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**Manipulation of autophagy by bacterial pathogens impacts host  
immunity**

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30 **Abstract**

31

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

42

## 42 **Introduction**

43 Maintaining cellular homeostasis requires a coordinated control of metabolic pathways. Thus  
44 the cell needs anabolic processes to build new components, and catabolic processes to discard  
45 long-lived or damaged components. Autophagy is one of these catabolic processes that leads  
46 to lysosomal degradation of unnecessary or damaged proteins, lipids and organelles (Ohsumi,  
47 2014; Rubinsztein et al., 2012). The term “autophagy” describes mainly three different  
48 processes: macroautophagy, microautophagy and chaperon-mediated autophagy. In this  
49 review we will focus on macroautophagy (hereafter called autophagy), which is characterized  
50 by the sequestration of cargo into double-membrane vesicles called autophagosomes (Miller  
51 and Celli, 2016).

52 Autophagy can be divided into selective and non-selective autophagy: in non-selective  
53 autophagy a random portion of the cytoplasm is engulfed to degrade long-lived components  
54 or to provide nutrients during starvation, while selective autophagy targets selected  
55 components for degradation. This includes organelle-specific autophagy, such as removal of  
56 mitochondria called mitophagy, and xenophagy which is the removal of invading bacteria  
57 (Deretic et al., 2013; Huang and Brumell, 2014).

58 Intracellular bacteria grow and replicate inside host cells. These bacteria can be divided  
59 into two groups: those that replicate in the cytosol, such as *Listeria* or *Shigella*, and those that  
60 replicate in membrane-derived pseudo-organelles called pathogen-containing vacuoles  
61 (PCVs), such as *Legionella*, *Mycobacteria* or *Salmonella*. PCVs facilitate the replication,  
62 survival and dormancy of intracellular bacteria. For their formation bacteria exploit the host’s  
63 membrane system, in particular the dynamic machineries involved in exo/endocytic traffic  
64 and autophagy. To enhance survival and proliferation in PCVs, bacteria secrete effector  
65 proteins that inhibit their degradation in lysosomes and facilitate the acquisition of membrane  
66 sources and nutrients (Ray et al., 2009). In order to ensure survival, intracellular bacteria also  
67 need to counteract self-defense mechanisms of the host cell. In animals, cellular self-defense  
68 (also known as cell-autonomous immunity) synergizes with the whole-body protection  
69 provided by “conventional” immunity (innate and adaptative immunity) to grant resistance to  
70 pathogens. While professional immune cells patrol the body in search of pathogens, cell-  
71 autonomous immunity guards both individual immune and non-immune cells against the  
72 immediate threat of infection. The current paradigm shows that cell-autonomous immunity  
73 against intracellular pathogens is based on the tight and continuous control of the composition  
74 and behaviour of distinct cellular compartments, such as the cytosol, organelles, endosomes,

75 autophagosomes and lysosomes (Randow et al., 2013). As the autophagy machinery removes  
76 intracellular pathogens by directing them to lysosomal degradation, xenophagy has emerged  
77 as an important cellular self-defense process to protect host cells against intracellular bacteria.  
78 In this case, engulfment of bacteria by autophagosomes triggers an integrated response within  
79 the infected cell, leading to pathogen elimination while professional immune cells are warned  
80 about the threat.

81 As autophagy is an important cellular self-defense mechanism, bacteria evolved various  
82 mechanisms to modulate autophagy such as inhibiting autophagosome formation, self-  
83 masking with host proteins to avoid recognition, escaping targeting to autophagosomes or  
84 blocking fusion of autophagosomes with lysosomes (Huang and Brumell, 2014; Escoll et al.,  
85 2016). As autophagy is linked to immune responses, bacteria-induced modulation of  
86 autophagy impacts also innate and adaptive immune responses (Kuballa et al., 2012).

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

### 91 **Autophagosome formation, elongation and maturation is a complex process involving** 92 **conserved host factors**

93 The pathway leading to autophagy includes signal induction, nucleation and elongation of the  
94 phagophore to form the autophagosome, closure of the autophagosome and fusion with  
95 lysosomes (Parzych and Klionsky, 2014). More than 35 Autophagy-related proteins (ATG-  
96 proteins) that have metazoan orthologues have been described in yeast to coordinate  
97 autophagosome formation and maturation (Ohsumi, 2014) (Figure 1).

