg-dependent pathway

The principal morphological feature of autophagy is the formation of endomembranous organelles called autophagosomes (Box 1). An ensemble of Atg factors¹ (Box 1), with Atg6/Beclin 1, Atg1/Ulk1, and Atg8/LC3 being key regulators, drives the formation in the cytosol of the autophagic isolation membrane (phagophore). A phagophore, often rendered in models as a stand alone crescent in the cytoplasm, is transiently connected to and derived from phosphatidylinositol 3-phosphate (PI3P)-positive domains of the endoplasmic reticulum (ER) termed omegasomes¹. Other compartments such as the Golgi, mitochondria, and plasma membrane-derived endocytic organelles contribute as well¹. The latest studies demonstrate that the ER-derived autophagosomes form at the ER-mitochondria contact sites¹². A phagophore sequesters the captured cytoplasmic cargo destined for autophagic disposal, and upon elongation and closure, an autophagosome is formed. At this stage, the corresponding endomembranes are commonly visualized as double membrane structures¹. The degradation of the captured cargo begins when autophagosomes mature into autolysosomes, delimited by a single membrane, through acidification and acquisition of lysosomal hydrolases¹.

A shared maturation pathway between autophagy and phagocytosis

Autophagy is often morphologically equated with the formation of double membrane autophagosomes in the cytoplasm. This is the result of a geometrical necessity requisite when a phagosome is derived from a pre-existing membranous intracellular compartment such as ER¹ rather than a requirement for double membrane per se (Box. 1). One well-studied exception to the double membrane precursors is the role of Atg factors in maturation of the standard phagolysosome. This process has been called LC3-associated phagocytosis (LAP)^{13,14} and occurs following uptake of various extracellular targets: particles coated with Toll-like receptors (TLR) agonists^{15,16}, phagocytosed dead cells¹⁷, live epithelial cells

engulfed via entosis by neighboring cells¹⁸, and Fc γ R-dependent uptake of immune complexes¹⁹. If a microbe or targets that can activate TLRs are taken up by conventional phagocytosis, the autophagic machinery enhances maturation of the conventional phagosomes indistinguishably from how it works with the internally formed autophagosomes. LAP utilizes Beclin 1-hVPS34 and LC3-conjugation systems. As expected, LAP is independent of Ulk1, since Ulk1 is needed to generate autophagosomes from internal ER membranes during starvation^{18,19}. The recapitulation of autophagic maturation steps in LAP is informative about the role of autophagy-associated membrane transactions such as PI3P generation, long known to be key for phagosomal maturation²⁰, whereas the role of LC3 may be a manifestation of the tethering and fusogenic properties of LC3^{21,22}.

Starvation and autophagy: IKKs and nutrient depletion as an alarmin

Competition for intracellular nutrients might have been one of the most primordial alarmin or danger signals available to the eukaryotic cell to detect microbial invasion and eliminate them through autophagy. Nutritional signalling has been experimentally linked to antimicrobial autophagy in response to bacterial invasion²³. (Fig. 1a-(v)). The metabolic regulation via mTOR (inhibiting autophagy) and AMPK (activating autophagy), covered in detail elsewhere¹, merges with immune signalling through IKK α and IKK β ^{24,25} (Box 2). The role of IKKs in autophagy initiation in response to starvation is independent of NF κ B action²⁴ and instead is associated with events involving TAB2/3-TAK1 (the upstream regulators of IKKs) and Beclin 1^{24,25}. TAB2 and TAB3 in the resting state bind Beclin 1 and repress its activity. TAB2 interacts with members of the Ulk complex and is phosphorylated by Ulk1²⁵. Following autophagy induction, TAB2 and TAB3 dissociate from Beclin 1, thus activating it, and also bind to TAK1²⁴. TAK1 in turn phosphorylates and activates AMPK. In sum, this triggers both branches of autophagy regulatory (protein and lipid) kinases - Ulk1/2 (via AMPK) and hVPS34 (via Beclin 1).

TBK-1 controls autophagy

Another immunologically significant IKK-related kinase, TBK-1, affects autophagy²⁶⁻²⁹. It is involved in eliminating intracellular microbes independently of its role in type I interferon activation³⁰. TBK-1 controls both the capture of autophagic cargo (e.g. microbes) via specialized adaptors²⁶⁻²⁹ and the maturation of autophagosomal organelles into degradative compartments²⁹ (Box 2). Thus, there is a sequential engagement of IKKs, with IKK α / β acting at the initiating stages and TBK-1 in the completion of autophagy. The exocyst, a dynamic multiprotein platform best known for its role in vesicular trafficking and TBK-1-STING localization and activity^{31,32}, also regulates autophagy³³. When centered on the component Exo84, exocyst assembles autophagy initiation-relevant complexes containing Ulk1, Beclin 1, Atg14L and VPS34³³. When centered on the component Sec5, exocyst controls STING and TBK-1 relocalization from the ER to sites important for activation of immune processes, i.e. IRF3 and type I IFN stimulation^{31,32}. The role of IKK and TBK-1 signalling in autophagy is of growing significance and in keeping with a key position of TBK-1 that now includes interactions with autophagic factors²⁶⁻²⁹, as further highlighted in systems biology analyses of innate immunity interactome for type I interferon³⁴.

Autophagy is controlled by PRRs, cytokines, and reactive nitrogen and oxygen species

Although is an ancient defense mechanism. However, the present-day antimicrobial autophagy in mammalian cells is well integrated with other innate and adaptive immune regulatory systems as summarized in Box 2. Furthermore, whereas autophagy is a built-in self-defense available in principle to any eukaryotic cell, the magnitude of its contribution in complex organisms is cell type dependent, as illustrated by the neuron-specific significance of autophagy against HSV-1 but a lack of its role in keratinocytes of the vaginal mucosa³⁵. Autophagy is controlled by nearly all classes of PRRs and responds to PAMPs and DAMPs. It is also regulated by cytokines and receptors modulating innate and adaptive immunity. These relationships are summarized in Box 2. Reactive oxygen species (ROS) and systems that generate them affect autophagy^{13,36–39}. NADPH oxidase and ROS are engaged in LAP during *Salmonella* phagocytosis¹³. NADPH oxidase and autophagic machinery are connected via a regulatory protein Rubicon. By jumping from the autophagy inhibitory complex to NADPH oxidase activating complex, Rubicon activates both bactericidal (autophagy and ROS) mechanisms simultaneously³⁸. Nitric oxide inhibits autophagy by inactivating JNK-1 and IKK- β via their direct S-nitrosylation³⁹. This prevents JNK-1-dependent Bcl-2 phosphorylation³⁹ and its dissociation from the BH3 domain of Beclin^{140,41} and inhibits IKK- β -associated²⁴ AMPK-dependent autophagy initiation^{39,42}.

First principal role: direct elimination of microbes

Autophagy intercepts microbes at different stages of invasion

Antimicrobial autophagy is a sequential set of barriers against incoming microbes (Fig. 1a). To deploy autophagy, mammalian cells utilize guidance systems detecting the presence, location and extent of the cytoplasmic penetration by a pathogen. This begins with conventional pattern recognition receptors (PRRs)⁴³ acting at any stage, and, if microbe penetrates the cytosol, ends with autophagic adaptors⁴⁴ involved in elimination of microbes. In their antimicrobial role, these autophagic adaptors are referred to as Sequestosome 1/p62-like receptors (SLRs)⁴⁵. SLRs physically recruit the autophagic machinery while recognizing the molecular tags (ubiquitin, galectin, membrane phospholipid modifications) found on the invading microbe or on damaged host membranes associated with the microbe^{26,27,46–49} (Fig. 1a–vi).

PRRs can elicit autophagic responses at different stages of host-microbe encounters (Fig. 1a). With bacteria, this includes early TLR- and Nod-dependent detection of the released microbial products (e.g. PAMPs). Autophagy stimulation can occur before (Fig. 1a-i)^{50,51}, during adhesion and pathogen-induced uptake of bacteria by the host cell (Fig. 1a-ii), or during active phagocytosis of microbes by macrophages (Fig. 1a-iii)^{15,52,53}. At stages past bacterial uptake, autophagy is elicited via pathogen-induced damage to the newly formed parasitophorous vacuoles (Fig. 1a-iv)^{26–28,49,54,55} and upon escape of bacteria into the cytosol (Fig. 1a-v)^{47,56–58}. The engagement of autophagy with viruses involves various stages of their life cycle (Fig. 1a-vi)^{7,16,35,37,59–65}. Two terms have been coined to refer to the aspects of the above continuum of antimicrobial autophagy: xenophagy refers to the uptake of intracellular microbes into double membrane autophagosomes¹⁰, whereas LC3-

associated phagocytosis (LAP)^{13,14} refers to an engagement of the autophagic machinery while the bacterium is confined within the nascent phagosome.

Microbes defend themselves

The significance of autophagy in protecting the cytosol from microbial invasion is underscored by microbial countermeasures (Box. 3)⁶⁶. These mechanisms often involve targeting of Beclin 1 by microbial factors to block autophagy^{67,59}, prevent autophagosomal maturation^{61,65} or even in some cases activate autophagy to generate nutrients for microbes⁶⁸. In other instances, an autophagosomal membrane can be perforated to prevent acidification⁵⁵ or mAtg8s are proteolytically cleaved to irreversibly remove C-terminal lipid modifications⁶⁹, whereas if all else fails, epitopes or tags for recognition by SLRs can be masked^{56,57,70}.

TLRs and autophagy cooperate in responses to PAMPs

TLR-ligands activate autophagy via specific molecular cascades (Box 2). Stimulation of TLRs induces autophagy, revving up this defense in advance of microbial invasion^{50,51} (Fig. 2a(a)), possibly promoting generation of antimicrobial peptides for eventual delivery to the parasitophorous vacuoles⁷¹, or focusing autophagic response to the points of microbial entry¹⁵ (Fig. 2) as during LAP¹⁵ (Fig. 2a). Autophagy stimulated via TLRs enhances antigen presentation in conjunction with LAP^{16,72}. Autophagy scoops cytosolic PAMPs for delivery to endosomal lumens where they can come in contact with the correct end of endosomal TLRs and stimulate other responses, e.g. type I IFN, as in the case of TLR7 recognition of cytosolic ssRNA virus replication intermediates in pDCs⁶⁰ (Fig. 2a-(c) – or/and Fig. 1b(viii-1)).

