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## Review Article: Special Edition

# From Christian de Duve to Yoshinori Ohsumi: More to autophagy than just dining at home

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## ABSTRACT

Christian de Duve first coined the expression “autophagy” during his seminal work on the discovery of lysosomes, which led to him being awarded the Nobel Prize in Physiology or Medicine in 1974. The term was adopted to distinguish degradation of intracellular components from the uptake and degradation of extracellular substances that he called “heterophagy”. Studies until the 1990s were largely observational/morphological-based until in 1993 Yoshinori Ohsumi described a genetic screen in yeast undergoing nitrogen deprivation that led to the isolation of autophagy-defective mutants now better known as ATG (AuTophagy-related) genes. The screen identified mutants that fell into 15 complementation groups implying that at least 15 genes were involved in the regulation of autophagy in yeast undergoing nutrient deprivation, but today, 41 yeast ATG genes have been described and many (though not all) have orthologues in humans. Attempts to identify the genetic basis of autophagy led to an explosion in its research and it's not surprising that in 2016 Yoshinori Ohsumi was awarded the Nobel Prize in Physiology or Medicine. Our aim

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here is not to exhaustively review the ever-expanding autophagy literature (>60 papers per week), but to celebrate Yoshinori Oshumi's Nobel Prize by highlighting just a few aspects that are not normally extensively covered. In an accompanying mini-review we address the role of autophagy in early-diverging eukaryote parasites that like yeast, lack lysosomes and so use a digestive vacuole to degrade autophagosome cargo and also discuss how parasitized host cells react to infection by subverting regulation of autophagy.

### Induction of autophagy: the ATG1/ULK1/2 initiation complex and the increasingly controversial roles of ATGs

Traditionally, starvation has been the classical way to stimulate autophagy [Fig. 1], since degradation of autophagosome cargo by lysosomes generates nutrients that enable the cell to survive until fresh “food” comes on-line [1]. Consistent with nutrient sensing being a key regulator of autophagy, when rapamycin (inhibitor of mTOR) treatment was found to promote the translocation of the ULK1 complex from the cytosol to certain domains of the endoplasmic reticulum (ER) (or closely attached structures) and induce autophagy [2,3], the role of mTORC1 kinase-mediated phosphorylation of the ATG1/ULK1/2 complex (ULK1/2, ATG13, FIP200 and ATG101) in suppressing induction of autophagy when nutrients (for example amino acids) are abundant, became clear [4–6]. By contrast, upon energy restriction, ULK1 is activated via phosphorylation by AMP-activated kinase (AMPK) that at the same time phosphorylates and inhibits mTORC1 [7,8]. Moreover, in addition to phosphorylation-mediated activation, ULK1-induction also occurs upon acetylation in response to amino acid, glucose, or growth factor deprivation [9]. However, the ever-expanding roles of autophagy in other cellular processes (differentiation [10], clearing of aberrant aggregates and damaged organelles [11], metabolism [12], innate and adaptive immunity [13–15] – see below) over and above nutrient deprivation has led to the realisation that there are ways of inducing autophagy independent of the ATG1/ULK1/2 complex. In addition, many early-diverging eukaryote parasites lack genes orthologous to ATG1 (see accompanying mini review), yet they still induce autophagy.

Further complicating matters, recent studies [16] have also challenged the orthodoxy that the ATGs (ATG3/AT5/ATG7) involved in lipid conjugation of ATG8/LC3 drive autophagosome formation in mammals by showing that mouse embryonic fibroblasts (MEF) lacking ATG-3, -5 or -7 (but not upstream elements ATG14 or FIP200) were still capable of forming isolation membranes. However, their ability to generate mature closed autophagosomes was impaired suggesting that these ATG elements may act to promote autophagosome closure and consequent degradation of the inner autophagosomal membrane, invoking a mechanism of inward fission by which vesicular enzymes can degrade membranes [16,17]. Interestingly, a role has previously also been suggested for LC3 in autophagosome closure and fusion with lysosomes [18]. The new studies were performed under basal or starvation conditions and so they do not necessarily rule out a role for the ATG lipid conjugation system in

autophagosome biogenesis during selective autophagy, where LC3-II is crucial for tethering specific cargoes for degradation [17]. Nevertheless, together with the increasing realisation of roles for ATG proteins both in non-canonical mechanisms of autophagy and also in functions completely independent of autophagy [14,19–21], care should be taken with genetic studies implicating the mechanistic involvement of autophagy on the basis of disruption of ATG signalling.

### The type III phosphatidylinositol 3-kinase Vps34

Activation of ULK1/ATG1 cooperates with Vps34 in autophagy initiation. Vps34 is usually found dimerized to Vps15, but a second complex including Beclin 1 (ATG6) is abundant in mammalian cells. Furthermore, Vps34/Vps15/Beclin 1 can form a tetramer complex with either ATG14 (complex I), or with UVRAG/Vps38 (complex II), where complex I regulates autophagosome formation and complex II the fusion of autophagosomes with lysosomes [22]. In the early steps of starvation-induced autophagy, VMP1-Beclin 1-mediated production of PIP<sub>3</sub> in the ER [4,23,24], recruits double-FYVE-domain containing protein 1 (DFCP1) to the so-called omega-some, which is an autophagosomal precursor to allow cooperation with WD-repeat domain phosphoinositide-interacting (WIPI)-2, that promotes development of omega-somes into isolation membranes or autophagosomes [25]. EEA1 is another FYVE-domain protein that is involved in Rab5 and Rab7 activation on early and late endosomes, the latter of which plays a key role in the maturation of autophagosomes and in autophagosome fusion with lysosomes [26]. As Oshumi's original genetic screen was measuring cytosol to vacuole transport (cvt), the finding that some Rabs, small GTPases that regulate vesicular traffic, are also involved in regulating vacuolar protein sorting (Vps) is not unexpected and today, in addition to the aforementioned Rab5 and Rab7, several other Rabs (Rab1, Rab9A, Rab11, Rab23, Rab32, and Rab33B) and Rab-effector proteins are known to participate in autophagosome formation and autophagosome maturation [27–29].