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

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

122 Phagophore elongation is then mediated by two ubiquitination-like conjugation systems of  
123 ATG proteins. First, ATG7 (E1-like protein) and ATG10 (E2-like protein) conjugate ATG12  
124 and ATG5, promoting phagophore elongation (Mizushima et al., 1998; Shintani et al., 1999).  
125 ATG12-ATG5 forms a complex with ATG16L1, which associates with the expanding  
126 phagophore membrane and gets released after the autophagosome is formed (Mizushima et al.,  
127 2001; 2003). The ATG12-ATG5-ATG16L1 complex is essential for the formation of the  
128 second ubiquitin-like conjugation system, where the protein light chain 3 (LC3; Atg8 in  
129 yeast) is conjugated to phosphatidylethanolamine (PE) in the phagophore membrane. This  
130 conjugation system relies on ATG7 (E1-like protein) and ATG3 (E2-like protein) that activate  
131 LC3. In addition, ATG4B cleaves the carboxy-terminus of LC3 and exposes a glycine residue.  
132 Activated LC3 is directed by ATG12-ATG5-ATG16L1 to the expanding phagophore, where  
133 the E3-like activity of ATG12-ATG5 conjugates PE to the previously exposed glycine residue  
134 of LC3 in the phagophore membrane. Once the phagophore is closed and the autophagosome  
135 is formed, the ATG12-ATG5-ATG16L1 complex is released from the autophagosome.  
136 ATG4B has also been shown to deconjugate a proportion of the LC3-PE complexes from  
137 mature autophagosomes, facilitating LC3 recycling for the formation of new autophagosomes  
138 (Fujita et al., 2008; Tanida et al., 2004). It is thought that recycling of LC3 is an important  
139 step in the maturation of fusion-capable autophagosomes. It has also been shown that LC3

140 mediates the hemifusion of vesicles and controls the size of autophagosomes in yeast  
141 (Nakatogawa et al., 2007; Xie et al., 2008).

142 Finally, a series of fusion events of autophagosomes with endosomes and lysosomes leads  
143 to their maturation into degrading autolysosomes. In mammalian cells the fusion of  
144 autophagosomes with lysosomes requires the small GTPase RAB7 (Ypt7 in yeast), the  
145 autophagosomal SNARE protein syntaxin 17, the lysosomal SNARE protein VAMP8  
146 (vesicle-associated membrane protein 8) and several lysosomal membrane proteins, such as  
147 the lysosomal-associated membrane glycoprotein 2 (LAMP2). After autophagosome-  
148 lysosome fusion the cargo is degraded by the lysosomal hydrolases now present in the  
149 autolysosome, (Eskelinen et al., 2002; Itakura et al., 2012; Jager et al., 2004; Tanaka et al.,  
150 2000).

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

### 169 **Crosstalk between autophagy and the innate immune response**

170 The first step of innate immune responses is the recognition of a pathogen that is mediated by  
171 a variety of pattern-recognition receptors (PRRs) like Toll-like Receptors (TLRs), RIG-I-like  
172 receptors (RLRs) and NOD-like receptors (NLRs) that bind pathogen-associated molecular

173 patterns (PAMPs) such as bacterial lipopolysaccharide (LPS), peptidoglycan, lipoproteins,  
174 flagellin or nucleic acids. As autophagic degradation of pathogens aids PAMP recognition by  
175 PRRs, autophagy is believed to have an important role in innate immunity. Additionally,  
176 binding of PAMPs to PRRs stimulates autophagy (Delgado et al., 2009).

177 *Toll-like Receptors (TLRs)*: TLRs are membrane-bound receptors found at the surface of  
178 the cell or in endosomal compartments. TLRs respond to LPS, lipoteichoic acid, flagellin and  
179 bacterial nucleic acids (Deretic, 2012). After binding their cognate ligand, TLRs activate  
180 proinflammatory responses by triggering the production of cytokines such as tumor necrosis  
181 factor- $\alpha$  (TNF- $\alpha$ ), Interleukin-6 (IL-6) and IL-1 $\beta$  (Figure 2).

182 Phagocytosed and cytosolic PAMPs are sequestered by autophagosomes and are delivered  
183 to the endosomally located and lumenally oriented TLRs for PAMP recognition. Therefore  
184 autophagy takes part in the first steps of TLR activation (Desai et al., 2015). On the other side,  
185 autophagy can also be induced upon TLR activation, through a MyD88/TRIF adaptor  
186 dependent process and their interaction with BECN1 (Shi et al., 2008). TLR-autophagy  
187 crosstalk is supported by several other findings: TLR4 stimulates PI3KC3-dependent  
188 formation of cytosolic LC3 aggregates, enhancing the elimination of mycobacteria from  
189 macrophages (Xu et al., 2007). Downstream of TLR4 activation, the TANK binding kinase 1  
190 (TBK1) also links the TLR signalling pathway and autophagy as TBK1 phosphorylates the  
191 autophagic receptor OPTN, enhancing LC3 binding affinity and consequently the autophagic  
192 clearance of cytosolic *Salmonella enterica* (Wild et al., 2011). TLR7 activation has also been  
193 shown to promote autophagic degradation of *Mycobacterium bovis* BCG (Bacillus Calmette-  
194 Guerin) (Delgado et al., 2008), and TLR2 induces autophagy in an ERK-dependent  
195 mechanism during *Listeria monocytogenes* infection (Anand et al., 2011). Altogether these  
196 studies highlight that TLR activation elicits autophagy, thus activating cell-autonomous  
197 immunity after PAMP detection.