NLRs interact with Atgs to localize autophagy

The cooperation in antimicrobial defense between Nod like receptors (NLRs) and autophagy is conserved from flies⁷³ to humans^{52,53}. Atg16L1 and Nod2, encoded by Crohn's disease (CD) risk loci⁷⁴, interact⁵². Atg16L1 also interacts with Nod1⁵² (Fig. 2b). Nod1 and Nod2 are necessary to induce autophagy in response to the presence of muramyl peptides in the cytosol⁵². Nod1 and Nod2 direct autophagic machinery by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry⁵². The Nod2-assisted localization of Atg16L1 at the plasma membrane fits with the general role of Atg16L1 in formation of autophagic precursors from the plasma membrane⁷⁵. Cells from donors homozygous for the CD-conferring *ATG16L1*300T* allele have diminished capacity for autophagy induction in response to Nod2 agonist muramyl dipeptide. A Nod2 truncation in CD, which renders it completely cytosolic, retains Atg16L1 in the cytosol instead of facilitating its recruitment to bacterial entry sites thus precluding timely and site-specific autophagic control. NLRs other than Nods interact with the autophagic machinery. NLRX1 and its interacting partner mitochondrial Tu translation elongation factor, which associates with Atg5-Atg12 and Atg16L1, promote autophagy⁷⁶. NLRC4, NLRP3, NLRP4, and NLRP10 interact with Beclin 1⁷⁷. In the case of NLRP4, recruitment of NLRP4 to the plasma membrane during phagocytosis of group A streptococci leads to its transient dissociation from Beclin 1⁷⁷. This permits initiation of Beclin 1-mediated autophagic responses⁷⁷. Collectively, NLRs may

induce autophagy by gathering autophagy factors in the vicinity of the incoming microbe (or resident mitochondria) followed by autophagy activation events.

Cyclic-di-GMP sensors, cytosolic DNA sensing, RLRs and autophagy

The conserved signaling molecules cyclic dinucleotides (e.g. cyclic di-GMP) released by bacteria can directly bind STING to induce TBK-1 signaling and type I IFN production⁷⁸. When cyclic di-GMP or di-AMP are introduced into cytosol, they also activate autophagy²⁸ (Fig. 2c(i)). STING does transduce signals from cytosolic DNA receptors to induce autophagy. Infection with dsDNA viruses (HSV-1, HCMV) induces autophagy^{79,80} dependent on STING⁸⁰. Akin to the findings with viruses^{79,80} (Fig. 2c(ii)), bacterial DNA released during infection can induce autophagy via STING²⁸ (Fig. 2c(iii)). It remains to be tested whether cGAMP (cyclic GMP-AMP)⁸¹ transduces the recognition of cytosolic bacterial and host DNA to induce autophagy. This second messenger, cGAMP, is generated by DNA-receptor/cGAMP synthase to activate type I IFN pathway via STING⁸², and may in parallel promote autophagy. Bacterial DNA leaches out from a subpopulation of *M. tuberculosis* phagosomes through pores introduced by ESX-1²⁸. Targeting of the DNA by autophagy occurs in a manner associated with ubiquitin tags and was shown to depend on SLRs²⁸. As its side effect, the pathogen's cytosolic DNA elicits, via STING and TBK-1, type I IFN response that suppresses production of the key anti-tuberculosis cytokine IL-1 β and leads to pathology during later stages of tuberculosis⁸³. DNA-dependent clearance of bacteria may be associated with an effort by the host cell to clear DNA mistaken for viral, whereas the release of DNA by the bacteria may be a pathogen's strategy to elicit an unproductive (in the context of tuberculosis) type I IFN inflammation (Fig. 2c(iii)). In keeping with this, only a minor fraction of all *M. tuberculosis* phagosomes becomes labeled with LC3⁺²⁸ whereas bacterial DNA is targeted via autophagy to autolysosomes for clearance²⁸ (Fig 2c(iv)).

RIG-I-like receptors (RLRs) signaling is a prominent exception to the rule that autophagy is induced by PRRs. A typical observation with RLRs and autophagy is one of a negative regulatory relationship, with a pattern of reciprocal activation and repression between autophagy and RLR-driven type I IFN responses^{76,84}.

SLRs clear invading microbes from the cytosol

When a pathogen makes it past the autophagy barriers directed by conventional PRRs, it can still be captured in the cytosol. These processes depend on specialized adaptors called SLRs (Fig. 3a)⁴⁵. SLRs (Fig. 3a) are defined as autophagic receptors/adaptors for capture of microbes in contact with the cytosol. SLRs have been named as a group^{45,85} after the archetypical protein termed Sequestosome 1/p62. In addition to Sequestosome 1/p62⁸⁶ and NBR1⁸⁷, which have similar domain organization, the extended definition of SLRs include Optineurin²⁷ and NDP52²⁶. SLRs (i) act as adaptors in antimicrobial autophagy^{26–28,46,47,57,88}, (ii) contain one or more cargo recognition domains (CRDs)⁴⁵ with ubiquitin^{26–28,46,47,57,88} and galectin^{49,89} thus far identified as tags associated with microbial targets, (iii) have one of the LC3/Atg8 interacting region motifs LIR (D/E-D/E-D/E-W/F/Y-X-X-L/I/V)⁴⁴ or CLIR (LVV, thus far identified in NDP52)⁹⁰; (iv) can affect

inflammation^{47,91,92}; (v) can be consumed during autophagy⁴⁴; and (vi) are subject to modulation of their autophagic activities by phosphorylation of LIRs or CRDs^{27,29,93}.

The tags on microbial targets come in different varieties with ubiquitin and galectin being the best characterized (Fig. 3b). All known SLRs contain ubiquitin-binding domains (UBDs)⁹⁴ with different specificities for ubiquitin chains (UBA, UBZ, UBAN)⁹⁴ which decorate invading microbes (Fig. 3b-i). CRDs in SLRs can bind tags other than ubiquitin. A hook-like CRD in NDP52 enables it to interact with Galectin 8⁸⁹ (Fig2b-ii). Galectin 8 bridges NDP52 with cytoplasmically exposed β -galactoside glycans on pathogen-damaged host membranes via Galectin 8's carbohydrate binding domains⁴⁹. These compact domains are shaped as bent β -sandwich structures consisting of two antiparallel β -sheets with 6 and 5 strands, presenting its concave surface to β -galactoside glycans whereas the convex surface binds the hook-like CRD of NDP52⁸⁹ (Fig. 3b-ii). The number of SLRs and the type of unique or repetitive structures recognized as tags may expand as research continues.

Cooperative processes in clearance of cytosolic microbes

The process of autophagic clearance of microbes penetrating into the cytosol is a tight cooperation between several components (Fig. 3c). This includes host DAMPs, microbial PAMPs, and multiple SLRs. DAMPs are exemplified by freshly exposed glycans upon damage to host membranes as in the case of *Salmonella*^{49,89}. A PAMP may be a microbial polymer such as DNA in contact with the cytosol as in the case of *M. tuberculosis*²⁸. As a part of the process, ubiquitin chains are generated around the microbe^{26–28,46,47,57,88} (Fig. 3b-i). This may involve specialized E3 ligases such as LRSAM1⁹⁵ (Fig. 3b). LRSAM1 has the ability to recognize *Salmonella* by the virtue of its LRR (leucine rich repeat), a domain often recognizing microbial ligands⁹⁵. LRSAM1 self-ubiquitinates, and ubiquitinates yet to be defined microbial targets in vitro⁹⁵. In vivo, E3 ligases may alternatively or in addition ubiquitinate host molecules associated with microbes.

A further cooperation exists between recognition of the initial tears in microbe-harboring vacuoles and ubiquitination, as exemplified in the case of *Salmonella* (Fig. 3c). In addition to LRSAM1's auto-ubiquitination or ubiquitination of bacterial targets, LRSAM1 interacts directly with NDP52⁹⁵. NDP52 (Fig. 3a) is an SLR with triple specificity recruited to the site of microbial penetration via: (i) binding of its hook-like CRD to Galectin-8- β -galactoside complex on perforated host membranes^{49,89}; (ii) association of the NDP52 region 127–255 to the LRR domain of LRSAM1⁹⁵; and (iii) binding of its BUZ domain to ubiquitin chains²⁶ placed on the target by E3 ligases. The downstream autophagic machinery is brought in via the NDP52 CLIR motif that binds to LC3C, requisite for acquisition of other mAtg8s and autophagy of *Salmonella*⁹⁰. NDP52 action is nonredundantly strengthened by other SLRs recruited to *Salmonella*: Optineurin (partitioning with NDP52 in microdomains surrounding *Salmonella*) and Sequestosome 1/p62 (not partitioning with NDP52)²⁷. These SLRs bind ubiquitin through their UBD domains with somewhat varied specificities for ubiquitin chains⁹⁴, and bring via their conventional LIR motifs other mAtg8s (e.g. the remaining LC3s and GABARAPs) to execute autophagosome formation. The links of SLRs to LC3 and ubiquitin are further enhanced by phosphorylation of the LIR in Optineurin and the UBD in Sequestosome 1/p62 by TBK-1. TBK-1 is recruited to these

sites via binding to NDP52²⁶, Optienurin²⁷, and to Rab8b²⁹, a small GTPases regulating membrane trafficking. To complete the gestalt, the cargo capture and autophagy is coordinated with maturation into autolysosomes by Rab8b²⁹.

