Manipulation of Autophagy by Bacterial Pathogens Impacts Host Immunity

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Abstract

Autophagy is a highly conserved catabolic process, degrading unnecessary or damaged components in the eukaryotic cell to maintain cellular homeostasis, but it is also an intrinsic cellular defence mechanism to remove invading pathogens. A cross-talk between autophagy and innate or adaptive immune responses has been recently reported, whereby autophagy influences both innate and adaptive immunity, like the production and secretion of pro-inflammatory cytokines or MHC class II antigen presentation to T cells. Pathogenic bacteria have evolved diverse strategies to manipulate autophagy, mechanisms that also impact host immune responses at different levels. Here we discuss the influence of autophagy on self-autonomous, innate and adaptive immunity and then focus on how bacterial mechanisms that shape autophagy may impact the host immune system.

Introduction

Maintaining cellular homeostasis requires a coordinated control of metabolic pathways. Thus the cell needs anabolic processes to build new components, and catabolic processes to discard long-lived or damaged components. Autophagy is one of these catabolic processes that leads to lysosomal degradation of unnecessary or damaged proteins, lipids and organelles (Ohsumi, 2014; Rubinsztein *et al.*, 2012). The term 'autophagy' describes mainly three different processes: macroautophagy, microautophagy and chaperone-mediated autophagy. In this review we will focus on macroautophagy (hereafter called autophagy), which is characterized by the sequestration of cargo into double-membrane vesicles called autophagosomes (Miller and Celli, 2016).

Autophagy can be divided into selective and non-selective autophagy: in non-selective autophagy a random portion of the cytoplasm is engulfed to degrade long-lived components

or to provide nutrients during starvation, while selective autophagy targets selected components for degradation. This includes organelle-specific autophagy, such as removal of mitochondria called mitophagy, and xenophagy which is the removal of invading bacteria (Deretic *et al.*, 2013; Huang and Brumell, 2014).

Intracellular bacteria grow and replicate inside host cells. These bacteria can be divided into two groups: those that replicate in the cytosol, such as *Listeria* or *Shigella*, and those that replicate in membrane-derived pseudo-organelles called pathogen-containing vacuoles (PCVs), such as *Legionella*, *Mycobacterium* or *Salmonella*. PCVs facilitate the replication, survival and dormancy of intracellular bacteria. For their formation bacteria exploit the host's membrane system, in particular the dynamic machineries involved in exo/endocytic traffic and autophagy. To enhance survival and proliferation in PCVs, bacteria secrete effector proteins that inhibit their degradation in lysosomes and facilitate the acquisition of membrane sources and nutrients (Ray *et al.*, 2009). In order to ensure survival, intracellular bacteria also need to counteract self-defence mechanisms of the host cell. In animals, cellular self-defence (also known as cell-autonomous immunity) synergizes with the whole-body protection provided by 'conventional' immunity (innate and adaptative immunity) to grant resistance to pathogens. While professional immune cells patrol the body in search of pathogens, cell-autonomous immunity guards both individual immune and non-immune cells against the immediate threat of infection. The current paradigm shows that cell-autonomous immunity against intracellular pathogens is based on the tight and continuous control of the composition and behaviour of distinct cellular compartments, such as the cytosol, organelles, endosomes, autophagosomes and lysosomes (Randow *et al.*, 2013). As the autophagy machinery removes intracellular pathogens by directing them to lysosomal degradation, xenophagy has emerged as an important cellular self-defence process to protect host cells against intracellular bacteria. In this case, engulfment of bacteria by autophagosomes triggers an integrated response within the infected cell, leading to pathogen elimination while professional immune cells are warned about the threat.

As autophagy is an important cellular self-defence mechanism, bacteria evolved various mechanisms to modulate autophagy such as inhibiting autophagosome formation, self-masking with host proteins to avoid recognition, escaping targeting to autophagosomes or blocking fusion of autophagosomes with lysosomes (Escoll *et al.*, 2016; Huang and Brumell, 2014). As autophagy is linked to immune responses, bacteria-induced modulation of autophagy impacts also innate and adaptive immune responses (Kuballa *et al.*, 2012).

Here, we will first discuss the general knowledge about autophagosome formation, elongation and maturation, the cross-talk between autophagy and immunity and then focus on the mechanisms used by bacterial pathogens to manipulate autophagy and how these autophagy subversions by bacteria impact the host immune response.

Autophagosome formation, elongation and maturation is a complex process involving conserved host factors

The pathway leading to autophagy includes signal induction, nucleation and elongation of the phagophore to form the autophagosome, closure of the autophagosome and fusion with lysosomes (Parzych and Klionsky, 2014). More than 35 Autophagy-related proteins (ATG-proteins) that have metazoan orthologues have been described in yeast to coordinate autophagosome formation and maturation (Ohsumi, 2014) (Fig. 4.1).

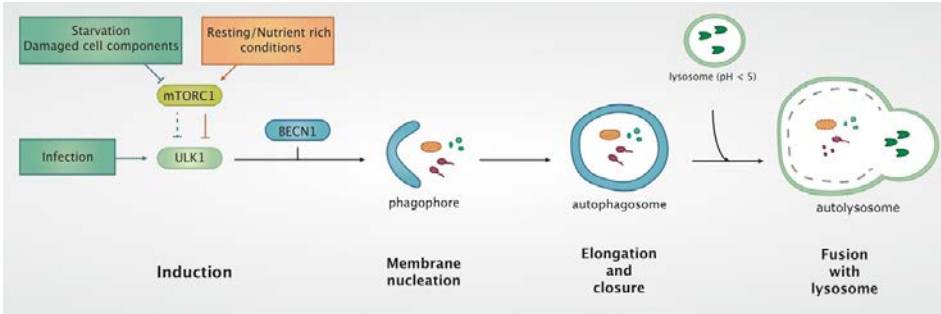

Figure 4.1 Schematic description of the autophagic pathway. Autophagy is triggered by starvation, damaged cellular components or cellular recognition of bacteria. These events activate the ULK1 complex. Under nutrient-rich conditions, mTORC1 inhibits autophagosome formation by repressing the ULK1 complex, however participation of mTORC1 in bacterial-induced autophagy is unclear. Once the ULK1 complex is activated, autophagosome formation begins with the recruitment of BECN1 and the nucleation of a small membrane called the phagophore, which starts to engulf the undesired material. The phagophore expands to form the autophagosome, a double-membrane compartment engulfing cyoplasmic targets (proteins, organelles or pathogens). This mature autophagosome then fuses with a lysosome for cargo degradation.