### cAMP-dependent PKA and autophagy regulation

Over 10 years ago using the yeast, *Saccharomyces cerevisiae* it was demonstrated that the cAMP-dependent PKA signalling pathway is involved in controlling autophagy [30]. Interestingly, ATG1, ATG13 and ATG18, were all identified as potential substrates for PKA and PKA-mediated phosphorylation of ATG1 regulated its association with the pre-autophagosomal

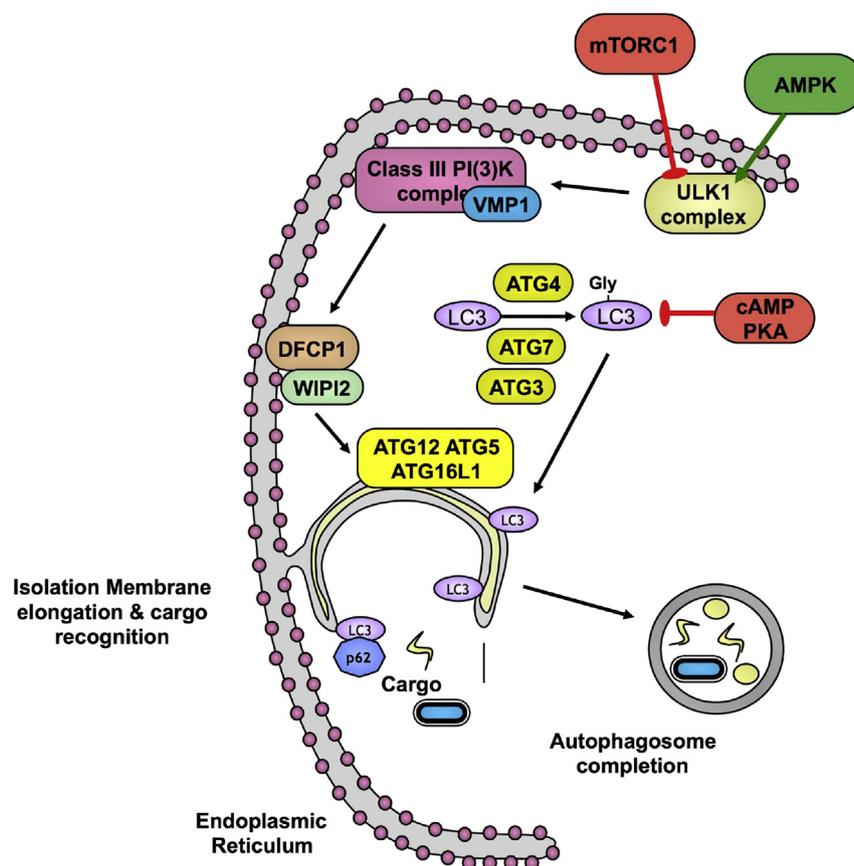


Fig. 1 The molecular events involved in membrane initiation, elongation and completion of the autophagosome. The major membrane source of the autophagosome is thought to be the endoplasmic reticulum (ER), as electron tomography has demonstrated a direct connection with ER cisternae and autophagosomal membranes [164,165]. However, and as evidenced by ATG9, a multispanning membrane protein that traffics between the trans-Golgi network, endosomes and autophagosome precursors being shown to be essential for autophagy [166], several additional sources (mitochondrial, plasma and nuclear) have been implicated [71,167,168]. Following induction of autophagy, the ULK1 complex (ULK1-ATG13-FIP200-ATG101) translocates to the ER and transiently associates with vacuole membrane protein 1 (VMP1) resulting in the activation of the class III PI3K, Vps34 and recruitment of the double FYVE domain-containing protein (DFCP1), WD repeat domain phosphoinositide-interacting protein 2 (WIPI2) and the ATG12-ATG5-ATG16L1 complex to the outer surface of the isolation membrane. Elongation of the isolation membrane requires the sequential action of the protease ATG4, the E1-like enzyme ATG7 and the E2-like enzyme ATG3 to generate, via the E3-like activity of the ATG5-ATG12-ATG16 complex, the phosphatidylethanolamine (PE)-conjugated form of microtubule-associated protein light chain 3 (LC3-II; a homolog of yeast ATG8). This is associated with both the outer and inner membrane of the isolation membrane where it can tether the autophagic cargo to the isolation membrane through specific adaptor proteins such as p62/SQSTM1, that bind polyubiquitinated protein aggregates targeting them for degradation in the autolysosomes, through its association with LC3-II in the forming autophagosome [63]. The mechanisms of autophagosome closure and fusion are less clear but it has been proposed that ATG proteins and LC3 may play an important role in closure of the autophagosome [16–18]. Following dissociation of ATG16, dynein may be recruited via interaction with LC3 to promote trafficking to the perinuclear region of the cell for fusion with lysosomes [18].

structure otherwise known as PAS [31]. Subsequently, 18 candidate PKA regulators of different steps of autophagy were identified demonstrating that cAMP-fluxing and phosphorylation via PKA has a profound impact on regulation of autophagy [32].

Thus, in mammalian cells (originally neurones) the ATG8 orthologue LC3 was found to be phosphorylated by PKA at S12 and this reduced LC3 recruitment to autophagosomes, illustrating that similarly to the yeast system, a rise in cAMP levels

that activates PKA diminishes autophagy [33]. This led to the proposal of the existence of a pool of pre-phosphorylated LC3 that gets rapidly recruited to autophagosomes in response to nutrient deprivation or mitochondrial injury with dephosphorylation of LC3 allowing cells to rapidly switch from basal to induced autophagy.