198 However, autophagy may also down-regulate TLR-induced responses. Inhibition of  
199 autophagy in macrophages and dendritic cells (DCs) through 3MA (3-methyladenine) or  
200 through siRNA knock-down of autophagy genes, leads to an increase in cytokine release in  
201 response to TLR3 or TLR4 agonists (Harris, 2011; Saitoh et al., 2008). ATG16L1-deficient  
202 mice were shown to excessively activate caspase-1 in response to TLR4 stimulation by LPS,  
203 which led to increased IL-1 $\beta$  and IL-18 production. This suggests that ATG16L1 might have  
204 a regulatory function on the TLR4 signalling pathway and its depletion/malfunction may lead  
205 to increased inflammation (Saitoh et al., 2008). Mature IL-1 $\beta$  protein can also be engulfed by



206 autophagosomes in macrophages stimulated with TLR ligands, showing another way by  
207 which autophagy can downregulate the production and secretion of cytokines (Harris et al.,  
208 2011) (Figure 2). Thus, autophagy influences TLR signalling and consequently impacts TLR-  
209 mediated cytokine production and secretion.

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

219 *cGAMP synthase (cGAS)*: Bacterial or aberrant cytosolic DNA are also recognized by  
220 direct binding to cGAS, a cytosolic protein that generates cyclic dinucleotides (CDNs) such as  
221 cGAMP within the host cytosol (Tao et al., 2016). Host cell-generated CDNs activate the  
222 downstream stimulator of interferon genes (STING), a receptor that can be also activated by  
223 CDNs of intracellular bacteria (Burdette et al., 2011). STING controls the activation of above-  
224 mentioned TBK1, which upon nucleic acid sensing and cGAMP synthesis triggers both IFN  
225 regulatory factor 3 (IRF3) phosphorylation and type I IFN production. STING induced type I  
226 IFN production occurs during infection by intracellular bacteria such as *Mycobacterium*  
227 *tuberculosis* (Watson et al., 2015). On the other hand, the autophagy activator protein ULK1  
228 phosphorylates STING, inhibiting sustained type I IFN activation in response to dsDNA  
229 (Konno et al., 2013) (Figure 2). Furthermore, cGAS binding to BECN1 facilitates autophagic  
230 removal of cytosolic dsDNA and reduces excessive type I IFN responses (Liang et al., 2014).

231 *NOD-like receptors (NLRs)*: NLRs are a class of receptors initiating a quick and potent  
232 inflammatory cytokine response to PAMPs. Upon sensing PAMPs, cytosolic NLR-receptors  
233 form a signalling complex called the inflammasome. The inflammasome consists of several  
234 oligomerized NLRs that bind Caspase-1 directly or through the adaptor protein called  
235 apoptosis-associated speck-like protein (ASC), which contains a caspase recruitment domain  
236 (CARD). These complexes cleave the protein precursor pro-Caspase-1 into p10 and p20  
237 subunits, activating Caspase-1. Active Caspase-1 then cleaves the presynthesized pro-IL-1 $\beta$   
238 into the active form of the cytokine IL-1 $\beta$ , which is secreted (Rodgers et al., 2014). IL-1 $\beta$

239 secretion relies on two different PRR activating signals: the NF- $\kappa$ B-dependant expression of  
240 pro-IL-1 $\beta$  through TLR activation and a signal activating Caspase-1 through inflammasomes  
241 to cleave pro-IL-1 $\beta$  (Eder, 2009) (Figure 2). The inflammasome also cleaves pro-IL-18 into  
242 active IL-18 and orchestrates the programmed cell death known as pyroptosis (Miao et al.,  
243 2011). Therefore, the inflammasome promotes inflammation by controlling the secretion of  
244 the strong proinflammatory cytokines IL-1 $\beta$  and IL-18 and avoiding that cells become a niche  
245 for the pathogens by activating pyroptotic cell death. These two cytokines are responsible for  
246 the recruitment of myeloid cells, including neutrophils, to sites of inflammation (Rider et al.,  
247 2011).