Autophagy induction can be triggered by different signals ranging from nutrient limitation (non-specific autophagy) to the recognition of pathogens or damaged cellular components (Deretic *et al.*, 2013). Most signals for the initiation of autophagy converge through the functions of the mammalian target of rapamycin complex 1 (mTORC1). Under nutrient-rich conditions the mTORC1 complex phosphorylates Unc-51-like kinase 1 (ULK1) and ATG13, which together with the FAK family kinase-interacting protein of 200kDa (FIP200) form the ULK1 complex. When mTORC1 phosphorylates ULK1 and ATG13, the complex cannot be recruited to the phagophore assembly site (PAS), therefore inhibiting autophagosome formation (Ganley *et al.*, 2009; Mizushima, 2010).

Under starvation or stress, mTORC1 becomes inactive and thus its inhibitory action on the ULK1 complex ceases which in turn phosphorylates FIP200 rendering the complex active (Ganley *et al.*, 2009). The activated ULK1 complex then translocates to the PAS and recruits other proteins such as beclin1 (BECN1, named in yeast Atg6) , ATG14-like protein (ATG14L) and phosphoinositide 3-kinase regulatory subunit 4 (PIK3R4; vacuolar protein sorting associated 15 Vps15 in yeast). Initiation of phagophore nucleation also results in engagement of PI3-kinase class III (PI3KC3; Vps34 in yeast), which produces PI3P at the phagophore and leads to the recruitment of WD-repeat domain phosphoinositide-interacting 1 (WIPI1; Atg18 in yeast) and WIPI2 (Atg21 in yeast). Like a cascade, these proteins recruit further downstream ATG proteins that aid phagophore assembly (Nair *et al.*, 2010; Polson *et al.*, 2014). The PAS needs a source of membranes to form the phagophore-expanding autophagosome. It has been shown that these are mainly mitochondrial-associated ER-membranes (MAMs) (Hamasaki *et al.*, 2013) although several other membranes have also been proposed as sites for nucleation of the phagophore, such as plasma membrane-derived vesicles, mitochondria and the Golgi apparatus (Tooze and Yoshimori, 2010).

Phagophore elongation is then mediated by two ubiquitination-like conjugation systems of ATG proteins. First, ATG7 (E1-like protein) and ATG10 (E2-like protein) conjugate

ATG12 and ATG5, promoting phagophore elongation (Mizushima *et al.*, 1998; Shintani *et al.*, 1999). ATG12–ATG5 forms a complex with ATG16L1, which associates with the expanding phagophore membrane and gets released after the autophagosome is formed (Mizushima *et al.*, 2001; 2003). The ATG12–ATG5–ATG16L1 complex is essential for the formation of the second ubiquitin-like conjugation system, where the protein light chain 3 (LC3; Atg8 in yeast) is conjugated to phosphatidylethanolamine (PE) in the phagophore membrane. This conjugation system relies on ATG7 (E1-like protein) and ATG3 (E2-like protein) that activate LC3. In addition, ATG4B cleaves the carboxy-terminus of LC3 and exposes a glycine residue. Activated LC3 is directed by ATG12–ATG5–ATG16L1 to the expanding phagophore, where the E3-like activity of ATG12–ATG5 conjugates PE to the previously exposed glycine residue of LC3 in the phagophore membrane. Once the phagophore is closed and the autophagosome is formed, the ATG12–ATG5–ATG16L1 complex is released from the autophagosome. ATG4B has also been shown to deconjugate a proportion of the LC3–PE complexes from mature autophagosomes, facilitating LC3 recycling for the formation of new autophagosomes (Fujita *et al.*, 2008; Tanida *et al.*, 2004). It is thought that recycling of LC3 is an important step in the maturation of fusion-capable autophagosomes. It has also been shown that LC3 mediates the hemifusion of vesicles and controls the size of autophagosomes in yeast (Nakatogawa *et al.*, 2007; Xie *et al.*, 2008).

Finally, a series of fusion events of autophagosomes with endosomes and lysosomes leads to their maturation into degrading autolysosomes. In mammalian cells the fusion of autophagosomes with lysosomes requires the small GTPase RAB7 (Ypt7 in yeast), the autophagosomal SNARE protein syntaxin 17, the lysosomal SNARE protein VAMP8 (vesicle-associated membrane protein 8) and several lysosomal membrane proteins, such as the lysosomal-associated membrane glycoprotein 2 (LAMP2). After autophagosome–lysosome fusion the cargo is degraded by the lysosomal hydrolases now present in the autolysosome (Eskelinen *et al.*, 2002; Itakura *et al.*, 2012; Jager *et al.*, 2004; Tanaka *et al.*, 2000).

For selective autophagy, such as xenophagy, an additional step of cargo selection is required, a process that is mediated by cargo receptors and adaptor proteins. Mammalian cargo specific receptors usually contain a LC3-interacting region (LIR) motif allowing the recruitment LC3-containing autophagosomes to the cargo (Liu *et al.*, 2012; Novak *et al.*, 2009; Polson *et al.*, 2014). In addition, many cargoes are ubiquitinated and then they are recognized by ubiquitin-binding protein adaptors that also contain LIRs. One of those proteins is p62 that, among other functions, directs bacteria to autophagosomes. Together with the adaptor proteins NDP52 and optineurin (OPTN), p62 is involved in *S.* Typhimurium recognition and its direction to autophagosomes (Boyle and Randow, 2013). NDP52 and p62 also target *Shigella flexneri* and *Listeria monocytogenes* to autophagosomes. Whereas p62 and NDP52 are recruited together during *S. flexneri* infection, p62 and NDP52 are recruited independently during *L. monocytogenes* infection, suggesting that *Shigella* and *Listeria* induce different pathways for selective autophagy (Mostowy *et al.*, 2011). Another example is the neighbour of BRCA1 gene 1 protein (NBR1) that is known to target *Francisella tularensis* to autophagy (Chong *et al.*, 2014) and also participates in the targeting of *S. flexneri* (Mostowy *et al.*, 2011), but it is not required for targeting of *S.* Typhimurium to autophagy (Zheng *et al.*, 2009). These studies highlight that selective autophagy uses distinct protein adaptors to target different types of bacteria.