A special type of autophagy is called mitophagy, where damaged mitochondria are degraded and this process is regulated by PINK1 (PTEN-induced putative kinase 1) and

Parkin, so called because mutations in these genes are involved in Parkinson's disease [34]. PINK1 recruits the ubiquitin ligase Parkin to damaged mitochondria that become polyubiquitylated and degraded by mitophagy. Raising cAMP levels by forskolin stimulation of adenylate cyclase, or over-expression of PKA both inhibited recruitment of Parkin to depolarized mitochondria, again consistent with cAMP-dependent PKA acting as a negative regulator of autophagy, in this case mitophagy [35]. A mitochondrial membrane protein MIC60 is phosphorylated by PKA at S528 and PKA-mediated phosphorylation of MIC60 blocks assembly of the PINK1 complex and loss of PINK1 on mitochondria.

Oxidative stress induces both autophagy and an anti-oxidant transcriptional response mediated by the Nrf2 transcription factor: coordination of these processes is mediated, at least in part by the scaffold protein p62/SQSTM1 that links autophagy to Keap1, an adapter of the ubiquitin ligase complex that (negatively) regulates Nrf2 [36]. mTORC1-dependent phosphorylation of p62 on S351 causes greater p62 affinity for Keap1 and as a consequence, Keap1 together with phosphorylated p62 is degraded by autophagy and Nrf2 is stabilized and can translocate to the nucleus to transcribe anti-oxidant genes. PKA also phosphorylates p62, but at S24 interfering with its ability to homopolymerise and initiate autophagy, indicating again how cAMP-dependent PKA can act as a negative regulator of autophagy [37]. In yeast there is a large (circa 500 proteins) network of potential PKA regulators [32] and therefore, there are many potential ways for cAMP-dependent PKA to act as a negative regulator of autophagy.

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### Stress activated c-Jun N-terminal Kinase (JNK) and regulation of autophagy

At the time of writing a key word search "JNK & autophagy" identified over 500 papers in PubMed, so we will not try to review the ensemble contribution of JNK to autophagy, but just highlight a few examples. As discussed above, Parkin and PINK1 mutations are associated with Parkinson's disease, but a third gene coding for DJ-1 also known as PARK7 is mutated [34,38]. DJ-1/PARK7 is a member of the C56 peptidases that function as sensors and modulators of reactive oxygen species, so it's not surprising that a stress activated kinase like JNK is involved in mediating DJ-1/PARK7's effect on autophagy. In normal growth conditions DJ-1/PARK7-regulated autophagy involves JNK due to DJ-1/PARK7 binding directly to MEKK1 to regulate JNK activity and increase c-Jun-driven Beclin 1 transcription [39,40]. Beclin 1 (BECN1) is the mammalian orthologue of ATG6 and Beclin 1 can interact with either Vps34 [41], or mitochondrial located Bcl-2, and because of this Beclin 1 plays a critical role in the regulation of both autophagy and cell death. Thus, in Parkinson's disease, loss of DJ-1/PARK7 would result in reduced JNK and c-Jun signalling, ablated BECN1 expression and dampened Vps34 activity, contributing to reduced autophagic clearing of damaged mitochondria.

When autophagy is induced by nutrient deprivation Bcl-2 only weakly binds to Beclin 1, but under conditions where autophagy is inhibited by nutrient excess, binding to Beclin 1 is increased [42]. This reflects that upon nutrient deprivation multi-site phosphorylation of Bcl-2 leads to disruption of the

Bcl-2/Beclin 1 complex resulting in "liberation" of Beclin 1 to form a complex with Vps34 and Vps15 and induce autophagy [41]. JNK1 signalling is responsible for the multi-site phosphorylation of Bcl-2 and as a consequence JNK1 signalling promotes autophagy [43]. However, as yeast lack a Bcl-2 orthologue this mode of autophagy induction is specific to mammalian cells. Since diverse stress stimuli trigger JNK1-mediated autophagy it argues that the different cellular stress-response programmes mediated by JNK1 signalling and regulation of autophagy are intricately intertwined [43].

The second JNK isoform JNK2 promotes stressed-induced mitophagy, but in contrast to JNK1 that targets Bcl-2, JNK2 directs the small mitochondrial form of ARF (smARF) for degradation [44]. smARF is a short isoform of the tumour suppressor ARF that localizes exclusively to mitochondria, whose abundance in steady-state conditions is extremely low due to constant degradation by the proteasome [45]. By exploiting MEFs deleted for *jnk1* and *jnk2* one observes that loss of JNK2, but not JNK1, leads to stabilization of smARF, but interestingly, the kinase activity of JNK2 was not necessary for the increase in smARF abundance. Rather, mitochondria-located JNK2 appears to act as a scaffold that promotes the ubiquitylation and proteasomal degradation of smARF via an unknown mechanism that involves p62/SQSTM1, since in *jnk2*<sup>-/-</sup> MEFs lysosomal degradation of p62/SQSTM1 is enhanced [45]. Increased levels of JNK2 thus protect p62 from degradation to promote mitophagy, while promoting degradation of smARF.

In addition to being involved in autophagy due to its LIR (LC3-interacting region), KIR (Keap1-interacting region) and UBA (ubiquitin-associated) domains (as discussed above), p62/SQSTM1 also has PB1 (Phox and Bem1), Zn (zinc finger) and TB (Traf6 binding) domains allowing it to participate in diverse signalling pathways [46]. One new pathway is Wnt/planar cell polarity (PCP) signalling due to the core component VANGL2 being able to bind p62/SQSTM1 [47]. Cancer cells have long been known to suppress autophagy and as VANGL2 expression is unregulated in breast cancer, its binding of p62/SQSTM1 might have been expected. However, counter-intuitively, VANGL2 recruits, and promotes the phosphorylation of, JNK leading to c-Jun-driven transcription of BECN1 and augmented Beclin 1 and eventually, greater Vps34 activity that would induce autophagy (as discussed above). However, JNK binding to VANGL2 was shown to occur close to the LC3 recruitment motif [47,48] and although not examined, it raises the possibility that VANGL2 binding to p62/SQSTM1 ablates LC3 recruitment to block autophagy and this would provide a molecular rationale for unregulated VANGL2 expression in breast cancer. Clearly, due to the multi-binding motif character of p62/SQSTM1 this scaffold is involved in many signalling pathways over and above its role in autophagy and caution is therefore required in pronouncing on specific p62/SQSTM1 functions.