Other methods of targeting cytosolic microbes

Autophagy factors can directly target microbial proteins as in the case of *Shigella*^{56,58}. Atg5 binds directly to a bacterial surface protein VirG (Fig. 3d-i). The Atg5-VirG interaction occurs only when the VirG recognition epitope is not masked by another *Shigella* protein – IcsB (Box 3), and requires an Atg5-binding partner Tecpr1 (Tectonin domain-containing protein) that interacts with WIPI-2⁵⁸. WIPI-2 is one of the four mammalian PI3P-binding Atg18 paralogs, and plays a role in phagophore formation. Of note, the control of *icsB*-mutant *Shigella* still requires Nod action (Nod1) to induce autophagy and limit intracellular growth⁵², illustrating the cooperative nature of different layers of autophagic defenses against a microorganism^{52,58}. This is complemented by ubiquitin and SLR contribution to *Shigella* clearance^{47,88}. In addition, cardiolipin unique to bacteria and mitochondria is targeted directly by autophagy factors⁹⁶. Cardiolipin binds IRGM⁹⁶, Beclin 1⁹⁷, and LC3⁹⁸ (Fig. 3d-ii).

SMURF1, a HECT domain E3 ligase, is required for antiviral response to Sindbis virus and HSV-1⁹⁹. Similarly to another E3 ligase Parkin, SMURF1 is involved in mitophagy⁹⁹. Surprisingly, the SMURF1 HECT domain (and hence its E3 ligase activity) is dispensable for these activities (tested in mitophagy), and instead its phospholipid-binding domain C2 is necessary (Fig. 3d-iii). In keeping with membrane phospholipids or their derivatives as potential tags or signaling intermediates, diacylglycerol has also been implicated in bacterial autophagy⁴⁸. Thus, the presence of an E3 ligase domain in an autophagy-targeting factor does not necessarily guarantee that it places ubiquitin tags on the cargo.

Second principal role: control of inflammation

Genetics links with inflammatory, autoimmune, and immunodeficiency disorders

The role of autophagy in inflammatory diseases has been initially established through genome-wide association studies (GWAS)⁷⁴. The GWAS studies have exposed links between *ATG16L1* and *IRGM* with Crohn's disease (CD), a common form of inflammatory bowel disease (IBD)⁷⁴, and have been replicated with exceptional reproducibility in numerous follow-up population studies. A recent GWAS analysis of 75,000 cases indicates an overlap between susceptibility loci for IBD and mycobacterial infections¹⁰⁰. This expands a potential lead that autophagy as a process and *IRGM* as a genetic susceptibility locus play a role in both CD and tuberculosis (ref____). Other links have been reported between additional autophagy loci and genetic predispositions for chronic inflammatory disorders and autoimmune diseases (Fig. 1b(i)). A link between CD and a newly identified autophagy targeting factor for viral and mitochondrial clearance, SMURF1⁹⁹, has been reported¹⁰⁰. Polymorphisms in the *ULK1* gene have been linked to CD¹⁰¹. In addition to single nucleotide polymorphisms, *IRGM* is an example of gene dosage (copy number variants) correlation with predisposition to CD in human populations¹⁰². One of the common *IRGM* polymorphisms in CD leads to an escape from negative regulation of IRGM

expression by a microRNA¹⁰³. *IRGM* may be a factor in systemic lupus erythematosus (SLE)¹⁰⁴. GWAS data link *ATG5* variants to SLE^{105,106}. Rheumatoid arthritis (RA) is associated with variations in the *PRDMI-ATG5* intergenic region¹⁰⁷. In a relationship to clinical markers of RA, autophagy promotes presentation of citrullinated antigens¹⁰⁸, correlating with the presence of autoantibodies against citrullinated proteins (e.g. vimentin) as a hallmark of RA. Citrullin is enzymatically generated from arginine residues by peptidylarginine deaminase, an enzyme that may be enriched in autophagosomes¹⁰⁸ (Fig. 1c(v)). A new human autophagy locus, first functionally identified in *C. elegans* screens as *epg5*, has been linked to the complex Vici syndrome that includes immunodeficiency¹⁰⁹. Thus, autophagy shows clinical relevance through genetic links with immunological and inflammatory disorders.

Autophagy regulates innate immune response mediated by nucleic acid-sensing TLRs

Autophagic machinery can amplify PAMP-TLR signalling. For example, autophagy enhances delivery of cytosolic PAMPs to TLRs, as shown for TLR7, enabling recognition of cytosolic viral replication intermediates and IFN- α production by plasmacytoid dendritic cells (pDCs)⁶⁰. The role of autophagy in PAMP-PRR amplification loops can backfire and lead to aberrations and autoimmunity. In the case of TLR9, normally responding to microbial unmethylated CpG DNA, autophagic machinery can enhance aberrant self-DNA presentation. This occurs via intracellular trafficking events following B cell receptor (BCR) stimulation with self DNA-containing antigens¹¹⁰. Autophagy promotes a merger of the DNA-containing antigen-loaded BCR compartment with the TLR9-containing endosomes and leads to B cell hyper-responses¹¹⁰ (Fig. 1b-viii-1). This phenomenon is mirrored in pDCs by a similar process akin to LAP, whereby the autophagic machinery enhances particulate self DNA-immune complexes and TLR9 trafficking to signaling-competent compartments resulting in increased IFN- α production¹⁹ (Fig. 1b-viii-2). These anomalies, spurred by autophagic machinery in two key cell types (B cells and pDCs) could synergize and lead to differentiation of autoimmune plasma and memory cells (Fig. 1b-viii 1+2).

Autophagy factors can downregulate RLR and type I IFN signalling

There are many examples (Fig. 1b-ii) where autophagy factors directly inhibit formation or suppress activation of proinflammatory protein complexes: Atg5-Atg12 complex negatively regulates RLR signalling by direct binding to CARD domains of RIG-I and IPS-1 (also known as MAVS, VISA, or CARDIF)⁸⁴ (Fig. 1b-ii). Rubicon, a Beclin 1-hVPS34 interacting protein, inhibits CARD9, BCL10, and MALT1 (the CBM complex) by binding to CARD9, thus terminating RIG-I or Dectin-1 induced pro-inflammatory signaling¹¹¹ (Fig. 1b-iii). Atg9 negatively controls trafficking of STING and suppresses activation of TBK-1 in type I interferon signalling in response to double stranded DNA¹¹² (Fig. 1b-iv). Absence of autophagy amplifies RLR signaling via increased IPS-1 levels due to accumulation of mitochondria, whereas increased pools of depolarized mitochondria in the absence of mitophagy are a source of ROS that also enhance RLR outputs³⁷ (Fig. 1b-v). NLRX1 promotes autophagy and inhibits RLR-dependent induction of type I IFN signalling and inflammation⁷⁶ (Fig. 1b-vi). These phenomena may represent feedback loops whereby autophagy is positioned to downregulate type I IFN responses following a period of their productive induction or elevating the threshold for their activation (Fig. 1b-vii-1).

Alternatively, autophagic interference with RLR signalling could reflect competition for limited resources such as TBK-1, shared between type I IFN signalling and autophagy pathways^{27–29,34} (Fig. 1b-vii-2). These phenomena, in addition to other independent factors not discussed here, could explain why autophagy paradoxically enhances replication of certain viruses.

Autophagy suppresses inflammasome activation

The functional recognition of anti-inflammatory function of autophagy has its roots in the findings of increased IL-1 β and IL-18 production, and associated intestinal inflammation in a mouse model of CD with defective Atg16L1¹¹³. The inflammasomes are a group of cytosolic innate immunity complexes responding to PAMPs and endogenous alarmins/DAMPs by triggering proteolytic processing and secretion of pivotal pro-inflammatory mediators such as IL-1 β and IL-18¹¹⁴. An inflammasome consists of pro-caspase 1, ASC adaptor, and one of the sensor proteins from the NLR family (e.g. NLRP1, NLRP3, NLRC4/IPAF, NLRP6 or NLRP12) or the PYHIN family (AIM2 or IF116)¹¹⁴. Upon recognition of an agonist (which can be microbial, endogenous, or an environmental agent), inflammasome components coalesce leading to caspase-1-dependent pro-IL-1 β processing in the cytosol into mature IL-1 β and its secretion¹¹⁴. A number of convergent reports^{115–119} show that autophagy plays a negative role in inflammasome activation. Under sterile conditions, autophagy cleanses cytoplasm ridding the cellular interior of debris, aggregates, and defunct organelles that can act as endogenous inflammasome agonists. It turns out that basal level of autophagy control the set point for inflammasome activation^{115,116}. If autophagy is blocked, this leads to accumulation of depolarized mitochondria that leak endogenous inflammasome agonists such as mitochondrial DNA (detected at least in part by AIM2) and ROS (activating NLRP3 inflammasome)^{115 116} (Fig. 1b-***iii). Additional studies suggest that pro-IL-1 β or other inflammasome components may be substrates for autophagic degradation possibly as a failsafe mechanism to block unwarranted inflammasome activation¹¹⁷ (Fig. 1b-x ****). Importantly, autophagy may, following a period of physiological inflammasome activation, downregulate the process by removing aggregated inflammasome components¹¹⁹. This depends on Sequestosome 1/p62 recruitment to K63-ubiquitinated ASC¹¹⁹ (Fig. 1b-xi ****). A role for exocyst has been implicated in the overall process¹¹⁹, but it could be self-starting, since Sequestosome 1/p62 associates with the E3 ligase TRAF6⁹¹ that ubiquitinates Beclin 1 to initiate autophagy¹²⁰, although this has not been explored (Fig. 1b-xi ****). In sum, basal autophagy protects cells from inadvertent inflammasome activation as a source of damaging sterile inflammation whereas deficient autophagy causes increased IL-1 β levels, in keeping with the IL-1 β -based inflammation in an Atg16L1 murine model of CD (Fig. 1b-xii ****)¹¹³.