Cross-talk between autophagy and the innate immune response

The first step of innate immune responses is the recognition of a pathogen that is mediated by a variety of pattern-recognition receptors (PRRs) like Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) that bind pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide (LPS), peptidoglycan, lipoproteins, flagellin or nucleid acids. As autophagic degradation of pathogens aids PAMP recognition by PRRs, autophagy is believed to have an important role in innate immunity. Additionally, binding of PAMPs to PRRs stimulates autophagy (Delgado *et al.*, 2009).

Toll-like receptors

TLRs are membrane-bound receptors found at the surface of the cell or in endosomal compartments. TLRs respond to LPS, lipotechoic acid, flagellin and bacterial nucleic acids (Deretic, 2012). After binding their cognate ligand, TLRs activate proinflammatory responses by triggering the production of cytokines such as tumour necrosis factor-α (TNF-α), interleukin 6 (IL-6) and IL-1β (Fig. 4.2).

Phagocytosed and cytosolic PAMPs are sequestered by autophagosomes and are delivered to the endosomally located and luminally oriented TLRs for PAMP recognition. Therefore autophagy takes part in the first steps of TLR activation (Desai *et al.*, 2015). On the other side, autophagy can also be induced upon TLR activation, through

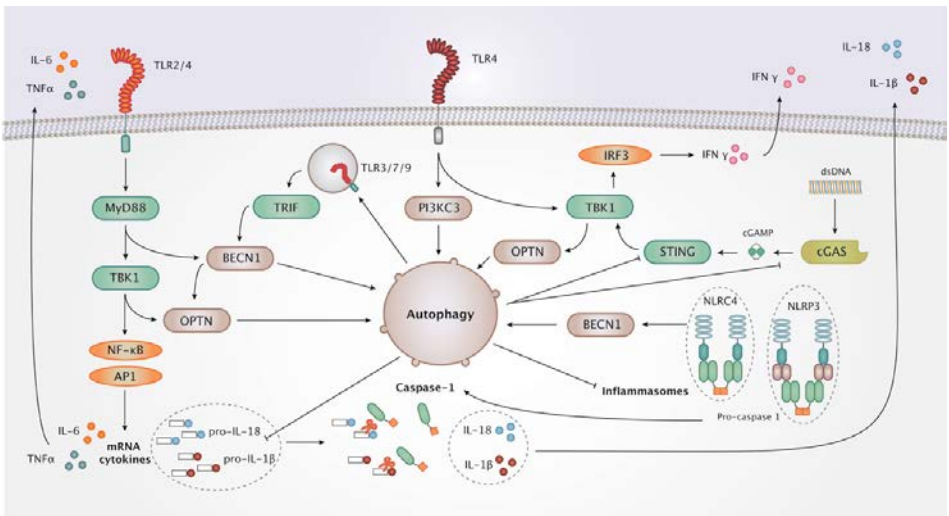

Figure 4.2 Cross-talk of autophagy and innate immunity. Activation of PRRs, such as TLRs, inflammasomes and cGAS, triggers signalling pathways (green components) that lead to activation of inflammatory transcription factors (orange components) and autophagy proteins that activate autophagy (brown components). Activation of inflammatory transcription factors leads to transcription and translation of cytokines such as TNF-α, IL-6 and IFN-γ, which are secreted, but also pro-IL-1β and pro-IL-18, which are then processed by inflammasome-activated caspase-1 into secreted IL-1β and IL-18. In turn, autophagy aids TLRs in meeting their cognate ligands, while it negatively regulates cytokine production by repressing cGAS synthesis of cGAMP and degrading precursor and mature forms of IL-1β and IL-18, and inflammasome components (dashed lines).

a MyD88/TRIF adaptor dependent process and their interaction with BECN1 (Shi *et al.*, 2008). TLR–autophagy cross-talk is supported by several other findings: TLR4 stimulates PI3KC3-dependent formation of cytosolic LC3 aggregates, enhancing the elimination of mycobacteria from macrophages (Xu *et al.*, 2007). Downstream of TLR4 activation, the TANK binding kinase 1 (TBK1) also links the TLR signalling pathway and autophagy as TBK1 phosphorylates the autophagic receptor OPTN, enhancing LC3 binding affinity and consequently the autophagic clearance of cytosolic *Salmonella enterica* (Wild *et al.*, 2011). TLR7 activation has also been shown to promote autophagic degradation of *Mycobacterium bovis* BCG (bacillus Calmette–Guérin) (Delgado *et al.*, 2008), and TLR2 induces autophagy in an ERK-dependent mechanism during *Listeria monocytogenes* infection (Anand *et al.*, 2011). Altogether these studies highlight that TLR activation elicits autophagy, thus activating cell-autonomous immunity after PAMP detection.

However, autophagy may also down-regulate TLR-induced responses. Inhibition of autophagy in macrophages and dendritic cells (DCs) through 3MA (3-methyladenine) or through siRNA knockdown of autophagy genes, leads to an increase in cytokine release in response to TLR3 or TLR4 agonists (Harris, 2011; Saitoh *et al.*, 2008). ATG16L1-deficient mice were shown to excessively activate caspase-1 in response to TLR4 stimulation by LPS, which led to increased IL-1β and IL-18 production. This suggests that ATG16L1 might have a regulatory function on the TLR4 signalling pathway and its depletion/malfunction may lead to increased inflammation (Saitoh *et al.*, 2008). Mature IL-1β protein can also be engulfed by autophagosomes in macrophages stimulated with TLR ligands, showing another way by which autophagy can down-regulate the production and secretion of cytokines (Harris *et al.*, 2011) (Fig. 4.2). Thus, autophagy influences TLR signalling and consequently impacts TLR-mediated cytokine production and secretion.