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### Autophagy: infection versus inflammation?

Ancient organisms likely exploited autophagy as a primordial protective mechanism against pathogens that depleted cellular nutrients before evolving, in parallel with

sophisticated immune responses, specific host autophagy-based defence processes termed ‘xenophagy’, to clear bacteria, parasites and viruses by targeting pathogen-containing phagocytic vacuoles for autophagolysosomal destruction [14,49]. Reflecting the evolutionary versatility of these processes, whilst the diameter of autophagosomes involved in metabolic turnover are typically 0.5–1.5  $\mu\text{m}$  in diameter [50], group A *Streptococcus*-containing autophagosome-like vacuoles can be as large as 10  $\mu\text{m}$ , due to the RAB7-dependent fusion of small isolation membranes [51]. Nevertheless, such evolution has resulted in integration of the autophagy machinery designed to maintain homeostasis of cell metabolism, function and survival with innate and adaptive immune response networks. This allows efficient coordination of pathogen-sensing and via phagolysosomal maturation, antimicrobial activity; for example, the cell surface microbial sensor, SLAM recruits the Beclin 1/class III phosphoinositide 3-kinase (PI3K) complex to phagosomes containing Gram-negative bacteria, facilitating phagolysosomal fusion and activation of the anti-bacterial NADPH oxidase (NOX2) complex [52]. Moreover, the generation of proinflammatory cytokine responses and fusion of pathogen-containing phagosomes with lysosomes to promote MHC class II antigen presentation [53], links innate and adaptive immunity, resulting in induction of protective cellular (Th polarisation) and humoral responses.

Moreover, as increasingly recognised [14,19–21], components of the autophagy machinery exhibit additional roles in infection, either functioning independently of autophagy as in the regulation of viral replication or alternatively, being recruited to deal with pathogens in a non-canonical manner that does not involve autophagosome formation as exemplified by LC3-associated phagocytosis (LAP); exploitation of ULK1/PI3K Class III complexes to form pathogen-containing single or double membrane vacuoles or recruitment of IFN $\gamma$  effector molecules to the parasitophorous vacuole. For example, in macrophages infected with *Toxoplasma gondii*, the parasite-derived membrane, termed the parasitophorous vacuole, undergoes destruction through a mechanism that involves ATG5-dependent recruitment of the IFN $\gamma$  effectors, immunity-related GTPase proteins (IRGs) guanylate-binding proteins (GBPs) leading to the death of the parasite-infected cell [54–56].

Nevertheless, the mechanisms utilised by cells to target pathogens such as intracellular bacteria to autophagolysosomal compartments are notably similar to those utilised for the selective autophagy of endogenous cargo. For example, cellular cargo is directed to the autophagosomes by molecular tags (e.g. polyubiquitin) that are recognised by adaptor proteins such as p62/SQSTM1 containing an LC3-interacting region (LIR; the core consensus sequence is [W/F/Y]xx[L/I/V], but several unconventional sequences have also been described) allowing them to deliver designated cargo to nascent LC3-containing isolation membranes [49]. Likewise, ubiquitin-p62 signalling is required for the targeting of intracellular bacteria such as *Salmonella enterica* serotype typhimurium (*S. typhimurium*), *Shigella flexneri* and *Listeria monocytogenes* to the autophagosomes [57].

The various microbial-sensing mechanisms (NLRs, TLRs, DAMPs, Cytokine Receptors) and cargo receptors (such as p62/

SQSTM1, NBR1, NDP52, OPTN and TAX1BP1 that bind LC3) are well described elsewhere [14,15,48,56,58–60]. Thus, we will focus on how autophagy balances the need to clear pathogens with its homeostatic resolution of inflammation to prevent inflammatory disease, with emphasis on the counter-regulatory TLR, inflammasome, and mTOR signalling that impacts on autophagy function: in particular, we will describe some of the mechanisms by which pathogens subvert the autophagy machinery to evade the immune response and hence may inform on potential targets to develop novel therapies to treat aberrant inflammatory disorders and associated comorbidities associated with metabolic syndrome (obesity) and ageing.

### TLR-induced autophagy and its subversion by pathogens

Autophagy plays key roles in TLR-associated immunity, notably in killing of intracellular pathogens, antigen presentation and T cell polarisation: however, this critical and complex cellular homeostatic mechanism also controls inflammation, acting to antagonise inflammasome signalling and to limit and resolve inflammation [14,15,61,62]. TLR4 and -7/8 mediate the strongest induction of autophagosome formation [63], with TLR-dependent autophagy generally occurring over 2–24 h, perhaps suggesting involvement of a transcription programme to promote surveillance of infectious agents that may have escaped into the cytosol [61,64]. Reflecting this, although the Gram-negative agent of tularaemia, *Francisella tularensis* that is recognised by, and acts to subvert TLR2 signalling, escapes the phagosome, it can still be targeted to autophagosomes due to its recognition by NALP3 (Nacht domain-, leucine-rich repeat-, and PYD-containing protein 3), a member of the NOD-like receptor (NLR) family: indeed, it has been suggested that such Pattern Recognition Receptors (PRRs) can provide docking sites for the formation of the isolation membrane [65]. Interestingly, therefore, in an apparent mechanism to minimise the opportunities for microorganisms to subvert the phagosome, TLR-mediated autophagosome formation can be more rapidly matured by involvement of LAP, as this process appears to occur without the formation of a double membrane phagophore [64]. TLR-mediated autophagy can be further modulated by cytokines with type I IFNs promoting autophagic clearance of viruses such as HSV1, while IFN $\gamma$  can directly up-regulate autophagosome formation for clearance of mycobacterium [63]. Autophagy is likewise generally promoted by TNF $\alpha$  (particularly under conditions of low NF- $\kappa$ B activation) and Th-1 cytokines, but antagonised by those produced by Th2 cells [66,67].