248 NLRs that respond to PAMPs in macrophages and activate Caspase-1 by inflammasome  
249 assembly are NLRP1, NLRP3, NLRC4, NLRP7, NLRP12 and AIM2 (absent in melanoma 2)  
250 (Latz et al., 2013). Interestingly, a vast amount of data strongly suggests that both machineries,  
251 autophagy and inflammasome, are highly interconnected and influence each other. Remnants  
252 of autophagosomes that were degraded during *S. flexneri* infection of epithelial cells are  
253 decorated with components of both, the autophagy and the inflammasome machineries.  
254 Moreover, macrophages of *Caspase-1*<sup>-/-</sup> mice display an excessive accumulation of  
255 autophagosomes during *S. flexneri* infection, suggesting that Caspase-1 affects either  
256 autophagosome formation or maturation (Dupont et al., 2009; Suzuki et al., 2007). Activation  
257 of AIM2 with poly(dA:dT) or NLRP3 activation with uric acid crystals or nigericin leads to  
258 an increase in autophagosome formation in macrophages (Shi et al., 2012). Autophagosomes  
259 have also been shown to sequester and degrade inflammasome components and cytokine  
260 precursors such as pro-IL-1 $\beta$  (Harris et al., 2011; Shi et al., 2012). Association between  
261 autophagy and inflammasome proteins was also shown in resting cells, where NLRP4 and  
262 NLRC4 formed a complex with BECN1, suggesting that NLRP4 sensing of bacteria leads to  
263 the initiation of BECN1-mediated autophagic responses (Jounai et al., 2011). Autophagy and  
264 inflammation are also regulated by NOD1 and NOD2, a Caspase-1 independent class of NLR  
265 proteins. NOD1 and NOD2 are intracellular sensors of peptidoglycan that induce autophagy  
266 by interacting with ATG16L1 and regulating IL-1 $\beta$  and IL-18 production through NF- $\kappa$ B  
267 (Philpott et al., 2013).

268 Key evidence for NLR signalling in anti-bacterial responses was gained from the analysis of  
269 mouse susceptibility to *L. pneumophila*. Indeed, only A/J mice are susceptible to infection  
270 due to a partial loss-of-function mutation in the Naip5 inflammasome, a component of the

271 flagellin-NLRC4 pathway (Molofsky et al., 2006; Zamboni et al., 2006). Autophagosomes of  
272 macrophages from *L. pneumophila*-resistant C57BL/6J mice matured quickly and prevented  
273 efficient *L. pneumophila* replication when compared to autophagosomes of *L. pneumophila*-  
274 permissive Naip5 mutant A/J macrophages. This observation is reinforcing the idea that  
275 inflammasome activation and autophagy are intertwined processes during bacterial infection  
276 (Amer and Swanson, 2005). Additional results obtained in primary mouse macrophages  
277 support a model in which both Caspase-1 and NLR components of inflammasomes are co-  
278 ordinally responding to *L. pneumophila* infection depending on the bacterial burden (Byrne  
279 et al., 2013). According to this model, NLRC4 is complexed with autophagy component  
280 BECN1 in resting macrophages, inhibiting autophagy. Low levels of bacteria lead flagellin-  
281 bound NAIP5 to recruit NLRC4 to a complex containing the pro-Caspase-1 protein,  
282 derepressing autophagy. When the capacity of autophagy to eliminate intracellular bacteria is  
283 exceeded, Caspase-1 triggers pyroptosis to eliminate the pathogen's niche while initiating a  
284 potent inflammatory response (Byrne et al., 2013).

### 285 **Autophagy crosstalks with adaptive immune responses**

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

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

298 The classical concept suggests that intracellular antigens get processed and loaded onto  
299 MHC class I cells while extracellular ones go on MHC class II cells. However, as autophagy  
300 delivers cytoplasmic constituents for lysosomal degradation and MHC class II molecules are  
301 loaded with lysosomal products, autophagy also supports the processing of endogenous  
302 antigens for presentation by MHC class II (Dengjel et al., 2005; Nimmerjahn et al., 2003;

303 Schmid et al., 2007; Zhou et al., 2005). Therefore, antigen-presenting cells such as  
304 macrophages and DCs can use the autophagy machinery to fuse autophagosomes containing  
305 bacterial-derived antigens with autolysosomes, which are afterwards loaded into MHC class II  
306 molecules for antigen presentation to CD4<sup>+</sup> T cells (Crotzer and Blum, 2009; Dengjel et al.,  
307 2005). This antigen-presentation enhancing effect of autophagy has been used to significantly  
308 upgrade the efficiency of the BCG vaccine (Jagannath et al., 2009). Autophagy also regulates  
309 exogenous antigen processing for presentation by MHC class II *via* the modification of the  
310 content and fate of phagosomes after LC3 recruitment to the phagosomal membranes  
311 (Shibutani et al., 2015).