RIG-I-like receptors

RLRs sense cytosolic dsRNA or DNA, and thus recognize also nucleic acids from pathogens. PAMP recognition by RLRs as RIG-I and MDA5 triggers the production of type I interferon (IFN) by infected cells, as has been shown for cells infected with the intracellular pathogen *Legionella pneumophila* (Monroe *et al.*, 2009). This type I IFN activation pathway can be directly suppressed by several autophagy factors. For example, autophagy-defective *Atg5*$^{-/-}$ cells exhibit enhanced RLR signalling and increased IFN secretion, mostly due to the accumulation of dysfunctional mitochondria and increased generation of reactive oxygen species (ROS) which were largely responsible for the enhanced RLR signalling in *Atg5*$^{-/-}$ cells (Tal *et al.*, 2009).

cGAMP synthase

Bacterial or aberrant cytosolic DNA are also recognized by direct binding to cGAMP synthase (cGAS), a cytosolic protein that generates cyclic dinucleotides (CDNs) such as cGAMP within the host cytosol (Tao *et al.*, 2016). Host cell-generated CDNs activate the downstream stimulator of interferon genes (STING), a receptor that can be also activated by CDNs of intracellular bacteria (Burdette *et al.*, 2011). STING controls the activation of above-mentioned TBK1, which upon nucleic acid sensing and cGAMP synthesis triggers both IFN regulatory factor 3 (IRF3) phosporylation and type I IFN production. STING induced type I IFN production occurs during infection by intracellular bacteria such as *Mycobacterium tuberculosis* (Watson *et al.*, 2015). On the other hand, the autophagy

activator protein ULK1 phosphorylates STING, inhibiting sustained type I IFN activation in response to dsDNA (Konno *et al.*, 2013) (Fig. 4.2). Furthermore, cGAS binding to BECN1 facilitates autophagic removal of cytosolic dsDNA and reduces excessive type I IFN responses (Liang *et al.*, 2014).

NOD-like receptors

NLRs are a class of receptors initiating a quick and potent inflammatory cytokine response to PAMPs. Upon sensing PAMPs, cytosolic NLRs form a signalling complex called the inflammasome. The inflammasome consists of several oligomerized NLRs that bind caspase-1 directly or through the adaptor protein called apoptosis-associated speck-like protein (ASC), which contains a caspase recruitment domain (CARD). These complexes cleave the protein precursor pro-caspase-1 into p10 and p20 subunits, activating caspase-1. Active caspase-1 then cleaves the presynthesized pro-IL-1β into the active form of the cytokine IL-1β, which is secreted (Rodgers *et al.*, 2014). IL-1β secretion relies on two different PRR activating signals: the NFκB-dependant expression of pro-IL-1β through TLR activation and a signal activating caspase-1 through inflammasomes to cleave pro-IL-1β (Eder, 2009) (Fig. 4.2). The inflammasome also cleaves pro-IL-18 into active IL-18 and orchestrates the programmed cell death known as pyroptosis (Miao *et al.*, 2011). Therefore, the inflammasome promotes inflammation by controlling the secretion of the strong proinflammatory cytokines IL-1β and IL-18 and avoiding that cells become a niche for the pathogens by activating pyroptotic cell death. These two cytokines are responsible for the recruitment of myeloid cells, including neutrophils, to sites of inflammation (Rider *et al.*, 2011).

NLRs that respond to PAMPs in macrophages and activate caspase-1 by inflammasome assembly are NLRP1, NLRP3, NLRC4, NLRP7, NLRP12 and AIM2 (absent in melanoma 2) (Latz *et al.*, 2013). Interestingly, a vast number of data strongly suggests that both machineries, autophagy and inflammasome, are highly interconnected and influence each other. Remnants of autophagosomes that were degraded during *S. flexneri* infection of epithelial cells are decorated with components of both the autophagy and the inflammasome machineries. Moreover, macrophages of caspase-$1^{-/-}$ mice display an excessive accumulation of autophagosomes during *S. flexneri* infection, suggesting that caspase-1 affects either autophagosome formation or maturation (Dupont *et al.*, 2009; Suzuki *et al.*, 2007). Activation of AIM2 with poly(dA:dT) or NLRP3 activation with uric acid crystals or nigericin leads to an increase in autophagosome formation in macrophages (Shi *et al.*, 2012). Autophagosomes have also been shown to sequester and degrade inflammasome components and cytokine precursors such as pro-IL-1β (Harris *et al.*, 2011; Shi *et al.*, 2012). Association between autophagy and inflammasome proteins was also shown in resting cells, where NLRP4 and NLRC4 formed a complex with BECN1, suggesting that NLRP4 sensing of bacteria leads to the initiation of BECN1-mediated autophagic responses (Jounai *et al.*, 2011). Autophagy and inflammation are also regulated by NOD1 and NOD2, a caspase-1 independent class of NLR proteins. NOD1 and NOD2 are intracellular sensors of peptidoglycan that induce autophagy by interacting with ATG16L1 and regulating IL-1β and IL-18 production through NFκB (Philpott *et al.*, 2013).