Autophagy also contributes to antigen processing and presentation, effectively linking innate and adaptive responses [13–15,59,68]. In particular, autophagy provides a mechanism for targeting cytosolic antigens to MHC class II molecules and explains why endogenous peptides from cytosolic and nuclear proteins and viral antigens are expressed on MHC class II molecules and elicit CD4+ T cell responses [69,70]. Likewise, autophagy can enhance antigen donor cell cross-presentation to CD8+ T cells [70], DC cross-

presentation of phagocytosed antigens to CD4<sup>+</sup> T cells [53] and MHC class I presentation of intracellular antigens to CD8<sup>+</sup> T cells [71]. That TLR-stimulated autophagy is associated with enhanced autophagy-dependent display of peptides on MHCII molecules is reflected by the ability of LPS (or rapamycin to block mTOR) to promote presentation of mycobacterial antigen on MHC class II molecules [72]. The participation of autophagy in cross-presentation has also been observed *in vivo* during HSV and *Listeria* infection [53] as DC-specific deletion of ATG5 results in defects in priming of CD4<sup>+</sup> T-cell responses and mice succumb more rapidly to lethal disease after intravaginal HSV infection. Analysis of these DCs revealed defects in phagosome-to-lysosome fusion and the cross presentation of MHC class II molecules of phagocytosed antigens containing TLR ligand, despite the ATG5-deficient DCs displaying normal migration, endocytic and phagocytic activity and cross-presentation of peptides on MHC class I molecules.

Nevertheless, whilst induction of autophagy is clearly critical to the immune response to pathogens, the complex signalling interplay between metabolic and inflammatory pathways and autophagy has for many years shrouded its role in TLR-mediated inflammation in controversy [14,15,73,74]. However, the increasing evidence that autophagy and inflammasome signalling are counter-regulatory and inflammasomes can be degraded by autophagy [73,75,76], goes some way to reconciling apparently contradictory data and indicates that in addition to its anti-microbial actions, autophagy plays critical roles in limiting pathological inflammation and also in the homeostatic resolution of inflammation following pathogen clearance. Thus, whilst the TLR4 ligand, LPS is not generally sufficient, in the absence of other signals such as ATP, to trigger inflammasome activation in macrophages, it can induce inflammasome signalling in macrophages deficient in ATG16L1 or ATG7 [73,75,77]. By contrast, autophagy is enhanced in caspase 1 KO cells in response to *S. flexneri* [78]. An intriguing example of how pathogens can exploit such counter-regulation of autophagy and inflammasome activation to evade the immune response is provided by recent studies on *Pseudomonas aeruginosa* [79,80]: thus, although recognition of *P. aeruginosa* induces autophagy via TLR4 and its adaptor TRIF, this is suppressed by the *P. aeruginosa*-mediated activation of the NLR4 inflammasome resulting from its ROS-mediated release of mitochondrial DNA. Such NLR4-associated caspase-1 activity cleaves TRIF, resulting in inhibition of autophagy and production of type I interferon production and consistent with the bacteria using this as an evasion mechanism, blockage of caspase-1 degradation of TRIF resulted in reduced IL-1 $\beta$  production but increased autophagy and bacterial clearance in a mouse model of *P. aeruginosa* infection [79,80].

Another major aspect that has likely contributed to the contradictory data on the roles of autophagy in inflammation reflects the emerging non-canonical and/or autophagy-independent functions of components traditionally considered to be restricted to the autophagy machinery. The adaptor/scaffold protein p62/SQSTM1 is one of the best characterised exponents of this multi-functionality due to its multi-module structure that contains PB1 (Phox and Bem1p-1), ZZ zinc finger, Traf-binding (TB) Site and UBA (ubiquitin associated)

domains that allow it to impact on a diverse range of cellular processes, including inflammation, neurogenesis, osteoclastogenesis and T cell differentiation [81–85]. In relation to inflammation, TLR induces upregulation and activation of p62, which cooperates, with Traf6 to coordinate pro-inflammatory cytokine secretion resulting from NF- $\kappa$ B activation. For example, although TLR-signalling in keratinocytes [86] induces p62-dependent autophagy to negatively regulate inflammation, p62 is strongly upregulated and, by acting as an atypical PKC scaffold (via its PB1 domain), generates a PKC $\epsilon$ /p62/Traf6 complex that promotes NF- $\kappa$ B activation to prime TLR-driven inflammatory responses [86]. This role of p62 is in keeping with its ability to activate NF- $\kappa$ B in autophagy-defective tumour cells [87,88] and the critical actions of the PKC- $\epsilon$ /p62/Traf6 complex in the activation of NF- $\kappa$ B in response to IL-1, TNF $\alpha$  and RANKL [89,90]. Presumably reflecting this dual functionality, by binding to Traf6 and interacting with its K63-ubiquitin tagged substrates (via its UBA domain), p62 intersects the two major cellular degradation pathways that, depending on context, allows it to traffic targets to the proteasome or the autophagosome for degradation.

