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Manipulation of Autophagy by Bacterial Pathogens Impacts Host Immunity

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3 # Manipulation of autophagy by bacterial pathogens impacts host
4 # immunity
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30

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).

Under starvation or stress, mTORC1 becomes inactive and thus its inhibitory action on the ULK1 complex ceases which in turn now 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) or Atg6 named in yeast, 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

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 nucleid 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, lipotechoic 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-α (TNF-α), Interleukin-6 (IL-6) and IL-1β (Figure 2).

182 Phagocytosed and cytosolic PAMPs are sequestered by autophagosomes and are delivered
183 to the endosomally located and luminally 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-1β 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-1β 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*$^{-/-}$ 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*$^{-/-}$ 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) phosporylation 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-1β
238 into the active form of the cytokine IL-1β, which is secreted (Rodgers et al., 2014). IL-1β

239 secretion relies on two different PRR activating signals: the NF-κB-dependant expression of
240 pro-IL-1β through TLR activation and a signal activating Caspase-1 through inflammasomes
241 to cleave pro-IL-1β (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-1β 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*$^{-/-}$ 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-1β (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-1β and IL-18 production through NF-κB
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-NLCR4 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 ordinately 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. $CD4^+$
292 helper T cells and cytotoxic $CD8^+$ 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 $CD8^+$ T
296 cells while MHC class II loading peptides are produced by lysosomes and presented to $CD4^+$
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 $CD4^+$ 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 $CD4^+$ 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+ 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 $Atg5^{-/-}$ mice, the $CD4^+$ and $CD8^+$ cells failed to undergo proliferation
328 upon T-receptor stimulation. $Atg5^{-/-}$ 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-α production
346 but enhanced IL-1β 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-1β and IL-6 increased while TNF-α 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*$^{-/-}$ mice displayed reduced autophagy
354 levels and a marked increase of cytokines, in particular IL-1β, TNF-α, IL-6 and IFN-γ, 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 conditons (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-α 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-1β 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-1β production in primary cells and *in vivo*. Interestingly, *in vitro* bacterial infections of
374 isolated splenic $CD11b^+$ macrophages from ATG16L1 T300A mice, led also to higher levels
375 of IL-1β. Collectively, this study shows that defective autophagy caused by a disease-
376 associated polymorphism of an autophagy gene leads to increased IL-1β production. However,
377 the mechanism linking ATG16L1 to IL-1β secretion was not elucidated (Lassen et al., 2014).

378 Furthermore, a recent report showed that injection of *B. burgdorferi* into the knees of
379 *Atg*7 $^{-/-}$ mice increased joint swelling and cytokine levels (IL-1β 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-1β 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-1β and IL-23, led
385 to the polarization of $CD4^+$ 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-1β 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-1β 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 *Lp*SPL 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 Δ*eis* mutant strain increased the production of TNF-α 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 Δ*eis* mutant to inhibit autophagy leads to the increased TNF-α 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 Δ*tlyA* strain displayed increased autophagy and showed increased IL-12p40 and reduced IL-
458 1β 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-α, IL-6 and IL-12p40
461 while it boosts the secretion of IL-1β and IL-10 in response to *M. tuberculosis* infection.

462 The bacterial effector *Lp*SPL 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-1β and lower secretion of TNF-α, 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 Δ*PE_PGRS47*-infected
477 DCs showed increased MHC class II antigen presentation compared to the WT strain, and
478 infection of mice with the Δ*PE_PGRS47* strain resulted in an increased number of bacterial-
479 antigen-specific $CD4^+$ 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 Δ*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-1β 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-1β (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-1β and TNF-α, respectively) and also on the production of
523 anti-inflammatory cytokines such as IL-10 or TGF-β. 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 *Lp*SPL, 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 cyoplasmic targets (proteins, organelles or pathogens). This mature autophagosome then fuses
648 with a lysosome for cargo degradation.