Autophagy suppresses calpain-dependent IL-1 α activation

Just like IL-1 β , IL-1 α is synthesized as a cytosolic proform. It is processed by calpain or other proteases and secreted actively from cells or released passively upon cell death¹²¹. Activation and secretion of IL-1 α involve caspase 1-dependent and caspase 1-independent processes¹²². Autophagy-defective (Atg5^{fl/fl} LysM-Cre⁺) macrophages generate excess IL-1 α ¹²¹. This phenotype has been observed both in vitro and in vivo^{28,121}. Surplus active IL-1 α secreted by autophagy deficient macrophage is inflammasome-independent and

calpain-dependent¹²¹. Calpain-processing of pro-IL-1 α is triggered by ROS released from accumulated depolarized mitochondria in autophagy-deficient cells¹²¹ (Fig. 1-xiii). The role of ROS from depolarized mitochondria is similar to inflammasome activation and IL-1 β secretion in autophagy-deficient cells¹¹⁵. However, excess IL-1 β released by Atg5^{fl/fl} LysM-Cre⁺ macrophages is independent of caspase 1, NLRP3 or ASC¹²¹. Thus ROS in autophagy-defective cells leads to activation of both inflammasome and calpain pathways that lead to excessive release of IL-1 β and IL-1 α , respectively^{115 121}. IL-1 α released from Atg5-defective macrophages has important consequences in vivo, as it leads to enhanced and prolonged Th17 response in the context of *M. tuberculosis* infection, contributing to lung tissue damage in the murine model of tuberculosis¹²¹ (Fig 1c-(iii)).

Autophagy and degradation of pro-inflammatory signaling factors

Autophagy factors inhibit Bcl10-containing complexes¹¹¹ whereas autophagy degrades Bcl10¹²³. Bcl10 is a CARD-domain member of L-CBM and M-CBM tripartite complexes transducing signals from ITAM-coupled receptors in lymphoid and myeloid immune cells. Autophagy degrades Bcl10 to reduce NF κ B activation following TCR activation in effector T cells¹²³. This occurs via Sequestosome 1/p62 association with K63-polyubiquitinated Bcl10 (Fig. 1b-iii-2). Bcl10-containing complexes are also inhibited upon binding of the autophagy regulator Rubicon to CARD9¹¹¹ (Fig. 1b-iii-1). Since Rubicon is a negative regulator of autophagic degradation, its translocation to Bcl10 complexes accomplishes two things: it directly inhibits M-CBM signalling and at the same time may enhance their autophagic degradation (Fig. 1b-iii-3). NF κ B signalling may also be downregulated by autophagy via p47/NSFL1C, a protein that possesses a UBD (UBA domain) and whose ortholog in yeast binds Atg8. This potential adaptor acts a negative regulator of IKK through lysosomal (presumably autophagic) degradation of polyubiquitinated NEMO (Fig. 1b-iii-4)¹²⁴.

Third principal role: antigen presentation and lymphocyte homeostasis

Autophagy promotes MHC II presentation of cytosolic and phagocytosed antigens

One of the unique capabilities of autophagy is that it acts as a bulk “topological inverter”, by transporting proteins from the cytosol into the lumen of antigen processing compartments^{63,125,126} (Fig. 1c-(i)). This property can be further artificially enhanced by generating protein fusions (e.g. influenza matrix protein 1) with LC3 to enhance MHC II presentation to CD4 T cells¹²⁷. The topological inversion principle is more general as it feeds cytosolic ligands to endosomal receptors such as viral RNA replication intermediates to luminal TLR7 (Fig. 1b (viii-1) or Fig. 2a-(c)).

The contributions of autophagy to MHC II presentation are not restricted to cytosolic proteins and extend via LAP to exogenous antigens. Autophagy augments MHC II processing and presentation of phagocytosed extracellular particulate antigens, as shown with PRR (via LPS)-stimulated LAP of OVA-latex beads in dendritic cells (DCs)¹⁶ (Fig. 1c (ii)-a). PRR-enhancement of these process is also reflected in NOD2 stimulation with bacterial MDP, which induces autophagy and increases MHC class II presentation and generation of antigen-specific CD4⁺ T cell responses⁵³ (Fig. 1c(ii)-b).

Autophagy role in MHC II presentation affects tolerance and adaptive immunity

Physiological effects of autophagy on MHC II presentation are broad in scope. An overarching role of autophagy in MHC II presentation is its proposed role in selection of naïve T cell repertoires (Fig. 1c (iii))¹²⁶. In a comprehensive study addressing this topic¹²⁶, both positive and negative selection of CD4 T cells but not CD8 T cells were affected in the autophagy-deficient thymus, with consequences for selection of appropriate TCR repertoires and elimination of autoreactive T cells. Transplantation of Atg5-deficient thymi caused autoreactive T cell infiltration of multiple organs and autoimmune colitis reminiscent of issues in CD¹²⁶. These effects have been ascribed to defective cytosolic antigen presentation by autophagy-deficient thymic epithelial cells¹²⁶. However, autophagy-dependent antigen presentation is also defective in DCs from CD patients expressing ATG16L1 or NOD2 disease risk variants⁵³. All of these issues may be compounding the defective direct autophagic control of intestinal bacteria^{52,53}. Contributions of autophagy to MHC II presentation in antimicrobial defense are experimentally detectable in the context of adaptive immunity against viral^{63,125} and bacterial⁷² infections. Related to the enrichment of peptidylarginine deaminase in autophagosomes¹⁰⁸, autophagy promotes presentation of citrullinated antigens of relevance for autoreactivity (Fig. 1c(iv)). This process is constitutive in DCs and macrophages, but in B cells occurs only after BCR stimulation¹⁰⁸ (Fig. 1c(iv)).

Autophagy downregulates signalling during antigen presentation

Autophagy helps disassemble immunological synapses (Fig. 1c-(v))¹²⁸. Knocking down ATG16L1 and IRGM in DCs leads to hyperstable interactions with T cells and increase in T cell activation (Fig. 1c(v))¹²⁸. This may be complemented at the T cell end by autophagic degradation of Bcl10 to downregulate TCR activation (Fig. 3b(iii)-2)¹²³. Thus, although autophagy initially promotes MHC II antigen processing it later on may downregulate the response. Autophagy negatively regulates MHC I presentation by competing with proteasome for degradation of newly synthesized cytosolic proteins (Fig. 1c-(vi))¹²⁹.

Autophagy role in T cell homeostasis

Autophagy in T cells affects them in both general homeostatic and specialized functional aspects (Fig. 1c(vii)-a). Autophagy and clearance of mitochondria are needed for normal hematopoietic stem cell maintenance and function¹³⁰ affecting primarily lymphocytes but not the myeloid lineage^{16,60}. Upon exit from the thymus, naïve T cells depend on autophagy for maturation including mitochondrial content reduction¹³¹. T cells require maintenance of an ER capable of Ca²⁺ homeostasis¹³². In the absence of autophagy, Ca²⁺ is sequestered in ER-like structures building up in the Atg7-deficient T cells. This curtails Ca²⁺ fluxes in response to physiological stimulation, e.g. TCR engagement¹³².

Autophagy and Th17 cell polarization

Autophagy affects cooperation between key immune cell types that lead to T cell polarization. Th17 phenotype is promoted by excessive secretion of IL-1 from autophagy-deficient macrophages¹²¹ (Fig. 1c(ix)). IL-17 is increased in the lungs of mice with Atg5 defect in myeloid cells during *M. tuberculosis* infection¹²¹. CD4⁺ T cells show increased IL-17 expression when lung leukocytes from mice with Atg5 deletion in the myeloid lineage

are re-stimulated with mycobacterial antigens *ex vivo*¹²¹. This is based at least in part on excessive IL-1 α release from autophagy-defective macrophage, and IL-1 α 's ability combination with IL-16 and TGF- β to drive IL-17 production in CD⁺ T cells in¹²¹. Th17 response can also be enhanced by the increased durability of immunological synapses between T cells and autophagy-defective DCs^{121,128} (Fig. 1c(iii)).

Autophagy in plasma cells and humoral immunity

Although autophagy appears not to be important for survival of the majority of mature B cells¹³³, lymphoid precursor stages are affected¹³⁴. Autophagy is needed for survival of a subset of self-renewing cells (called B1) that cannot be replenished from the adult bone marrow¹³³ (Fig. 1c(x)). Upon differentiation of B cell into plasma cells, autophagy plays a role in ER maintenance under conditions of high secretory demands. Somewhat paradoxically, absence of autophagy leads to runaway immunoglobulin secretion¹³⁵. Autophagy is important in survival and homeostasis of plasma cells (Fig. 1c(xi)), especially the preservation of the bone marrow plasma cell pool, and thus is of significance for the maintenance of long-lived humoral immunity¹³⁵.

Emerging fourth principal role: autophagy in secretion of immune mediators

Further afield from its intracellular domain of action, autophagy influences extracellular release of immunologically significant substrates (Fig. __). Autophagy supports regulated secretion of extracellular innate immunity effectors and mediators¹³⁶⁻¹³⁸, some of which are stored as pre-made secretory granules cargo. This is mirrored by the role of autophagy factors and LC3 in exocytosis of secretory lysosomes during osteoclastic bone resorption, possibly representing a form of LAP whereby LC3 helps lysosomes to fuse with the plasma membrane of the ruffled border¹³⁹ in the resorption lacuna in place of the fully formed phagosomes. Defective secretion from Paneth cells in Atg16L1 hypomorph mice correlates with the corresponding intestinal phenotype in CD patients¹³⁶. Autophagic processes in senescent cells affect secretion of IL-6 and IL-8 exported via the constitutive ER-to-Golgi secretory pathway¹⁴⁰. Autophagy reduces immunoglobulin secretion from plasma cells, and thus appears to be a mechanism that prevents excessive antibody production in the body¹³⁵.