Key evidence for NLR signalling in anti-bacterial responses was gained from the analysis of mouse susceptibility to *L. pneumophila*. Indeed, only A/J mice are susceptible to infection due to a partial loss-of-function mutation in the Naip5 inflammasome, a component of the flagellin-NLCR4 pathway (Molofsky *et al.*, 2006; Zamboni *et al.*, 2006). Autophagosomes

of macrophages from *L. pneumophila*-resistant C57BL/6J mice matured quickly and prevented efficient *L. pneumophila* replication when compared to autophagosomes of *L. pneumophila*-permissive Naip5 mutant A/J macrophages. This observation is reinforcing the idea that inflammasome activation and autophagy are intertwined processes during bacterial infection (Amer and Swanson, 2005). Additional results obtained in primary mouse macrophages support a model in which both caspase-1 and NLR components of inflammasomes are co-ordinately responding to *L. pneumophila* infection depending on the bacterial burden (Byrne *et al.*, 2013). According to this model, NLRC4 is complexed with autophagy component BECN1 in resting macrophages, inhibiting autophagy. Low levels of bacteria lead flagellin-bound NAIP5 to recruit NLRC4 to a complex containing the pro-caspase-1 protein, derepressing autophagy. When the capacity of autophagy to eliminate intracellular bacteria is exceeded, caspase-1 triggers pyroptosis to eliminate the pathogen's niche while initiating a potent inflammatory response (Byrne *et al.*, 2013).

Autophagy has recently been implied in maturation of natural killer cells (NKs). NK cells play an important role in early immune response through cytokine secretion and granule-mediated cytotoxicity of tumours and infected cells (Sun and Lanier, 2011). NK cells have been classified as members of one of the subsets (type 1) of innate lymphoid cells (ILCs), a recently identified group of cells from the lymphoid lineage that have emerging roles in mediating immune responses and in regulating tissue homeostasis (Walker *et al.*, 2013). Mice with a NK cell-specific deletion of ATG5 exhibited a reduced number of circulating NK cells compared to wild-type (WT) mice, whereas the amounts of peripheral T and B cells remained normal. Isolated NK progenitors from ATG5 deficient mice were unable to differentiate into immature NK or mature NK cells upon IL-15 exposure. The same effect was also observed by silencing ATG3 or ATG7 (O'Sullivan *et al.*, 2016; Wang *et al.*, 2016). Moreover lymphoid progenitors from *Atg5*$^{-/-}$ mice failed to generate mature type 2 and type 3 ILCs in the small intestine of recipient WT mice, showing the key role of autophagy in the development of ILCs *in vivo* (O'Sullivan *et al.*, 2016).

Autophagy cross-talks with adaptive immune responses

Considering the role of autophagy in the restriction and destruction of intracellular pathogens and its interplay with innate immunity, it is expected that autophagy is also a crucial process in adaptive immunity where it acts on the modulation of antigen processing and presentation to elicit the correct development and homeostasis of lymphocytes.

T cells are the main effectors of the adaptive immune system. They scan through the output of the proteolytic machineries of the cells to detect pathogen-derived peptides. CD4$^+$ helper T cells and cytotoxic CD8$^+$ T cells display a diverse receptor repertoire that allows them to recognize these peptides but this recognition is not direct as antigens need to be loaded to major histocompatibility class (MHC) molecules before being presented to T cells. MHC class I ligands are commonly generated by the proteasome and are presented to CD8$^+$ T cells while MHC class II loading peptides are produced by lysosomes and presented to CD4$^+$ T cells (Münz, 2010).

The classical concept suggests that intracellular antigens get processed and loaded onto MHC class I cells while extracellular ones go on MHC class II cells. However, as autophagy delivers cytoplasmic constituents for lysosomal degradation and MHC class II molecules are loaded with lysosomal products, autophagy also supports the processing of endogenous

antigens for presentation by MHC class II (Dengjel *et al.*, 2005; Nimmerjahn *et al.*, 2003; Schmid *et al.*, 2007; Zhou *et al.*, 2005). Therefore, antigen-presenting cells such as macrophages and DCs can use the autophagy machinery to fuse autophagosomes containing bacteria-derived antigens with autolysosomes, which are afterwards loaded into MHC class II molecules for antigen presentation to $CD4^+$ T cells (Crotzer and Blum, 2009; Dengjel *et al.*, 2005). This antigen-presentation enhancing effect of autophagy has been used to significantly upgrade the efficiency of the BCG vaccine (Jagannath *et al.*, 2009). Autophagy also regulates exogenous antigen processing for presentation by MHC class II via the modification of the content and fate of phagosomes after LC3 recruitment to the phagosomal membranes (Shibutani *et al.*, 2015).

T cells may up-regulate autophagy upon T-cell receptor (TCR) stimulation, a process that seems to be essential not only for T-cell proliferation but also for their survival as autophagy-defective $CD4^+$ cells are more susceptible to apoptosis (Kovacs *et al.*, 2012; Pua *et al.*, 2007). One important physiological process in which autophagy-dependent endogenous antigen presentation by MHC class II is essential is the education of naive CD4+ T cells in the thymus, where thymic epithelial cells (TECs) present self-antigens on MHC molecules for the induction of T-cell tolerance. TECs display constitutive starvation-independent high levels of autophagy which appear to be crucial for correct negative selection of T cells, elimination of autoreactive T cells and the correct development of self-tolerance (Aichinger *et al.*, 2013; Nedjic *et al.*, 2008).

However, autophagy pathways engage in far more aspects of adaptive immunity than antigen presentation, as they also affect lymphocyte selection, maturation, proliferation and survival. Defects in autophagy lead to serious damage in the lymphoid lineage: mice lacking ATG5 displayed a reduced number of B and T cells, suggesting that autophagy regulates the activity of lymphoid precursors. Even though lethally irradiated mice get repopulated with haematopoietic cells of $Atg5^{-/-}$ mice, the $CD4^+$ and $CD8^+$ cells failed to undergo proliferation upon T-receptor stimulation. $Atg5^{-/-}$ T cells managed to repopulate the thymus but experienced high levels of cell death that prevented the repopulation of the periphery (Pua *et al.*, 2007). ATG7-deficient cells failed to reconstitute the haematopoietic system of lethally irradiated mice and the production of lymphoid progenitors was also impaired in the absence of ATG7 (Mortensen *et al.*, 2011). Selective autophagy of mitochondria (mitophagy), also seems to be essential for the development of T cells as the number of naive T cells is significantly lower if mitophagy is impaired (Farfariello *et al.*, 2012; Pua *et al.*, 2009). Furthermore, the absence of autophagy during B cell differentiation appears to negatively impact the numbers of B1 cells while the overall number of B cells remains unchanged, a process that seems to involve BECN1 and ATG5 (Arsov *et al.*, 2011; Miller *et al.*, 2008). Finally, autophagy is capital for the maintenance of plasma cells, which require autophagy for sustainable immunoglobulin production (Pengo *et al.*, 2013).