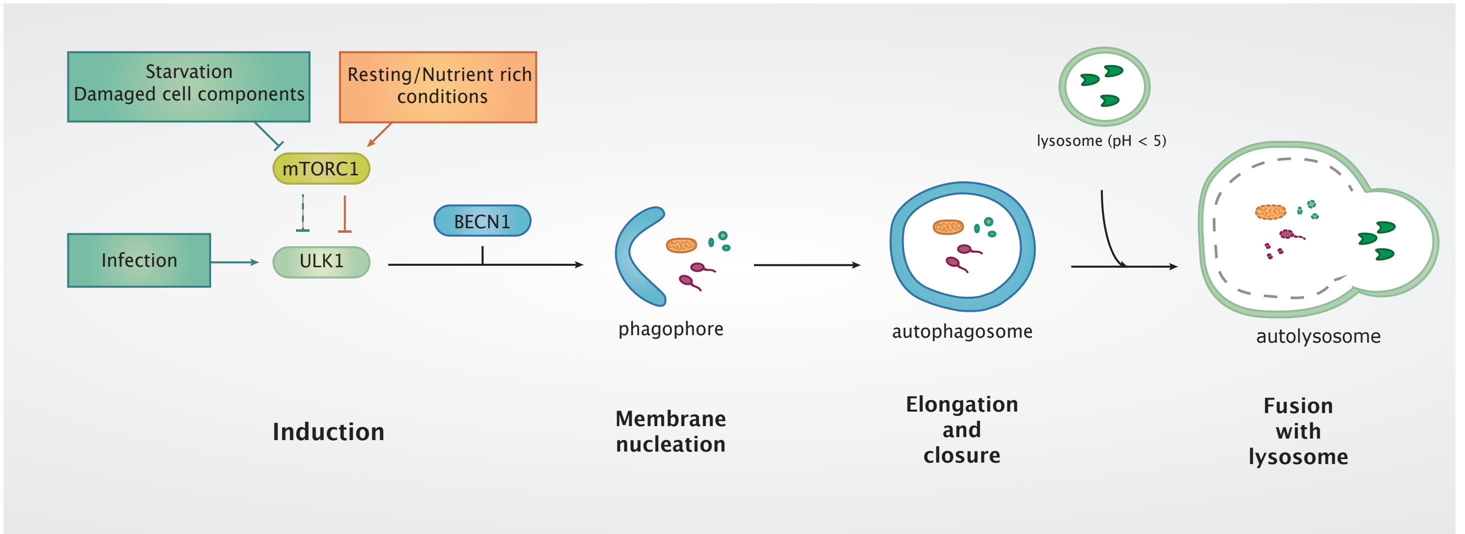
649 **Figure 2. Crosstalk of autophagy and innate immunity.** Activation of PRRs, such as TLRs,
650 inflammasomes and cGAS, triggers signalling pathways (green components) that lead to
651 activation of inflammatory transcription factors (orange components) and autophagy proteins
652 that activate autophagy (brown components). Activation of inflammatory transcription factors
653 leads to transcription and translation of cytokines such as TNF-α, IL-6 and IFN-γ, which are
654 secreted, but also pro-IL-1β and pro-IL-18, which are then processed by inflammasome-
655 activated Caspase-1 into secreted IL-1β 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-1β and IL-18, and
658 inflammasome components (dashed lines)