Autophagy may contribute to unconventional secretion of cytosolic proteins with extracellular immune functions. The exact mechanism of how this occurs is not fully understood. IL-1 β and IL-18 do not have signal peptides to enter the ER and follow the conventional secretory pathway (ER-Golgi-plasma membrane). Instead, they are delivered upon inflammasome activation to the extracellular environment via unconventional secretion. Although autophagy suppresses inflammasome activation under normal, e.g. nutrient-rich, conditions, autophagy can contribute to the unconventional secretion of IL-1 β under stress, e.g. nutrient starvation¹¹⁸. It is possible that this represents a double-latch mechanism to repress inflammasome under basal conditions but increase temporarily its physiological output in response to infection or DAMPs/PAMPs. Autophagy contributes to the unconventional secretion of IL-1 β ¹¹⁸. This also extends to IL-18 and HMGB1¹¹⁸. The unconventional secretion of these immune mediators depends on Atg factors and on a

specialized unconventional secretion regulator GRASP (GRASP55). GRASP is important not only in unconventional secretion but also affects the canonical starvation-induced autophagy in mammalian cells¹¹⁸.

The secretory role of autophagy is still in its embryonic phase regarding understanding both the molecular and cellular mechanisms and its full biological scope. It nevertheless extends the sphere of autophagy influence from the intracellular space to the extracellular milieu, where it affects tissue organization, remodelling, and delivery of extracellular mediators of immunity and inflammation.

Conclusions

Autophagy is a bona fide immunological process permeating many aspects of innate and adaptive immunity. Autophagy may have indeed evolved as one of the first antimicrobial defenses available to eukaryotic cells, shaped early on in evolution from what may have arisen as a metabolic and quality control pathway. Autophagy has its own set of PRRs, the SLR adaptors, to eliminate invading microbes whereas pathogens have evolved strategies to evade autophagic capture. As evolution progressed, nearly all innate immunity systems such as conventional PRRs and inflammasomes have become integrated with autophagy. In the chordate lineage, this has further extended to adaptive immunity as best documented in mammalian systems. In humans, a failure in parts of the autophagic apparatus can lead to inflammatory, autoimmune or general immunity disorders. The present knowledge of immunological autophagy is still in its infancy, and many interesting puzzles and important questions remain. As IKKs and TBK-1 regulate autophagy, why is this often at cross-purposes with type I interferon inflammation? Is ubiquitin the primary tag in recognizing incoming microbes by SLRs, or is it a result of failure to detect them early on by some other means? How are the steps in recognizing microbial entry and their targeting for autophagy integrated via host membrane damage, E3 ligases, and protein and lipid kinases? Have eukaryotic cells trained on mitochondria to learn how to deal with microbes, or are mitochondria and the present day mitophagy the result of an evolutionary battle between autophagy and the bacterium endosymbiont precursor to mitochondria? The expansion of autophagy role via its effects on secretion to the extracellular space in inflammation and tissue remodelling is an important development. In this context, the relationship of autophagy with inflammasome and unconventional secretion needs further work. Finally, while early progress has been made in adaptive immunity roles of autophagy, this area beckons more study. Some of the questions recently opened in this area are at the interface between innate and adaptive immunity and include networks between different types of immune cells. This includes the role of autophagy in Th17 polarization and possibly other T cell subsets along with general lymphocyte homeostasis and differentiation. In summary, autophagy and immunity are fully integrated and our continuing study of this interface will be a fruitful area of scientific inquiry for many years to come. In translational terms, autophagy is undoubtedly a target of opportunity for developing new treatments in inflammatory disorders and autoimmunity, and possibly as an anti-infective mechanism.

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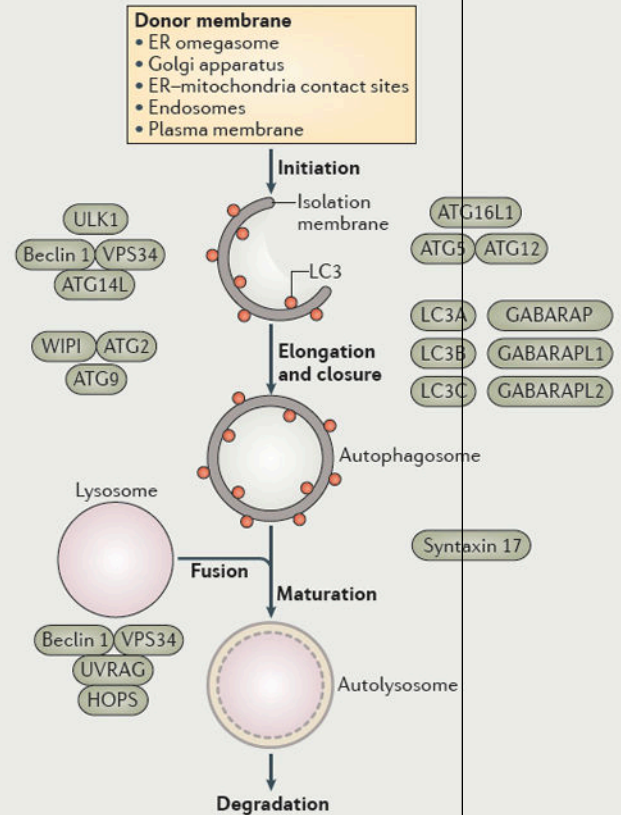
Box 1

Autophagy pathway

Autophagy is controlled by the regulatory serine/threonine protein kinases ULK1 and ULK2 (which are the mammalian homologues of the yeast autophagy-related protein 1 (ATG-1)) and by the lipid kinase activity of the class III phosphatidylinositol-3-phosphate kinase (PtdIns₃K) VPS34 (VPS-34 in yeast), which forms a complex with beclin 1 (which is the mammalian orthologue of yeast ATG-6) and ATG14-like protein (ATG14L). ULK1, ULK2 and the VPS34–beclin 1–ATG14L complex integrate upstream signals. These factors, which are engaged in the activation of autophagy during nutritional and immune responses, induce the downstream ATG conjugation cascade¹. This involves the association of the ATG5–ATG12 conjugate with ATG16L1. The ATG5–ATG12–ATG16L1 complex facilitates the addition of a phosphatidylethanolamine group to the carboxyl terminus of the mammalian paralogues of ATG-8: LC3A, LC3B (which has been used as a marker for the identification of autophagosomes in mammalian cells), LC3C, γ -aminobutyric acid receptor-associated protein (GABARAP), GABARAP-like 1 (GABARAPL1) and GABARAPL2 (REF. 1). Lipidated mammalian paralogues of ATG-8 function together with other factors to assemble, elongate and lead to the closure of nascent autophagic organelles¹ (see the figure). The lipid kinase VPS34, through its production of PtdIns₃ phosphate (PtdIns₃P), has a dual function in autophagy¹. During initiation, VPS34-derived PtdIns₃P is recognized by the PtdIns₃P-binding factors WD repeat domain phosphoinositide-interacting protein 1 (WIPI1)–WIPI4 (which are the mammalian paralogues of yeast ATG-18), and these factors cooperate with ATG2 and ATG9 to form the phagophore¹. During the final maturation of autophagosomes into autolysosomes, the attachment of a tail-anchored SNARE, syntaxin 17 (REF. 6), to the autophagosomal membrane enables fusion with lysosomes. At this stage, the VPS34–beclin 1 complex contains ultraviolet radiation resistance-associated gene protein (UVRAG) instead of ATG14L¹.

Autophagy requires the rapid remodelling of endomembranes in the cytoplasm, which involves the formation of transient PtdIns₃P-positive structures (known as omegasomes) on the endoplasmic reticulum (ER), the ER–mitochondria contact sites, the Golgi apparatus and the plasma membrane-derived endosomal organelles. This happens independently of transcriptional changes. However, transcriptional adjustments are necessary for sustained autophagy, for instance, to replenish ‘consumables’ such as LC3 and autophagic adaptors including sequestosome 1 (also known as p62), which are degraded along with the captured cargo in autolysosomes. Transcriptional adjustments are accomplished by transcription-positive regulators (for example, transcription factor EB (TFEB), forkhead box protein O3A (FOXO3A) and NFE2-related factor 2 (NRF2)) and transcription-negative regulators (for example, zinc finger protein with KRAB and SCAN domains 3 (ZKSCAN3; also known as ZNF306), which coordinate autophagy with lysosomal, proteasomal, lipolytic and oxidative stress response systems^{136,139}.

HOPS, homotypic fusion and vacuole protein sorting.



The Atg nomenclature is based on yeast genetics. It includes the Ser/Thr kinase Atg1 (mammalian Ulk1 and Ulk2), Atg6 (mammalian Beclin 1) in complexes with class III phosphatidylinositol 3-phosphate kinase (PI3K) Vps34 (mammalian hVPS34), Atg5-Atg12 conjugate in complex with Atg16 (mammalian Atg16L1), and Atg8 (mammalian orthologs, mAtg8s: LC3A, LC3B, LC3C, GABARAP, GABARAPL1, and GABARAPL2). Historically, LC3B has been used as a marker for identification of autophagosomes in mammalian cells. A hierarchical organization of mAtg8s has begun to emerge in conjunction with autophagic adaptors and recognition of intracellular microbes. Autophagy is controlled by the regulatory protein kinases Ulk1/2 plus the lipid kinase hVPS34, the latter being in a complex with Beclin 1 and Atg14L. They integrate upstream signals, and set in motion the downstream Atg conjugation cascade. This involves Atg5-Atg12/Atg16L1 assembly, which then acts as a functional equivalent of E3 ligases from the ubiquitin system. This results in a hallmark event of mAtg8s C-terminal

lipidation with phosphatidylethanolamine, the basis for a key assay in measuring autophagy. Lipidated mAtg8s, in cooperation with other factors, assemble, elongate, and lead to closure of nascent autophagic organelles. The lipid kinase hVPS34 participates once again during the final maturation stages of autophagosomes into autolysosomes. For this, it is still complexed with Beclin 1 but now contains UVRAG instead of Atg14L. Autophagy requires rapid remodeling of endomembranes in the cytoplasm independently of transcriptional changes. Appropriate transcriptional adjustments are necessary for sustained autophagy, e.g. to replenish “consumables” such as LC3 and autophagic adaptors including Sequestosome 1/p62, degraded along with the captured cargo in autolysosomes. Transcriptional adjustments are accomplished via global positive (TFEB, FoxO3A, NRF2) and negative (ZKSCAN3/ZNF306) regulators coordinating autophagy with lysosomal, proteasomal, and oxidative stress response systems.