Autophagy regulates cytokine production during bacterial infection

Considering the cross-talk between autophagy and PRR signalling, it can be hypothesized that autophagy influences cytokine production during bacterial infection. Indeed, several groups investigated the effect of autophagy inhibition on cytokine production, either during *in vitro* infection of isolated cells or *in vivo* infection of mice with bacterial pathogens

Table 4.1 Effects of autophagy on cytokine production upon bacterial infection

Bacteria	Infection	Host model	Mechanism of autophagy-inhibition	Effect on autophagy compared with WT mice or non-treated cells	Cytokines	Reference
Mycobacterium tuberculosis	*In vitro*	PBMCs	3-MA and siRNA knockdown of ATG7	Down-regulated ↓	TNF-α ↓, IL-1β ↑, IL-6 ↑	Kleinnijenhuis *et al*. (2011)
Borrelia burgdorferi	*In vitro*	PBMCs	3-MA, wortmannin	Down-regulated ↓	IL-1β ↑, IL-6 ↑, IL-23 ↑, IL-17 ↑, IFN-γ ↑	Buffen *et al*. (2016)
	In vivo	Patellae of mice	*Atg7*$^{-/-}$ mice		IL-1β ↑, IL-6 ↑	
	In vitro	BMDMs	*Atg7*$^{-/-}$ mice		IL-1β ↑	Buffen *et al*. (2013)
Pseudomonas aeruginosa	*In vivo*	Lung macrophages of infected mice	*Anxa2*$^{-/-}$ mice	Down-regulated ↓	TNF-α ↑, IL-6 ↑, IL-1β ↑ IFNγ ↑	Li *et al*. (2015)
Adherent Invasive *E. coli* (AIEC)	*In vitro*	THP-1 macrophage-like cells	siRNA knockdown of ATG16L1	Down-regulated↓	TNF-α ↑, IL-6 ↑	Lapaquette *et al*. (2012)
Salmonella Typhimurium	*In vivo*	Epithelial cells in terminal ileum and cecum of mice	*Atg16l1*$^{-/-}$ mice	Down-regulated ↓	IL-6 ↑, IL-1β ↑	Conway *et al.* (2013)
Shigella flexneri	*In vitro*	Splenic macrophages of mice	Atg16L1 T300A knock-in mice	Down-regulated ↓	Il-1β ↑	Lassen *et al*. (2014)
Legionella pneumophila	*In vitro*	BMDMs	Δ*legS2*	Up-regulated ↑	IL-10 ↓, IL-6 ↓, TNFα ↓ Il-1β ↑	Abu Khweek *et al*. (2016)
Mycobacterium tuberculosis	*In vitro*	BMDMs	Δ*eis*	Up-regulated ↑	TNFα ↑ IL-6 ↑	Shin *et al*. (2010)
	In vivo	Lungs and spleen of infected mice	Δ*tlyA*	Up-regulated ↑	IL-12p40 ↑ IL-1β ↓, IL-10 ↓	Rahman *et al*. (2015)

(Table 4.1). These studies have shown that inhibition of autophagy by 3MA decreased TNF-α production but enhanced IL-1β and IL-6 production in *M. tuberculosis*-infected peripheral blood mononuclear cells (PBMCs). In contrast, induction of autophagy by starvation had the opposite effect (Kleinnijenhuis *et al.*, 2011). Similar results were obtained for 3MA inhibition of autophagy during infection of PBMCs with *Borrelia burgdorferi*, the causative agent of Lyme disease, as IL-1β and IL-6 increased while TNF-α remained unaltered (Buffen *et al.*, 2013). In a mouse model of *Pseudomonas aeruginosa* infection, Li and colleagues showed that Annexin A2 (AnxA2) regulates autophagosome formation through the mTORC1–ULK1 signalling pathway (Li *et al.*, 2015) as infected *Anxa2*$^{-/-}$ mice displayed reduced autophagy levels and a marked increase of cytokines, in particular IL-1β, TNF-α, IL-6 and IFN-γ, in bronchoalveolar lavage fluid (BALF). Given the body of work available, it seems plausible to affirm that autophagy reduction induces an increase in cytokine production during bacterial infection.

Crohn's disease is an inflammatory disorder characterized by an excessive immune response to intestinal microbiota. The Thr300Ala polymorphism on ATG16L1 is associated with Crohn's disease, a fact that inspired several studies on cytokines production on ATG16L1 down-regulation conditions (Conway *et al.*, 2013; Lapaquette *et al.*, 2012; Lassen *et al.*, 2014). One of the first studies showed that knockdown of *Atg16l1* in THP-1 macrophage-like cells led to decreased autophagy and increased production of TNF-α and IL-6 during infection with adherent invasive *Escherichia coli* (Lapaquette *et al.*, 2012). In a later study, Conway and colleagues showed that *S.* Typhimurium was unable to associate with autophagosomes in *Atg16l1*-deficient epithelial cells and showed significantly higher levels of IL-1β and IL-6 compared to WT mice in the terminal ileum and caecum (Conway *et al.*, 2013). But the most informative study was carried out using a knock-in mouse model expressing the ATG16L1 T300A variant (Lassen *et al.*, 2014). As autophagy was reduced in multiple cell types from T300A knock-in mice compared to WT mice, the authors explored several aspects of the immune response to *S. flexneri* infection. Their results showed that the T300A polymorphism was associated with decreased antibacterial autophagy and increased IL-1β production in primary cells and *in vivo*. Interestingly, *in vitro* bacterial infections of isolated splenic CD11b^{+} macrophages from ATG16L1 T300A mice, led also to higher levels of IL-1β. Collectively, this study shows that defective autophagy caused by a disease-associated polymorphism of an autophagy gene leads to increased IL-1β production. However, the mechanism linking ATG16L1 to IL-1β secretion was not elucidated (Lassen *et al.*, 2014).