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## Autophagy and the adaptive immune response

Studies investigating the impact of autophagy genes, particularly ATG5 and ATG7, on the immune response have also shown them to play crucial roles in the maintenance of normal numbers of foetal haematopoietic stem cells, NK, CD4<sup>+</sup> and CD8<sup>+</sup> T cells [10,68,91] as well as B cells and plasma cells, and sustained production of immunoglobulin by the latter [92–94]. Autophagy also promotes maintenance of regulatory T cells (Tregs). Indeed, inhibition of autophagy results in aberrant Th2 responses and intestinal inflammation, perhaps as a consequence of the disruption of the autophagy-mediated counter-regulation of the mTOR-regulated metabolic signalling that determines Th lineage cell fate: thus, high mTOR activity/anabolic (glycolytic) metabolism is associated with priming of Th1, Th2 and Th17 responses whereas in addition to reflecting the metabolic status of naive cells, the catabolic (fatty acid oxidation) bias associated with the inhibition of mTOR promotes maintenance of Tregs and memory cells [68,95–98]. Intriguingly, autophagy appears to be differentially regulated in the various Th lineages with Th2 polarisation inducing formation of more autophagosomes than in Th1 differentiation, at least *in vitro* [68]. These findings that TCR signalling induces autophagosome accumulation [98,99] appear rather at odds with the high (counter-regulatory) mTOR signalling/glycolytic signalling required for cell growth and rapid clonal expansion. However, as the energy sensor, AMPK becomes activated following TCR signalling [98,100], this should induce autophagy, which may act to promote contraction of the immune response and transition to the memory phase (conditions where mTOR activity is low) [68,95–98]. Consistent with this, autophagy downregulates TCR-coupled NF- $\kappa$ B activation by the highly selective degradation of Bcl10 (but not its binding partner Malt1) [101,102] via the TCR-driven K63-polyubiquitination of Bcl10 and its subsequent binding to the autophagy adaptor p62/SQSTM1. Reminiscent of the dual roles of p62 in promoting TLR-stimulated NF- $\kappa$ B and resolving

inflammatory cytokine production by autophagy in keratinocytes and DCs [86,103], p62 binding to Bcl-10 was required both for NF- $\kappa$ B activation and its downregulation [101,102]: this dual functionality perhaps suggests that the accumulation of autophagosomes following TCR signalling reflects autophagic flux blockade [103] to promote priming of responses, that is alleviated by the fall in mTOR and rise in AMPK activation allowing rapid induction of autophagic flux during the contraction phase of the T cell response.

## Autophagy in inflammatory disease

In addition to its role in promoting maintenance of Treg populations, autophagy also acts to maintain central tolerance [10] by promoting the expression of self-antigens by MHC class II on thymic epithelial cells that is required for negative selection of autoreactive thymocytes. Reflecting this, genetic disruption of ATG5 in thymic epithelial cells results in dysfunctional selection of certain MHC class-II restricted T-cell specificities and autoimmunity [104]. Moreover, there are clear links between the variants of autophagy genes and (aberrant) immune responses [77,105] reflecting that loss of ATG16L1 function in mice results in enhanced TLR-mediated cytokine production by macrophages [77]. For example, polymorphisms in ATG16L1, such as the unstable T300A risk variant, have been associated with susceptibility to Crohn's disease, which is characterised by hyper-responsiveness to the gut microbiota. This T300A variant has also been associated with higher mortality following haemopoietic stem cell transplantation (HSCT), perhaps reflecting the ability of autophagy to prevent such Graft Versus Host Disease (GVHD) in animal models [106]. Likewise, GWAS (Genome Wide Association Studies) studies have linked several ATG5 polymorphisms with susceptibility to asthma and systemic lupus erythematosus (SLE), but not rheumatoid arthritis (RA), at least in the populations examined [107–110]. Furthermore, certain mutations in the cystic fibrosis transmembrane conductance receptor (CFTR) result in downregulation of autophagy [111], perhaps contributing to the chronic lung infections (*P. aeruginosa* and *Burkholderia cenocepacia*) associated with this disease and it is therefore promising that treatment with rapamycin may protect against this and suppress lung inflammation [112]. Unfortunately, treatment of patients with the antibiotic azithromycin, which had generally been administered long-term to improve disease outcome by reducing infection and chronic inflammation, was found to exacerbate this suppression of autophagy and predispose cystic fibrosis (CF) patients towards infection with non-tuberculous *Mycobacterium abscessus* resulting in chronic infection with this drug-resistant pathogen [113].

Advances in medicine, in concert with improved nutrition and sanitation over the last century, have resulted in a dramatic increase in life-span: however, this has not necessarily been accompanied by a corresponding increase in health and well-being due to the accumulation of cellular damage associated with the ageing process. This has been exacerbated by the modern Western life-style, that by incorporating a high fat diet (HFD) results in the dysfunction of metabolic homeostatic mechanisms, leading to age-associated co-morbidities such as