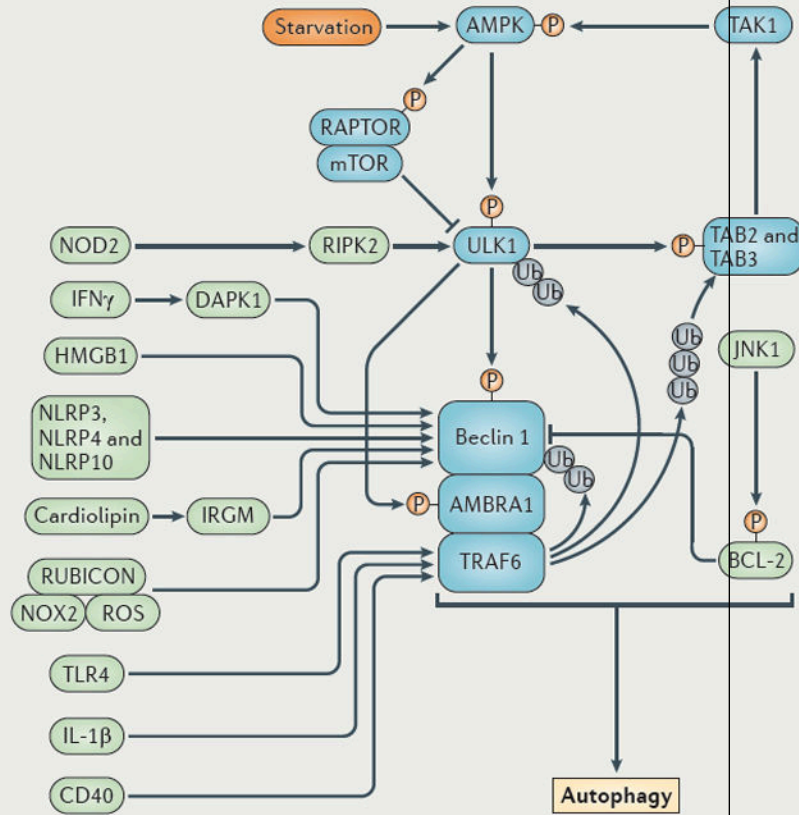
Box2

Immune signaling and autophagy

The model (see the figure) proposes that immune and nutritional signals converge to activate a similar cascade. Nutritional triggers, such as starvation, are transduced via mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK). Starvation inhibits mTOR and activates AMPK, which in turn activates the serine/threonine protein kinase ULK1 to phosphorylate beclin 1 (REF. 140), activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1)¹⁵⁰ and TAK1-binding protein 2 (TAB2)¹⁴¹. In the resting state, TAB2 and TAB3 bind beclin 1 and repress its activity^{30,140}. TAB2 activates TGFβ-activated kinase 1 (TAK1)³⁰ to further enhance AMPK activity. AMBRA1 (REF. 10), along with beclin 1 (REF. 107), recruits TNF receptor-associated factor 6 (TRAF6). TRAF6 functions as an E3 ubiquitin ligase, generating polyubiquitin chains that in turn stabilize and activate ULK1 (REF. 10) and beclin 1 (REF. 107), and that probably activate the TAB2 and TAB3-TAK1 complexes. Once fully amplified, the concomitant activation of ULK1 and beclin 1 leads to

autophagy through both branches of autophagy regulatory kinases — that is, the protein kinase ULK1 (via AMPK) and the lipid kinase VPS34 (via beclin 1) (not shown)¹⁰⁷.

Immune signals activate autophagy and engage at least some components of the pathways, including ULK1 (REF. 108), beclin 1 (REF. 142) and TRAF6 (REF. 107). Downstream of the TAB2 and TAB3-TAK1 complexes, IκB kinases (IKKs) affect autophagy independently of nuclear factor-κB¹⁴³. TANK-binding kinase 1 (TBK1) also controls autophagy^{14,77} and promotes the elimination of intracellular microorganisms independently of its role in type I interferon (IFN) activation (not shown)¹⁴⁴. TBK1 controls both the capture of autophagic cargo (for example, microorganisms) via specialized adaptor proteins^{14,35,36,45} and the maturation of autophagosomal organelles into degradative compartments^{14,77}. TBK1 may also contribute to autophagosome formation at stages earlier than autophagosome maturation⁷⁷.



BCL-2, B cell lymphoma 2; DAPK1, death-associated protein kinase 1; HMGB1, high-mobility group box 1 protein; IL-1β, interleukin-1β; IRGM, immunity-related GTPase family M protein; JNK1, Jun N-terminal kinase 1; NLRP, NOD-, LRR- and pyrin domain-containing protein; NOD2, nucleotide-binding oligomerization domain-containing protein 2; NOX2, NADPH oxidase 2; P, phosphorylation; RAPTOR, regulatory-associated protein of mTOR; RIPK2, receptor-interacting serine/threonine-protein kinase 2; ROS, reactive oxygen species; RUBICON, run domain beclin 1-interacting and cysteine-rich-containing protein; TLR4, Toll-like receptor 4; Ub, ubiquitylation.

IKK signaling and its upstream regulators TAB2, TAB3 and TAK1 control autophagy. TAB2 and TAB3 repress Beclin 1. Upon induction, TAB2 and TAB3 dissociate from Beclin 1, allowing it to activate autophagy. They also bind to TAK1. TAK1 in turn activates AMPK, which helps induce autophagy. PAMPs and PRRs can set in motion autophagosome formation. TLR4-MyD88 signaling leads to a direct ubiquitination of

Beclin 1 by TRAF6, which in turn dissociates Bcl-2 from Beclin 1 and disinhibits it. Whether signalling from TLRs engages IKKs and TBK-1 has not been experimentally addressed. HMGB1, an alarmin, derepresses Beclin 1 by displacing its negative regulator Bcl-2. It can additionally activate autophagy extracellularly via RAGE signalling. Other DAMPs/alarmins such as DNA complexes and ATP can activate autophagy. IL-1 β , a key pro-inflammatory cytokine induces autophagy in autophagic control of *M. tuberculosis* in infected macrophages. IL-1 β does it through MyD88 downstream of IL-1R and may involve TRAF6-dependent Beclin 1 ubiquitination. Th1 cytokines such as IFN- γ induce autophagy in effector cells including macrophages where they restrict mycobacteria. IFN- γ activation of autophagy involves a death associated protein kinase (DAPK)-dependent phosphorylation of Beclin 1 on Thr 119 located within its BH3 domain, which (congruently with other processes leading to Beclin 1 activation) dissociates its inhibitor Bcl-2. TNF- α stimulates Sequestosome 1/p62 adaptor-guided autophagy to restrict intracytosolic bacteria such as *Shigella* and *Listeria*. Th2 cytokines, such as IL-4 and IL-13 inhibit autophagy. IL-10 can also inhibit autophagy via Akt signalling. STAT3, which transduces signals downstream of various inputs including IL-6, can inhibit autophagy. This occurs not via transcriptional signalling, but rather by binding of the cytosolic STAT3 to protein kinase R (PKR). T cell receptor engagement and CD28 co-stimulation induce autophagy in T cells. CD40L-CD40 engagement between activated T cells and macrophages induces autophagy via TRAF6 to help eliminate *Toxoplasma gondii* parasitizing phagocytes.

Box 3**Microbial countermeasures against autophagy**

Microbes show a wide range of mechanisms to prevent, counter, or commandeer autophagy. *Listeria* avoids autophagic capture of bacteria via AktA and InlK, and *Shigella* via IcsB. These processes mask bacterial epitopes or host ubiquitin tags that can be recognized by the autophagic machinery, either directly or by recruiting cytosolic proteins. For the fraction of bacteria that do not escape autophagic capture, *Listeria* can block autophagosome acidification via its pore-forming toxin LLO and thus prevent its maturation into autolysosomes. *Legionella* RavZ, an effector injected into the host cytoplasm via bacterial type IV secretion system, inhibits host autophagy through irreversible mAtg8 deconjugation. This happens by proteolysis of the mAtg8 C-terminal tails removing the C-terminal glycine along with its PE modification. Some of the viral defense examples include HSV-1 protein ICP34.5, influenza M2 protein, and HIV Nef, all of which target Beclin 1 to inhibit autophagy altogether or just to block autophagosomal maturation. Nef binds to the evolutionary conserved domain of Beclin 1 at the same 267–299 region where the host protein GAPR-1 (Golgi-associated plant pathogenesis-related protein 1) binds to act as an endogenous inhibitor of Beclin 1. Mouse herpesvirus 68 encoded protein M11 (viral Bcl-2) inhibits Beclin 1 via its BH3 domain whereas KSHV FLIP blocks Atg3 in the LC3 lipidation cascade. HIV Nef, Hepatitis C virus NS3, and Measles virus Mev3 proteins interact, with a yet to be fully understood consequences, with IRGM, a factor that controls antimicrobial autophagy and confers genetic risks in Crohn's disease and tuberculosis. Finally, several microbes can also actively utilize autophagy for their life cycle based on an array of effects including membrane platforms for replication and metabolic effects, as previously reviewed. As a mechanistic example of the latter, the obligatory intracellular pathogen, *Anaplasma phagocytophilum*, uses its type IV secretion effector Ats-1 to bind Beclin 1 and activate autophagy to generate nutrients.