Furthermore, a recent report showed that injection of *B. burgdorferi* into the knees of *Atg7*$^{-/-}$ mice increased joint swelling and cytokine levels (IL-1β and IL-6) when compared to WT mice, suggesting that *B. burgdorferi*-induced joint inflammation is controlled by autophagy (Buffen *et al.*, 2016). Autophagy inhibition by wortmannin led to an increase in the production of IL-1β and IL-23 cytokines by human PBMCs infected with *B. burgdorferi* suggesting that the production of these cytokines is controlled by autophagy during *B. burgdorferi* infection *in vitro*. Increased production of IL-1β led to the polarization of CD4^{+} T cells to IL-17-producing Th17 cells, a specific subtype of T cells that is commonly elevated in patients with confirmed neuroborreliosis (Henningsson *et al.*, 2011) and which seem to be involved in the pathogenesis of Lyme arthritis (Burchill *et al.*, 2003). Collectively, these results show that autophagy controls *B. burgdorferi*-induced secretion

of cytokines such as IL-1β and IL-23, which in turn impact T-cell polarization during infection.

Thus, it seems that autophagy has a role limiting cytokine production during bacterial infection (especially IL-1β production). This resembles the cytokine production profiles observed during stimulation of cells with bacterial ligands in the presence of autophagy inhibitors discussed in previous sections.

Intracellular bacteria modulate autophagy in the infected cell

Infection by bacterial pathogens triggers autophagy in infected cells as a cell-autonomous defence mechanism aimed at degrading the invading pathogen. However, pathogenic bacteria evolved mechanisms to manipulate autophagy and counteract these host self defences. While certain intracellular bacteria induce and manipulate autophagy taking advantage of it, other bacteria inhibit autophagy in order to avoid xenophagy and lysosomal degradation (Escoll *et al.*, 2016).

Anaplasma phagocytophilum, *Yersinia pseudotuberculosis*, *Coxiella burnetii* and *F. tularensis* are intracellular pathogens that have evolved mechanisms to hijack autophagosomes during infection. These bacteria redirect the by-products of the autophagic degradation of cellular components for their own nutritional use, thereby promoting their replication (Steele *et al.*, 2015). They replicate within bacterial vacuoles decorated with autophagy components, such as LC3, and show defective replication in autophagy-deficient cells. Consequently, treatment of host cells with autophagy activators, increases bacterial replication rather than promoting bacterial clearance (Escoll *et al.*, 2016). *A. phagocytophilum* uses the secreted effector Ats-1 to promote autophagosome nucleation and utilization of the nutrients contained in the autophagosomes (Niu *et al.*, 2012). Similarly, it was elegantly shown by monitoring autophagy-derived radiolabelled amino acids that during *F. tularensis* infection a transfer from host proteins to invading bacteria takes place (Steele *et al.*, 2013).

Other bacteria, such as *M. tuberculosis* and *S.* Typhimurium, inhibit autophagy initiation upstream of autophagosome formation, thus evading xenophagy and pathogen degradation (Shin *et al.*, 2010; Tattoli *et al.*, 2012). *S. flexneri* evades autophagy recognition by masking the bacterial surface (Ogawa *et al.*, 2005).

An important observation is that some bacterial pathogens actively induce autophagy but, at the same time, block autophagosome maturation and fusion with the lysosome. *L. pneumophila* is one of these intracellular bacterial pathogens that uses this dual strategy. After phagocytosis, the establishment of the *Legionella*-containing vacuole (LCV) in the infected macrophage is accompanied by the acquisition of autophagy markers like LC3 at the LCV, showing that the LCVs rapidly become autophagosomes (Amer and Swanson, 2005). The secreted *L. pneumophila* effector LegA9 was shown to promote the recognition of the LCV by the autophagy machinery (Khweek *et al.*, 2013). In line with this observation, inhibition of autophagy in permissive A/J mouse macrophages reduces *L. pneumophila* survival at 2 hours post infection (Amer and Swanson, 2005; Amer *et al.*, 2005), supporting the idea that autophagy has a role in the promotion of the survival of *L. pneumophila* within the host cell early during infection (Amer *et al.*, 2005). Later during infection, *L. pneumophila* inhibits autophagy by secreting the effectors *Lp*SPL and RavZ (Choy *et al.*, 2012; Rolando *et al.*, 2016), which respectively inhibit autophagosome formation and maturation. This strategy delays the maturation of the LCV-containing autophagosome into autolysosomes,

thus gaining precious time for multiplication of the pathogen (Amer and Swanson, 2005; Joshi and Swanson, 2011).

Autophagy subversion by pathogenic bacteria shapes host immunity

As previously discussed, autophagy and immunity are strongly linked and coordinated processes. Considering that many pathogenic bacteria manipulate autophagy, it is expected that this strategy has direct consequences on immunity. Regarding cell-autonomous immunity, bacteria-induced inhibition of autophagy promotes evasion from xenophagy, a benefit for the pathogen as they escape lysosomal degradation. However, autophagy is also linked to 'conventional' immune responses, i.e. innate and adaptive immunity (Kuballa *et al.*, 2012), but this relationship and the impact of bacterial modulation of autophagy on immune responses remains poorly understood.