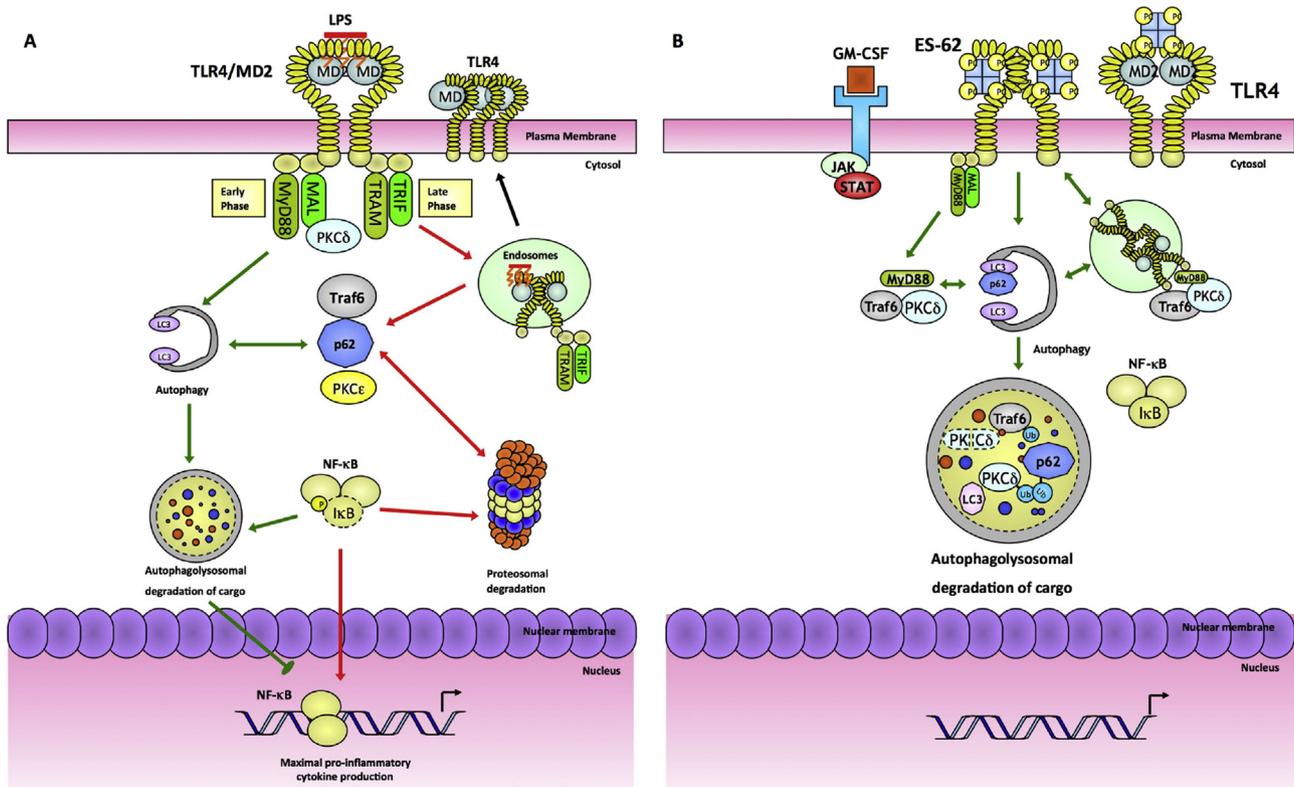
Type-2 diabetes (T2D), stroke and cardiovascular disease and cancers [114]. Meta-analysis of gene signatures associated with longevity and ageing of mouse hepatocytes [115], has indicated that inflammatory genes (e.g. acute phase, complement, PRR and SLE-related) are upregulated whilst those associated with metabolism and stress responses (e.g. xenobiotic (p450) metabolism as well as oxidative, mitochondrial and Nrf2-regulated stress response/cytoprotective genes) are downregulated during mid-life. By contrast, an inverse transcriptional signature was observed in long-lived mice that also validated their potential as biomarkers of ageing [115–117]. Certainly, chronic low-grade inflammation driven by (dysbiosis {dysregulation of microbiota}-driven) activation of IL-1R/TLR/MyD88 signalling and the NLRP3 inflammasome appear to play key roles in regulating such inflamm-ageing (chronic upregulation of inflammatory response with ageing) [114,118–122]. Consequently, mTOR, which acts to integrate metabolic and inflammatory signalling becomes dysregulated, particularly under conditions of HFD, and promotes ER stress and impairs insulin/IGF-1 signalling, increases glycolytic metabolism and premature ageing of haemopoietic stem cells (HSC) [74,123–125]. Thus, inflamm-ageing and dysregulated mTOR are increasingly recognised as important integrated triggers in the ageing process [74,114,123] and consistent with this, genetic and pharmacological (e.g. by rapamycin) downregulation of mTOR has been shown to reduce ageing and increase lifespan [74,123]. The protective, anti-ageing mechanisms resulting from reduced mTOR signalling are not well defined but increased autophagy [126] relieves ER stress and maintains mitochondrial integrity in response to reactive oxygen and nitrogen species (ROS; RNS) generated during dysregulated glucose metabolism and energy homeostasis [127]. Supporting this key role for autophagy, short-lived *S. cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster* mutants exhibit defects in autophagy [128] and mice with brain-specific ATG5 or ATG7 deletion, exhibit reduced lifespan [129]. Certainly, expression of the autophagy machinery (e.g. ATG5/7, Beclin 1 and Sirtuin 1) declines with age and this loss of autophagy function appears to impact directly on various parameters associated the ageing process, notably the accumulation of toxic protein aggregates, decreased mitochondrial function, increased (stress-induced) cell death, stem cell loss and induction of cellular senescence [128].

## Autophagy: a target with therapeutic potential

In addition to its role in regulating infection, inflammation and ageing, as well as the inflamm-ageing that underpins ageing-associated comorbidities like obesity and cardiovascular disease [128,130], autophagy has been implicated both in protecting against and promoting a wide range of diseases, including neurodegenerative and muscle disorders and cancers [73,130–132]. Indeed, the intense focus on developing novel therapies based on modulating autophagy reflects the wealth of evidence that genetic modifications of autophagy genes in mice and deficiencies/polymorphisms of these genes in humans can be correlated with human illnesses encompassing inflammatory diseases such as asthma, COPD and autoimmunity (inflammatory bowel diseases [IBD] and SLE)

through to neurological and neurodegeneration disorders (motor neuropathies, Parkinson's, Huntington's, dementia), diabetes, atherosclerosis and cancers (breast, ovarian, prostate, colorectal, lung and brain) as well as susceptibility to various infections (*Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Salmonella typhi* and *Salmonella paratyphi*). Promisingly, therefore, gene therapy involving *ATG7*, *Tfeb* and *BECN1* has resulted in amelioration of diseases including obesity, Parkinson's and Alzheimer's diseases and cystic fibrosis in mouse models. Moreover, a number of commonly used drugs can induce autophagy by their ability to target levels of  $IP_3/Ca^{2+}$  (Carbamazepine, Lithium, Verapamil); cAMP (Clonidine) and AMPK/mTORC1 (Metformin, Statins, Rapamycin): this raises the exciting possibility of their potential repurposing for diseases associated with defects in the regulation of autophagy (rather than in the autophagy machinery, *per se*) and indeed, mTOR inhibitors and Statins have already been repurposed for treatment of neurodegenerative diseases and infection with *M. tuberculosis*, respectively [130–132]. This rational approach to repurposing is complemented by intensive screening and development of small molecule regulators of autophagy for novel drug development [131–134].