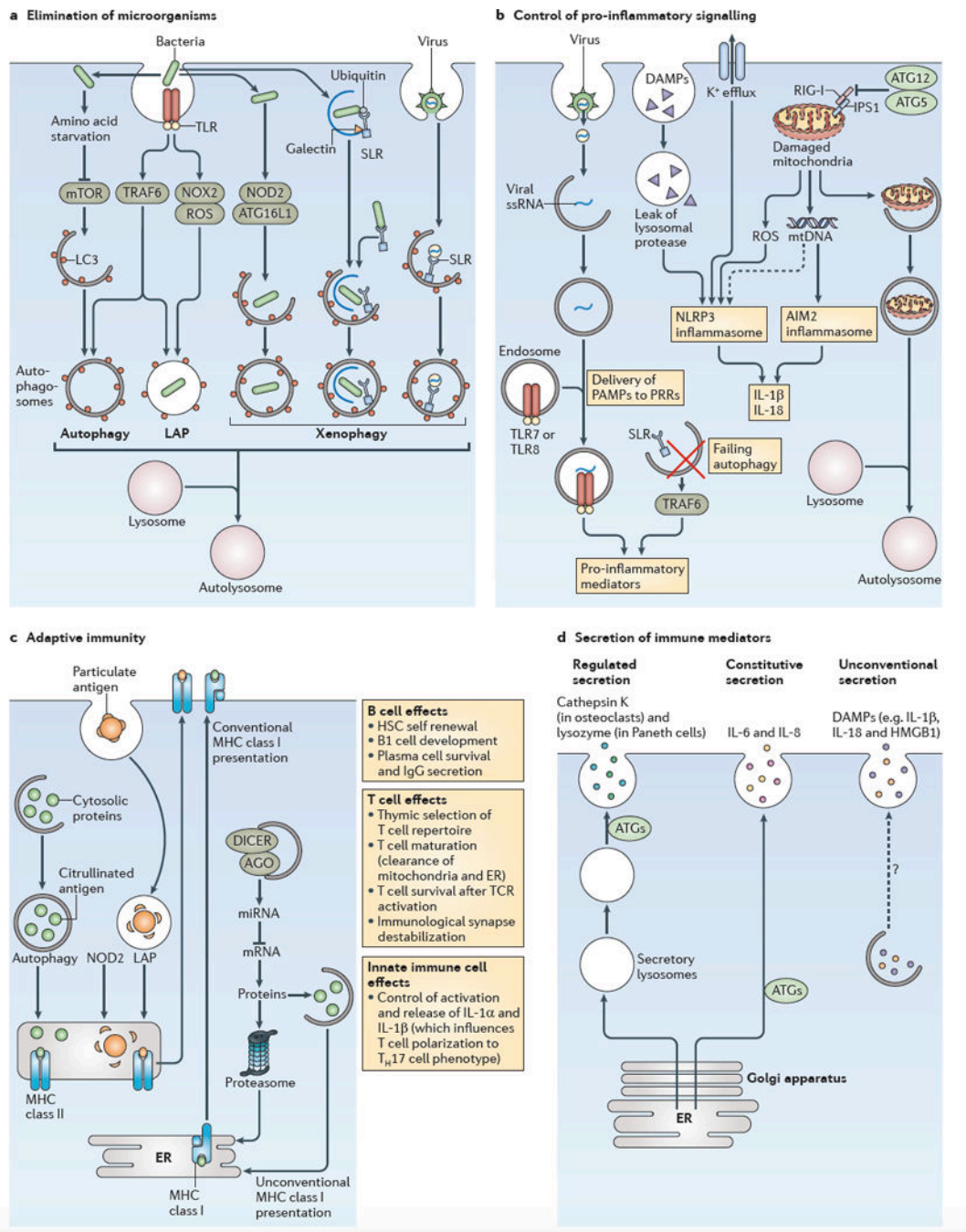


Figure 1. Four principal roles of autophagy in immunity

a | The role of autophagy in the elimination of microorganisms is shown. An incoming microorganism can induce autophagy by competing for nutrients or by stimulating innate immune receptors, such as Toll-like receptors (TLRs). When the microorganism is taken up by phagocytosis and remains in an intact vacuole, an autophagic process termed LC3-associated phagocytosis (LAP) can promote the maturation of autophagosomes into autolysosomes. Xenophagy of pathogens that enter the cytosol can be initiated by sequestosome 1-like receptors (SLRs) or other mechanisms, including nucleotide-binding

oligomerization domain-containing protein 2 (NOD2)–autophagy-related protein 16-like 1 (ATG16L1) interactions. **b** Several examples of the role of autophagy in the control of pro-inflammatory signalling (see also FIG. 3) are shown. Failure to remove SLRs by autophagy can increase the levels of these receptors and the levels of pro-inflammatory signalling. Autophagy can deliver cytoplasmic pathogen-associated molecular patterns (PAMPs) to endocytic TLRs and can stimulate their activity. NOD-like receptors (NLRs; such as NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3)) and RIG-I-like receptors (RLRs; such as absent in melanoma 2 (AIM2)) show complex positive and negative co-regulation with autophagy: the ATG5–ATG12 complex inhibits retinoic acid-inducible gene I (RIG-I) signalling, and autophagy limits inflammasome activation by removing damaged mitochondria, which then release the inflammasome activators reactive oxygen species (ROS) and mitochondrial DNA. **c** The role of autophagy in adaptive immunity is shown. Autophagy can increase the MHC class II presentation of cytoplasmic antigens, including self or viral antigens, as well as promoting the citrullination of antigens. LAP can enhance the processing of particulate antigens for MHC class II presentation. NOD2 enhances autophagic antigen presentation. Autophagy may directly or indirectly affect MHC class I presentation by competing with the proteasome for substrates, by influencing the peptidome pools through the control of levels of components of microRNA (miRNA) machinery (for example, argonaute (AGO) and DICER), or by supporting unconventional MHC class I presentation. In addition, autophagy affects the self-renewal of haematopoietic stem cells (HSCs), B1 cell development, plasma cell survival and IgG secretion. Autophagy affects T cell survival following T cell receptor (TCR) activation, and it destabilizes the immunological synapse. It also controls innate immune cell (such as macrophage) signalling through the release of interleukin-1 α (IL-1 α) and IL-1 β , which influence the polarization of T cells into T helper 17 (T_H17) cells. Autophagy also affects naive T cell repertoire selection in the thymus and the survival and function of maturing T cells by removing the mitochondria and endoplasmic reticulum (ER), thus ensuring calcium homeostasis. **d** The role of autophagy in the secretion of immune mediators is shown. Autophagy affects the quality of regulated secretion from pre-stored granules. Autophagy affects the quality and the quantity of the output of the constitutive secretory pathway (which is the conventional pathway of protein secretion via the ER, the Golgi apparatus and the plasma membrane). Autophagy supports a form of unconventional secretion that captures cytoplasmic proteins for extracellular release. Note that secretory protein cargo in the regulated and constitutive secretory pathways contains conventional leader peptides for co-translational import into the ER lumen, whereas protein cargo that enters the unconventional secretory pathway lacks leader peptides and does not enter the ER. Dashed arrow indicates that this pathway remains to be defined. DAMPs, damage-associated molecular patterns; HMGB1, high-mobility group box 1 protein; IPS1, IFN β promoter stimulator protein 1; mtDNA, mitochondrial DNA; mTOR, mammalian target of rapamycin; NOX2, NADPH oxidase 2; PRRs, pattern recognition receptors; ssRNA, single-stranded RNA; TRAF6, TNF receptor-associated factor 6.

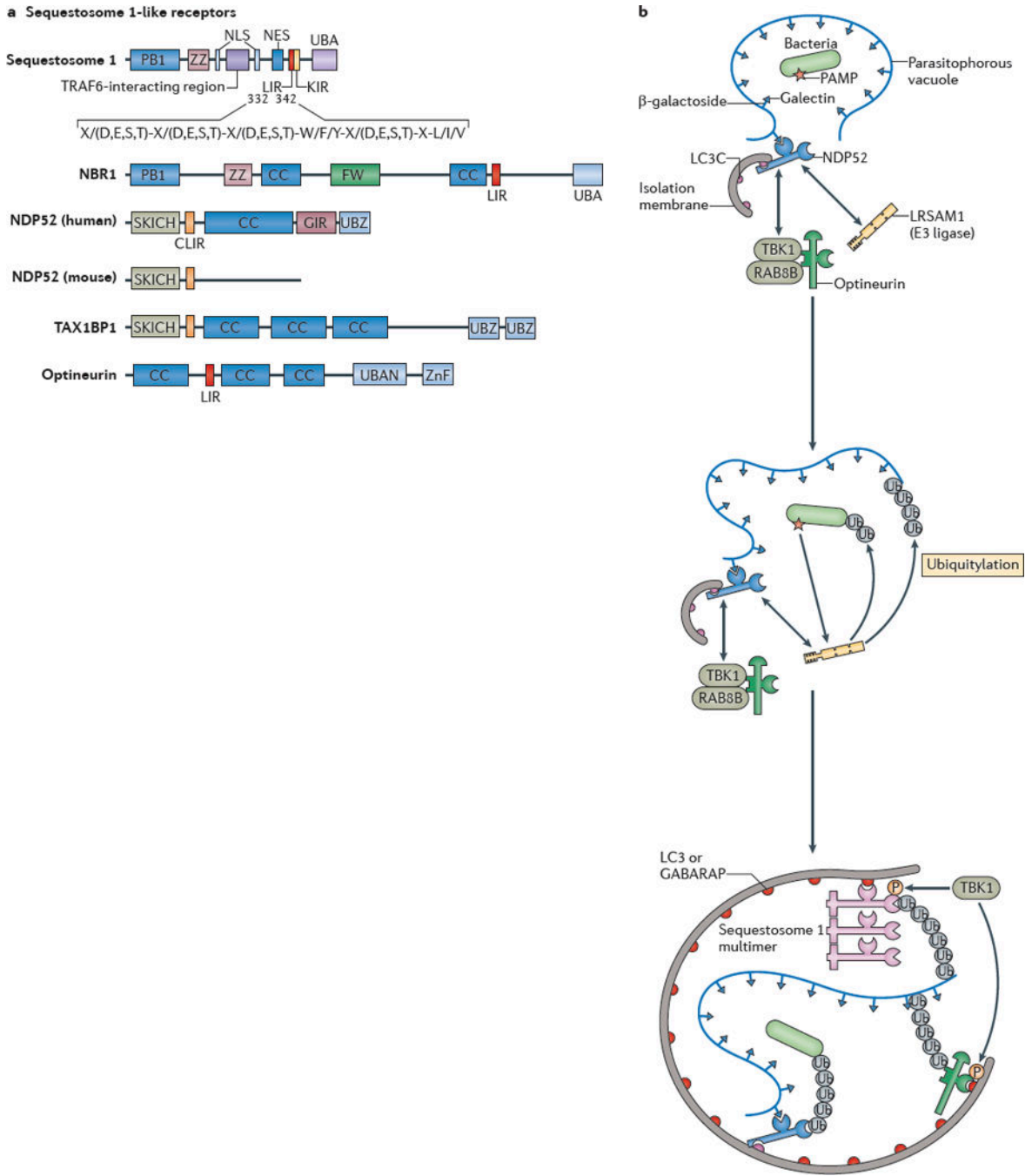


Figure 2. Autophagy-mediated clearance of intracellular pathogens
a) Protein domains of sequestosome 1-like receptors (SLRs) are shown. The LC3-interacting region (LIR) motif of an SLR binds to autophagy-related LC3 proteins through its consensus sequence at amino acids 332–342 in sequestosome 1 (also known as p62). The conserved residues are shown. X/(D,E,S,T) indicates that any amino acid (X) is allowed but that acidic (D,E) or phosphorylatable amino acids (S,T) are often present (usually at least one or more within the entire consensus sequence). The core LIR motif residues are aromatic pocket-filling W (or F or Y) residues and aliphatic pocket-filling L (or I or V). They form an