Manipulation of autophagy by pathogenic bacteria may impact cytokine production by innate immune cells, as autophagy is interconnected to PRR signalling. This may represent an underappreciated effect on innate immunity induced by bacteria that modulate autophagy. Indeed, as listed in Table 4.1, some studies have pointed to this possibility. For example, the *M. tuberculosis* protein Eis is involved in bacterial survival within the host. Macrophages infected with a mutant lacking *eis* displayed markedly increased accumulation of autophagy vacuoles and formation of autophagosomes *in vitro* and *in vivo*, suggesting that Eis down-regulates autophagy in the host cell (Shin *et al.*, 2010). Interestingly, infection of macrophages with a Δ*eis* mutant strain increased the production of TNF-α and IL-6 as compared to those measured after infection with the WT strain, suggesting that the loss of the capacity of the *eis* mutant to inhibit autophagy leads to the increased TNF-α and IL-6 production (Shin *et al.*, 2010). In addition, the virulence factor TlyA also inhibits autophagy and significantly contributes to the pathogenesis of *M. tuberculosis*. DCs infected with a Δ*tlyA* strain displayed increased autophagy and showed increased IL-12p40 and reduced IL-1β and IL-10 cytokine responses, which clearly contrasts with the immune responses induced by the WT strain (Rahman *et al.*, 2015). Collectively, these two studies suggest that inhibition of autophagy induced by Eis and TlyA reduces production of TNF-α, IL-6 and IL-12p40 while it boosts the secretion of IL-1β and IL-10 in response to *M. tuberculosis* infection.

The bacterial effector *Lp*SPL secreted by *L. pneumophila* inhibits autophagy in human cells by modulating the sphingolipid metabolism of the host cell during infection (Rolando *et al.*, 2016). It was also shown, that a mutant lacking this effector induces higher secretion of IL-1β and lower secretion of TNF-α, IL-6 and IL-10 compared to the WT strain during infection of BMDMs (Abu Khweek *et al.*, 2016). As these studies were carried out in different host cells (human and mice) and none of them tested the influence of *L. pneumophila*-induced inhibition of autophagy on cytokine production of infected cells directly, it is difficult to draw conclusions about how manipulation of autophagy by *L. pneumophila* influences cytokine production of infected macrophages; however, it is tempting to assume that there is a link.

As autophagy is also involved in antigen presentation to T cells, autophagy modulation by bacterial pathogens may also impact adaptive immunity. Indeed, a recent report showed that autophagy modulation by *M. tuberculosis* impacts adaptive immunity. A genome-wide

screening identified the protein PE_PGRS47 of *M. tuberculosis* as responsible for the inhibition of MHC class II antigen presentation of infected DCs as Δ*PE_PGRS47*-infected DCs showed increased MHC class II antigen presentation compared to the WT strain, and infection of mice with the Δ*PE_PGRS47* strain resulted in an increased number of bacterial-antigen-specific $CD4^+$ cells compared to WT-infected mice (Saini *et al.*, 2016). Interestingly, infection with a *M. tuberculosis* mutant lacking PE_PGRS47 showed increased autophagy when compared to the WT strain, suggesting that the bacteria use PE_PGRS47 to inhibit autophagy in the host cell. Moreover, the Δ*PE_PGRS47* strain was significantly attenuated *in vivo* and its defects in intracellular replication *in vitro* were restored to WT levels when autophagy was inhibited by 3MA treatment (Saini *et al.*, 2016). These results demonstrate that the *M. tuberculosis* protein PE_PGRS47 inhibits autophagy in the infected cells resulting in a reduction of MHC class II antigen presentation that impacts specific T-cell responses to infection.

Concluding remarks

Autophagy works as a cell-autonomous defence mechanism of immune and non-immune cells by removing invading pathogens immediately after infection. It is well established that bacteria-induced inhibition of autophagy promotes bacterial evasion from xenophagy and allows the pathogen to escape from lysosomal degradation (Huang and Brumell, 2014). Autophagy is also linked to conventional innate and adaptive immune responses but the impact of autophagy modulation by bacteria at these levels remains poorly understood.

Current data support a model where PRR signalling and autophagy cross-talk at different levels. In general, PRR activation through the recognition of bacterial ligands promotes autophagy (Delgado *et al.*, 2009). However, drugs or genetic approaches used to inhibit autophagy in infected cells or animals showed increased secretion of pro-inflammatory cytokines after stimulation with bacterial ligands or viable bacteria, suggesting that cells might also use autophagy to limit PRR-initiated immune responses as a negative-feedback loop during infection. Most of the available literature supports this model (Table 4.1), with data for inflammasome signalling and IL-1β secretion being very solid and reproducible through out the different studies.

One of the most compelling results is the role of ATG16L1 in Crohn's disease. Defects in ATG16L1, such as the T300A polymorphism carried by patients, lead to reduced autophagy in host cells during bacterial infection, reduced bacterial clearance and increased secretion of cytokines, mainly of IL-1β (Conway *et al.*, 2013; Lapaquette *et al.*, 2012; Lassen *et al.*, 2014). These studies highlight the existence of intrinsic mechanisms within host cells where autophagy and cytokine production are coordinated during host–pathogen interactions, suggesting that malfunction of autophagy might be one cause of inflammatory diseases with excessive cytokine production such as Crohn's disease. Finally, the investigation of the mechanisms used by bacteria to modulate autophagy identified some bacterial effectors inhibiting autophagy, thereby promoting bacterial survival during infection. However, only few studies addressed the question of how these bacterial-derived autophagy inhibitors directly impact immune responses during infection. Most is known for *M. tuberculosis*, in which the bacterial proteins Eis, TlyA and PE_PGRS47 have been shown to inhibit autophagy and to modulate cytokine production and MHC class II antigen presentation to T cells, shaping innate and adaptive immunity during *M. tuberculosis* infection.

Future work on this topic should be directed to uncover whether mechanisms exist that coordinate autophagy and cytokine production during infection. It would be important to investigate in parallel the impact of autophagy on the secretion of inflammasome-dependent/-independent cytokines (such as IL-1 and TNF-α, respectively) and also on the production of anti-inflammatory cytokines such as IL-10 or TGF-β. Additionally, an intriguing question yet to be answered is whether or not bacterial effectors that increase or reduce autophagy (such Ats-1 or *Lp*SPL, respectively) impact the innate and adaptive immune responses to infection. In depth knowledge on these questions will help to better understand bacterial infection and to better combat disease.

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