However, depending on the context, autophagy can be both cytoprotective and cytotoxic: thus, whilst its ability to promote cell death may be desirable in combating certain cancers, this could contribute to pathogenesis in a host of disorders ranging from diabetes to ischemic brain damage. Moreover, therapeutic induction of autophagy could potentially leave patients open to catastrophic infection. Thus, an alternative and safer approach may be to harness the therapeutic potential of organisms such as parasitic helminths (and in particular their secreted immunomodulators) that by acting to dampen down host inflammation and limit tissue pathology to promote both parasitic worm and host survival [135,136], appear to serendipitously suppress allergic and autoimmune inflammatory diseases as evidenced by the clear inverse relationship between the incidence of these parasitic worms and the prevalence of chronic inflammatory disorders globally [137–140]. The ability of these parasites to protect against metabolic syndrome and obesity [141–148] is also increasingly recognised. Perhaps pertinently, given that obesity and autoimmunity are reciprocal risk factors that impact on ageing and associated co-morbidities, evidence is also emerging that (at least some) of these anti-inflammatory



**Fig. 2 TLR4 signalling: subversion of autophagic flux by ES-62 promotes homeostatic resetting of inflammation.** Panel A. LPS-TLR4/MD2 signalling induces an early MyD88/Mal phase of NF-κB activation via the rapid proteasomal degradation of IκB-α and -β, followed by a more prolonged TRAM/TRIF endosomal signalling phase during which the expression of p62 is progressively increased due to blockage of autophagic flux, allowing it to further stimulate, via a Traf6-p62-PKCε complex, activation of NF-κB resulting in pro-inflammatory cytokine production. During resolution of inflammation, autophagic flux provides a negative feedback inhibition mechanism. Panel B. ES-62 specifically targets the autophagolysosomal degradation of PKCδ, and Traf6, key transducers required for full TLR activation: this is achieved by induction of autophagic flux (evidenced by the accumulation of p62 and LC3-II upon inhibition of autophagolysosomal degradation). Together with the accompanying suppression of Traf6-p62-PKCε-mediated NF-κB activation, this results in dampening of LPS-induced cytokine production.

actions may reflect the inhibition of mTOR and/or induction of autophagy to suppress (aberrant) inflammation [103,149,150].

Thus, we have shown that ES-62, a phosphorylcholine (PC)-containing immunomodulatory glycoprotein from the filarial nematode *Acanthocheilonema viteae*, which exhibits protection against disease in mouse models of allergic and autoimmune disease [135,139,150], can homeostatically reset inflammatory responses by partially downregulating MyD88 expression to subvert TLR signalling in effector cells of both innate and adaptive immunity [150–154]. ES-62 appears to do this by inducing highly selective autophagolysosomal degradation of MyD88 (but not its binding partner Mal) and, depending on the cell type, other TLR-associated signalling elements like PKC- $\alpha$  and - $\delta$  (mast cells) or PKC- $\delta$  and Traf6 (DCs) [103,150,151,155]. Specifically, whilst LPS/TLR4 signalling strongly upregulates p62 and LC3, this appears to be associated with a block in autophagic flux, allowing p62 to promote NF- $\kappa$ B-mediated cytokine responses: by contrast, ES-62 induces a low level of ATG7-dependent autophagic flux to sequester and degrade Traf6 and PKC- $\delta$  to dampen such LPS-driven pro-inflammatory responses [Fig. 2].

Importantly, ES-62 appears to preferentially mediate its effects during conditions of inflammation; for example, ES-62-induced autophagolysosomal degradation of MyD88 in DCs is enhanced in the presence of GM-CSF [103,150], a cytokine that plays important roles in chronic, MyD88-dependent inflammation and pathologies associated with metabolic syndrome [156]. In this way, ES-62 does not block induction of inflammatory responses necessary to combat infection but rather acts in a homeostatic way to prevent perpetuation of chronic hyper-inflammation [103,150]. Reflecting this, we have obtained microarray data ([135] and unpublished) showing that in synovial membranes from RA, but not osteoarthritis, patients, whilst ES-62 downregulates LPS-stimulated levels of the insulin signalling pathway (IGFR2, IRS2 and IGF-1) and inflammation-associated genes such as TLR4, Fc $\gamma$ RIIa, C1q and C3aR, chemokines CCL3 and 11 and caspase-1, it upregulates anti-oxidant/cytoprotective genes (including glutathione s-transferase, methionine adenosyltransferase, histamine methyltransferase and succinyl CoA ligase), several of which have been investigated for their impact in long-lived Ames mice [157,158]. This apparent inverse control of inflammation-promoting genes and anti-oxidant/cytoprotective pathways may suggest that in conjunction with its ability to suppress PI3K/AKT [159] (and hence presumably mTOR) signalling, induction of selective autophagy may be a central tenet of ES-62 protection. Intriguingly, therefore, a small molecule analogue of ES-62, termed 11a, is protective against the development of collagen-induced arthritis in a mouse model of RA, by virtue of its ability to downregulate MyD88 and NLRP3 expression and consequent IL-1 $\beta$  production, in an Nrf-2-dependent manner [160]. Given the role of p62/SQSTM1 in autophagolysosomal degradation of KEAP1, the negative regulator of Nrf-2, it is therefore tempting to speculate that the protective effects of ES-62 and a small molecule analogue SMA-11a involve induction of selective autophagy to effect differential degradation of key pathogenic signals and upregulation of cytoprotective pathways to effect inflammation resolution and tissue repair. Harnessing of this homeostatic regulatory

mechanism presumably evolved to promote parasite survival but may now be exploited therapeutically.

Finally, returning to the theme of food intake, an emerging factor is the recognition that dietary restriction, nutritional factors (e.g. caffeine or vitamin-D) and exercise may exert their health-promoting effects, at least in part by promoting autophagy [130]. Thus, autophagy appears to be essential to the extension of lifespan in *C. elegans* afforded by dietary restriction [161], as is the caffeine-induced reduction of steatosis in mice with non-alcoholic liver disease [162] and even the effects of exercise that counteract HFD-induced diabetes in mice [74,163].

## Conflicts of interest

None of the authors have conflicts of interest.

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