intermolecular parallel β -sheet with LC3 proteins or γ -aminobutyric acid receptor-associated proteins (GABARAPs). The CLIR motif, which is a LIR motif that is specific for LC3C, lacks the aromatic residue found in the LIR motif and, instead, uses hydrophobic contacts provided by additional aliphatic residues located between the W and L position anchors to stabilize interactions with LC3C. All human SLRs also contain a ubiquitin-binding domain (UBD): UBA (as found in sequestosome 1 and NBR1) is a three-helix bundle UBD that has affinity for monoubiquitin and K63 ubiquitin linkages; UBAN (as found in optineurin) is a parallel coiled-coiled dimer UBD that has specificity for linear ubiquitin chains; and UBZ (as found in nuclear dot protein 52 (NDP52)) is a zinc finger $\beta\beta\alpha$ -fold UBD that binds to monoubiquitin and polyubiquitin. **b** A model of cooperative action between different SLRs and E3 ligases in bacterial targeting for xenophagy is shown. The schematic shows a parasitophorous vacuole with glycosylated molecules (in this case β -galactosides) facing the lumen of the vacuole that contains a bacterium and that is experiencing membrane damage. This membrane tear exposes β -galactosides to galectins (for example, galectin 8) which in turn bind to the galectin-interacting region (GIR) motif of NDP52. NDP52 also directly interacts with the E3 ligase LRSAM1 and indirectly with the serine/threonine protein kinase TANK-binding kinase 1 (TBK1), which interacts with optineurin. The CLIR motif of NDP52 binds to LC3C, which is a proposed initiator in the LC3 and GABARAP cascade during bacterial xenophagy. LRSAM1 or other E3 ubiquitin ligases polymerize ubiquitin at molecular targets that are yet to be identified. The hypothetical model includes the putative recognition of bacterial pathogen-associated molecular patterns (PAMPs) by LRSAM1 through its leucine-rich repeat domain. Ubiquitin tags are recognized by UBDs of NDP52, optineurin and sequestosome 1. The LIR motif of optineurin is phosphorylated by TBK1 and this improves LC3 and GABARAP binding. The UBA of sequestosome 1 is also phosphorylated by TBK1 and this improves ubiquitin chain binding. As a consequence, the autophagic isolation membrane initiates at the appropriate location and grows to capture the bacterium and to eliminate it through autophagy. CC, coiled-coil domain; FW, four W domain (also known as the NBR1 domain); KIR, KEAP1 interacting region; NES, nuclear export signal; NLS, nuclear localization signal; P, phosphorylation; PB1, protein-binding domain 1; SKICH, skeletal muscle and kidney enriched inositol phosphatase carboxyl homology domain; TAX1BP1, TAX1-binding protein 1; Ub, ubiquitylation; ZnF, zinc finger domain; ZZ, ZZ-type ZnF domain.

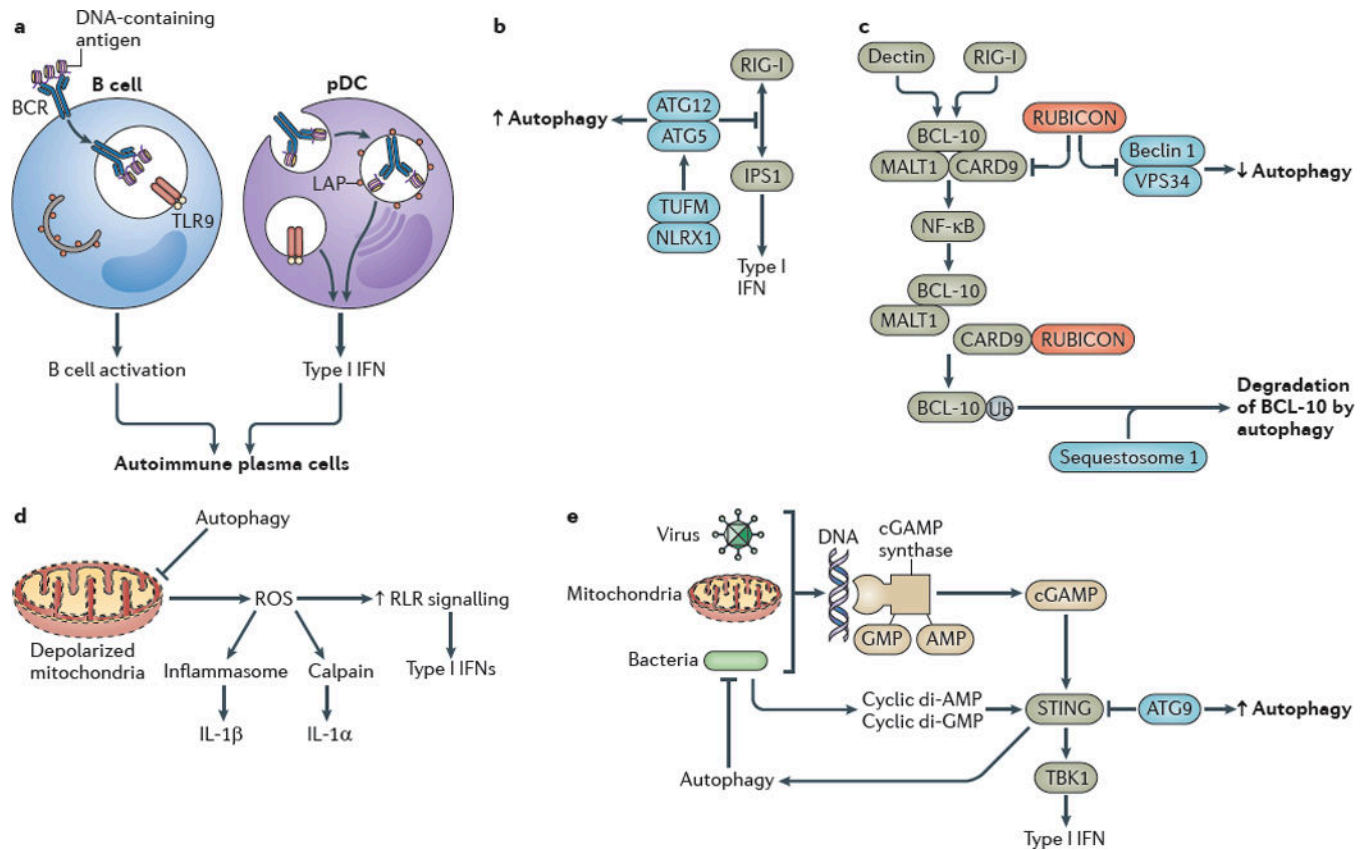


Figure 3. Autophagy controls inflammatory processes

a Autophagy promotes Toll-like receptor 9 (TLR9) signalling in B cells and type I interferon (IFN) production by plasmacytoid dendritic cells (pDCs). **b** The autophagy protein complex autophagy-related protein 5 (ATG5)–ATG12 inhibits RIG-I-like receptor (RLR) signalling by binding to the caspase recruitment domains of retinoic acid-inducible gene I (RIG-I) and IFN β promoter stimulator protein 1 (IPS1), which is the mitochondrial adaptor of RIG-I signalling. The NOD-like receptor X1 (NLRX1)-interacting partner mitochondrial Tu elongation factor (TUFM) associates with the ATG5–ATG12 complex to promote autophagy while inhibiting RLR-dependent type I IFN activation. **c** Autophagy factors negatively regulate the caspase recruitment domain-containing protein 9 (CARD9)–B cell lymphoma 10 (BCL-10)–mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) complex. RUBICON (run domain beclin 1-interacting and cysteine-rich-containing protein), which is a binding partner and a negative regulator of beclin 1, inhibits CARD9, whereas sequestosome 1 leads to the degradation of BCL-10. **d** Excessive production of reactive oxygen species (ROS) by depolarized mitochondria that are not cleared by autophagy enhance RLR signalling. **e** Viral, mitochondrial or bacterial DNA lead to the activation of stimulator of IFN genes protein (STING), probably through cGAMP synthase and cyclic GMP–AMP (cGAMP) production, which increases the type I IFN response. Autophagy removes sources of agonists that stimulate STING, whereas autophagic factors (for example, ATG9) inhibit the activation of STING by affecting its cytoplasmic translocation. Bacterial cyclic dinucleotides (di-AMP and di-GMP) can activate autophagy, thereby functioning as a regulatory loop that amplifies the removal of infectious or

endogenous irritants. BCR, B cell receptor; IL, interleukin; LAP, LC3-associated phagocytosis; NK- κ B, nuclear factor- κ B; TBK1, TANK-binding kinase 1; Ub, ubiquitylation.

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