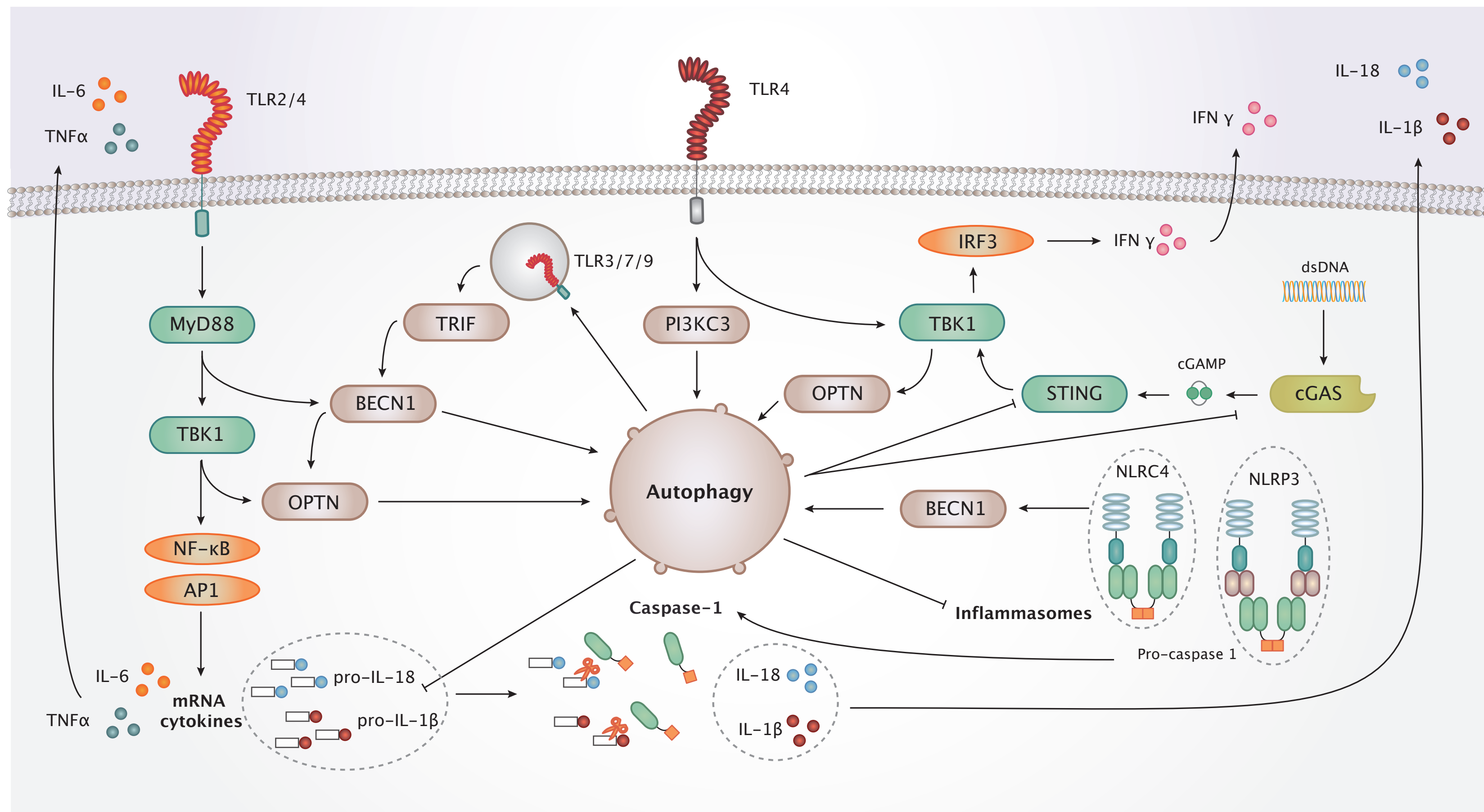
659

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
Mycobacterium tuberculosis	*In vitro*	PBMCs	3-MA and siRNA knockdown of ATG7	Downregulated ↓	TNF-α ↓ IL-1β ↑ IL-6 ↑	(Kleinnijenhuis et al., 2011)
Borrelia burgdorferi	*In vitro*	PBMCs	3-MA, wortmannin	Downregulated ↓	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	Downregulated ↓	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	Downregulated↓	TNF-α ↑ IL-6 ↑	(Lapaquette et al., 2012)
Salmonella typhimurium	*In vivo*	Epithelial cells in terminal ileum and cecum of mice	*Atg16l1*$^{-/-}$ mice	Downregulated ↓	IL-6 ↑ IL-1β ↑	(Conway et al., 2013)
Shigella flexneri	*In vitro*	Splenic macrophages of mice	Atg16L1 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**
Legionella pneumophila	*In vitro*	BMDMs	Δ*legS2*	Upregulated ↑	IL-10 ↓ IL-6 ↓ TNFα ↓ Il-1β ↑	(Abu Khweek et al., 2016)
Mycobacterium tuberculosis	*In vitro*	BMDMs	Δ*eis*	Upregulated ↑	TNFα ↑ IL-6 ↑	(Shin et al., 2010)
	In vivo	harvested lungs and spleen of infected mice	Δ*tlyA*	Upregulated ↑	IL-12p40 ↑ IL-1β ↓ IL-10 ↓	(Rahman et al., 2015)

660


Starvation
Damaged cell components
Resting/Nutrient rich conditions
mTORC1
Infection
ULK1
BECN1
phagophore
autophagosome
lysosome (pH < 5)
autolysosome
Induction
Membrane nucleation
Elongation and closure
Fusion with lysosome


IL-6
TNFα
TLR2/4
TLR4
IL-18
IFN γ
IL-1β
TLR3/7/9
IRF3
IFN γ
dsDNA
MyD88
TRIF
PI3KC3
TBK1
BECN1
OPTN
STING
cGAMP
cGAS
TBK1
NLRC4
NLRP3
OPTN
Autophagy
BECN1
NF-κB
AP1
Caspase-1
Inflammasomes
Pro-caspase 1
IL-6
pro-IL-18
IL-18
mRNA
cytokines
TNFα
pro-IL-1β
IL-1β