312 T cells may upregulate autophagy upon T-cell receptor (TCR) stimulation, a process that  
313 seems to be essential not only for T cell proliferation but also for their survival as autophagy-  
314 defective CD4<sup>+</sup> cells are more susceptible to apoptosis (Kovacs et al., 2011; Pua et al., 2007).  
315 One important physiological process in which autophagy-dependent endogenous antigen  
316 presentation by MHC class II is essential is the education of naïve CD4<sup>+</sup> T cells in the thymus,  
317 where thymic epithelial cells (TECs) present self-antigens on MHC molecules for the  
318 induction of T cell tolerance. TECs display constitutive starvation-independent high levels of  
319 autophagy which appear to be crucial for correct negative selection of T cells, elimination of  
320 autoreactive T cells and the correct development of self-tolerance (Aichinger et al., 2013;  
321 Nedjic et al., 2008).

322 However, autophagy pathways engage in far more aspects of adaptive immunity than  
323 antigen presentation, as they also affect lymphocyte selection, maturation, proliferation and  
324 survival. Defects in autophagy lead to serious damage in the lymphoid lineage: mice lacking  
325 ATG5 displayed a reduced number of B and T cells, suggesting that autophagy regulates the  
326 activity of lymphoid precursors. Even though lethally irradiated mice get repopulated with  
327 haematopoietic cells of *Atg5*<sup>-/-</sup> mice, the CD4<sup>+</sup> and CD8<sup>+</sup> cells failed to undergo proliferation  
328 upon T-receptor stimulation. *Atg5*<sup>-/-</sup> T cells managed to repopulate the thymus but  
329 experienced high levels of cell death that prevented the repopulation of the periphery (Pua et  
330 al., 2007). ATG7-deficient cells failed to reconstitute the haematopoietic system of lethally  
331 irradiated mice and the production of lymphoid progenitors was also impaired in the absence  
332 of ATG7 (Mortensen et al., 2011). Selective autophagy of mitochondria (mitophagy), also  
333 seems to be essential for the development of T cells as the number of naïve T cells is  
334 significantly lower if mitophagy is impaired (Farfariello et al., 2012; Pua et al., 2009).  
335 Furthermore, the absence of autophagy during B cell differentiation appears to negatively

336 impact the numbers of B1 cells while the overall number of B cells remains unchanged, a  
337 process that seems to involve BECN1 and ATG5 (Arsov et al., 2011; Miller et al., 2014).  
338 Finally, autophagy is capital for the maintenance of plasma cells, which require autophagy for  
339 sustainable immunoglobulin production (Pengo et al., 2013).

#### 340 **Autophagy regulates cytokine production during bacterial infection**

341 Considering the crosstalk between autophagy and PRR signalling, it can be hypothesized that  
342 autophagy influences cytokine production during bacterial infection. Indeed, several groups  
343 investigated the effect of autophagy inhibition on cytokine production, either during *in vitro*  
344 infection of isolated cells or *in vivo* infection of mice with bacterial pathogens (Table 1).  
345 These studies have shown that inhibition of autophagy by 3MA decreased TNF- $\alpha$  production  
346 but enhanced IL-1 $\beta$  and IL-6 production in *M. tuberculosis*-infected peripheral blood  
347 mononuclear cells (PBMCs). In contrast, induction of autophagy by starvation had the  
348 opposite effect (Kleinnijenhuis et al., 2011). Similar results were obtained for 3MA inhibition  
349 of autophagy during infection of PBMCs with *Borrelia burgdorferi*, the causative agent of  
350 Lyme disease, as IL-1 $\beta$  and IL-6 increased while TNF- $\alpha$  remained unaltered (Buffen et al.,  
351 2013). In a mouse model of *Pseudomonas aeruginosa* infection, Li and colleagues showed  
352 that Annexin A2 (AnxA2) regulates autophagosome formation through the mTORC1–ULK1  
353 signalling pathway (Li et al., 2015) as infected *Anxa2*<sup>-/-</sup> mice displayed reduced autophagy  
354 levels and a marked increase of cytokines, in particular IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IFN- $\gamma$ , in  
355 bronchoalveolar lavage fluid (BALF). Given the body of work available, it seems plausible to  
356 affirm that autophagy reduction induces an increase in cytokine production during bacterial  
357 infection.

358 Chron's disease is an inflammatory disorder characterized by an excessive immune  
359 response to intestinal microbiota. The Thr300Ala polymorphism on ATG16L1 is associated  
360 with Chron's disease, a fact that inspired several studies on cytokines production on  
361 ATG16L1 downregulation conditions (Conway et al., 2013; Lapaquette et al., 2012; Lassen et  
362 al., 2014). One of the first studies showed that knockdown of *Atg16l1* in THP-1 macrophage-  
363 like cells led to decreased autophagy and increased production of TNF- $\alpha$  and IL-6 during  
364 infection with adherent invasive *Escherichia coli* (Lapaquette et al., 2012). In a later study,  
365 Conway and colleagues showed that *S.Typhimurium* was unable to associate with  
366 autophagosomes in *Atg16l1*-deficient epithelial cells and showed significantly higher levels of  
367 IL-1 $\beta$  and IL-6 compared to wild-type (WT) mice in the terminal ileum and cecum (Conway  
368 et al., 2013). But the most informative study was carried out using a knock-in mouse model

369 expressing the ATG16L1 T300A variant (Lassen et al., 2014). As autophagy was reduced in  
370 multiple cell types from T300A knock-in mice compared to WT mice, the authors explored  
371 several aspects of the immune response to *S. flexneri* infection. Their results showed that the  
372 T300A polymorphism was associated with decreased antibacterial autophagy and increased  
373 IL-1 $\beta$  production in primary cells and *in vivo*. Interestingly, *in vitro* bacterial infections of  
374 isolated splenic CD11b<sup>+</sup> macrophages from ATG16L1 T300A mice, led also to higher levels  
375 of IL-1 $\beta$ . Collectively, this study shows that defective autophagy caused by a disease-  
376 associated polymorphism of an autophagy gene leads to increased IL-1 $\beta$  production. However,  
377 the mechanism linking ATG16L1 to IL-1 $\beta$  secretion was not elucidated (Lassen et al., 2014).

378 Furthermore, a recent report showed that injection of *B. burgdorferi* into the knees of  
379 *Atg7*<sup>-/-</sup> mice increased joint swelling and cytokine levels (IL-1 $\beta$  and IL-6) when compared to  
380 WT mice, suggesting that *B. burgdorferi*-induced joint inflammation is controlled by  
381 autophagy (Buffen et al., 2016). Autophagy inhibition by wortmannin led to an increase in the  
382 production of IL-1 $\beta$  and IL-23 cytokines by human PBMCs infected with *B. burgdorferi*  
383 suggesting that the production of these cytokines is controlled by autophagy during  
384 *B. burgdorferi* infection *in vitro*. Increased production of both cytokines, IL-1 $\beta$  and IL-23, led  
385 to the polarization of CD4<sup>+</sup> T cells to IL-17-producing Th17 cells, a specific subtype of T  
386 cells that is commonly elevated in patients with confirmed neuroborreliosis (Henningsson et  
387 al., 2011) and which seem to be involved in the pathogenesis of Lyme arthritis (Burchill et al.,  
388 2003). Collectively, these results show that autophagy controls *B. burgdorferi*-induced  
389 secretion of cytokines such as IL-1 $\beta$  and IL-23, which in turn impact T-cell polarization  
390 during infection.

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

### 395 **Intracellular bacteria modulate autophagy in the infected cell**

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

401 al., 2016).

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

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

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

434 Swanson, 2011).

### 435 **Autophagy subversion by pathogenic bacteria shapes host immunity**

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

444 Manipulation of autophagy by pathogenic bacteria may impact cytokine production by  
445 innate immune cells, as autophagy is interconnected to PRR signalling. This may represent an  
446 underappreciated effect on innate immunity induced by bacteria that modulate autophagy.  
447 Indeed as listed in Table 1 some studies have pointed to this possibility. For example, the  
448 *M. tuberculosis* protein Eis is involved in bacterial survival within the host. Macrophages  
449 infected with a mutant lacking *eis* displayed markedly increased accumulation of autophagy  
450 vacuoles and formation of autophagosomes *in vitro* and *in vivo*, suggesting that Eis  
451 downregulates autophagy in the host cell (Shin et al., 2010). Interestingly, infection of  
452 macrophages with a  $\Delta eis$  mutant strain increased the production of TNF- $\alpha$  and IL-6 as  
453 compared to those measured after infection with the WT strain, suggesting that the loss of the  
454 capacity of the  $\Delta eis$  mutant to inhibit autophagy leads to the increased TNF- $\alpha$  and IL-6  
455 production (Shin et al., 2010). In addition, the virulence factor TlyA also inhibits autophagy  
456 and significantly contributes to the pathogenesis of *M. tuberculosis*. DCs infected with a  
457  $\Delta tlyA$  strain displayed increased autophagy and showed increased IL-12p40 and reduced IL-  
458 1 $\beta$  and IL-10 cytokine responses, which clearly contrasts with the immune responses induced  
459 by the WT strain (Rahman et al., 2015). Collectively, these two studies suggest that inhibition  
460 of autophagy induced by Eis and TlyA reduces production of TNF- $\alpha$ , IL-6 and IL-12p40  
461 while it boosts the secretion of IL-1 $\beta$  and IL-10 in response to *M. tuberculosis* infection.

462 The bacterial effector *LpSPL* secreted by *L. pneumophila* inhibits autophagy in human  
463 cells by modulating the sphingolipid metabolism of the host cell during infection (Rolando et  
464 al., 2016). It was also shown, that a mutant lacking this effector induces higher secretion of  
465 IL-1 $\beta$  and lower secretion of TNF- $\alpha$ , IL-6 and IL-10 compared to the WT strain during



466 infection of BMDMs (Abu Khweek et al., 2016). As these studies were carried out in  
467 different host cells (human and mice) and none of them tested the influence of  
468 *L. pneumophila*-induced inhibition of autophagy on cytokine production of infected cells  
469 directly, it is difficult to draw conclusions about how manipulation of autophagy by  
470 *L. pneumophila* influences cytokine production of infected macrophages, however, it is  
471 tempting to assume that there is a link.

472 As autophagy is also involved in antigen presentation to T cells, autophagy modulation by  
473 bacterial pathogens may also impact adaptive immunity. Indeed, a recent report showed that  
474 autophagy modulation by *M. tuberculosis* impacts adaptive immunity. A genome-wide  
475 screening identified the protein PE\_PGRS47 of *M. tuberculosis* as responsible for the  
476 inhibition of MHC class II antigen presentation of infected DCs as  $\Delta$ PE\_PGRS47-infected  
477 DCs showed increased MHC class II antigen presentation compared to the WT strain, and  
478 infection of mice with the  $\Delta$ PE\_PGRS47 strain resulted in an increased number of bacterial-  
479 antigen-specific CD4<sup>+</sup> cells compared to WT-infected mice (Saini et al., 2016). Interestingly,  
480 infection with a *M. tuberculosis* mutant lacking PE\_PGRS47 showed increased autophagy  
481 when compared to the WT strain, suggesting that the bacteria use PE\_PGRS47 to inhibit  
482 autophagy in the host cell. Moreover, the  $\Delta$ PE\_PGRS47 strain was significantly attenuated *in*  
483 *vivo* and its defects in intracellular replication *in vitro* were restored to WT levels when  
484 autophagy was inhibited by 3MA treatment (Saini et al., 2016). These results demonstrate that  
485 the *M. tuberculosis* protein PE\_PGRS47 inhibits autophagy in the infected cells resulting in a  
486 reduction of MHC class II antigen presentation that impacts specific T-cell responses to  
487 infection.

#### 488 **Concluding remarks**

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

495 Current data support a model where PRR signalling and autophagy crosstalk at different  
496 levels. In general, PRR activation through the recognition of bacterial ligands promotes  
497 autophagy (Delgado et al., 2009). However, drugs or genetic approaches used to inhibit

498 autophagy in infected cells or animals showed increased secretion of pro-inflammatory  
499 cytokines after stimulation with bacterial ligands or viable bacteria, suggesting that cells  
500 might also use autophagy to limit PRR-initiated immune responses as a negative-feedback  
501 loop during infection. Most of the available literature supports this model (Table 1), with data  
502 for inflammasome signalling and IL-1 $\beta$  secretion being very solid and reproducible through  
503 out the different studies.

504 One of the most compelling results is the role of ATG16L1 in Chron's disease. Defects in  
505 ATG16L1, such as the T300A polymorphism carried by patients, lead to reduced autophagy  
506 in host cells during bacterial infection, reduced bacterial clearance and increased secretion of  
507 cytokines, mainly of IL-1 $\beta$  (Conway et al., 2013; Lapaquette et al., 2012; Lassen et al., 2014).  
508 These studies highlight the existence of intrinsic mechanisms within host cells where  
509 autophagy and cytokine production are coordinated during host-pathogen interactions,  
510 suggesting that malfunction of autophagy might be one cause of inflammatory diseases with  
511 excessive cytokine production such as Chron's disease. Finally, the investigation of the  
512 mechanisms used by bacteria to modulate autophagy identified some bacterial effectors  
513 inhibiting autophagy, thereby promoting bacterial survival during infection. However, only  
514 few studies addressed the question of how these bacterial-derived autophagy inhibitors  
515 directly impact immune responses during infection. Most is known for *M. tuberculosis*, in  
516 which the bacterial proteins Eis, TlyA and PE\_PGRS47 have been shown to inhibit  
517 autophagy and to modulate cytokine production and MHC class II antigen presentation to T  
518 cells, shaping innate and adaptive immunity during *M. tuberculosis* infection.

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

528

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534

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637

638

638 **Figure Legends**

639 **Figure 1. Schematic description of the autophagic pathway.** Autophagy is triggered by  
640 starvation, damaged cellular components or cellular recognition of bacteria. These events  
641 activate the ULK1 complex. Under nutrient-rich conditions, mTORC1 inhibits  
642 autophagosome formation by repressing the ULK1 complex, however participation of  
643 mTORC1 in bacterial-induced autophagy is unclear. Once the ULK1 complex is activated,  
644 autophagosome formation begins with the recruitment of BECN1 and the nucleation of a  
645 small membrane called the phagophore, which starts to engulf the undesired material. The  
646 phagophore expands to form the autophagosome, a double-membrane compartment engulfing  
647 cytoplasmic targets (proteins, organelles or pathogens). This mature autophagosome then fuses  
648 with a lysosome for cargo degradation.

649 **Figure 2. Crosstalk of autophagy and innate immunity.** Activation of PRRs, such as TLRs,  
650 inflammasomes and cGAS, triggers signalling pathways (green components) that lead to  
651 activation of inflammatory transcription factors (orange components) and autophagy proteins  
652 that activate autophagy (brown components). Activation of inflammatory transcription factors  
653 leads to transcription and translation of cytokines such as TNF- $\alpha$ , IL-6 and IFN- $\gamma$ , which are  
654 secreted, but also pro-IL-1 $\beta$  and pro-IL-18, which are then processed by inflammasome-  
655 activated Caspase-1 into secreted IL-1 $\beta$  and IL-18. In turn, autophagy aids TLRs in meeting  
656 their cognate ligands, while it negatively regulates cytokine production by repressing cGAS  
657 synthesis of cGAMP and degrading precursor and mature forms of IL-1 $\beta$  and IL-18, and  
658 inflammasome components (dashed lines)

659

**Table 1. Effects of autophagy on cytokine production upon bacterial infection**

Bacteria	Infection	Host model	Mechanism of autophagy-inhibition	Effect on Autophagy compared to WT mice or non-treated cells	Cytokines	Reference
<i>Mycobacterium tuberculosis</i>	<i>In vitro</i>	PBMCs	3-MA and siRNA knockdown of ATG7	Downregulated ↓	TNF-α ↓ IL-1β ↑ IL-6 ↑	(Kleinnijenhuis et al., 2011)
	<i>In vitro</i>	PBMCs	3-MA, wortmannin	Downregulated ↓	IL-1β ↑ IL-6 ↑ IL-23 ↑ IL-17 ↑ IFN-γ ↑	(Buffen et al., 2016)
<i>Borrelia burgdorferi</i>	<i>In vivo</i>	Patellae of mice	<i>Atg7</i> <sup>-/-</sup> mice		IL-1β ↑ IL-6 ↑	
<i>Pseudomonas aeruginosa</i>	<i>In vitro</i>	BMDMs	<i>Atg7</i> <sup>-/-</sup> mice	IL-1β ↑	IL-1β ↑	(Buffen et al., 2013)
	<i>In vivo</i>	Lung macrophages of infected mice	<i>Anxa2</i> <sup>-/-</sup> mice	Downregulated ↓	TNF-α ↑ IL-6 ↑ IL-1β ↑ IFNγ ↑	(Li et al., 2015)
Adherent Invasive <i>E. coli</i> (AIEC)	<i>In vitro</i>	THP-1 macrophage-like cells	siRNA knockdown of ATG16L1	Downregulated ↓	TNF-α ↑ IL-6 ↑	(Lapaquette et al., 2012)
<i>Salmonella typhimurium</i>	<i>In vivo</i>	Epithelial cells in terminal ileum and cecum of mice	<i>Atg16l1</i> <sup>-/-</sup> mice	Downregulated ↓	IL-6 ↑ IL-1β ↑	(Conway et al., 2013)
	<i>In vitro</i>	Splenic macrophages of mice	<i>Atg16l1</i> T300A knock-in mice	Downregulated ↓	IL-1β ↑	(Lassen et al., 2014)
Bacteria	Infection	Host model	Autophagy-regulation defective mutant	Effect on Autophagy compared to WT-strain	Cytokines	Reference
<i>Legionella pneumophila</i>	<i>In vitro</i>	BMDMs	$\Delta$ <i>legS2</i>	Upregulated ↑	IL-10 ↓ IL-6 ↓ TNFα ↓ IL-1β ↑	(Abu Khweek et al., 2016)
	<i>In vitro</i>	BMDMs	$\Delta$ <i>eis</i>	Upregulated ↑	TNFα ↑ IL-6 ↑	(Shin et al., 2010)
<i>Mycobacterium tuberculosis</i>	<i>In vivo</i>	harvested lungs and spleen of infected mice	$\Delta$ <i>tlyA</i>	Upregulated ↑	IL-12p40 ↑ IL-1β ↓ IL-10 ↓	(Rahman et al., 2015)





