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## ***Legionella* and mitochondria, an intriguing relationship**

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27 **ABSTRACT**

28 *Legionella pneumophila* is the causative agent of Legionnaires' disease, a severe pneumonia.  
29 *L. pneumophila* injects via a type-IV-secretion-system (T4SS) more than 300 bacterial  
30 proteins into macrophages, its main host cell in humans. Certain of these bacterial effectors  
31 target organelles in the infected cell and hijack multiple processes to facilitate all steps of the  
32 intracellular life cycle of this pathogen. In this review, discuss the interplay between  
33 *L. pneumophila*, an intracellular bacterium fully armed with virulence tools, and  
34 mitochondria, the extraordinary eukaryotic organelles playing prominent roles in cellular  
35 bioenergetics, cell-autonomous immunity and cell death. We present and discuss key findings  
36 concerning the multiple interactions of *L. pneumophila* with mitochondria during infection  
37 and the mechanisms employed by T4SS effectors that target mitochondrial functions to  
38 subvert infected cells.

39

## 39 MAIN TEXT

### 40 1. Introduction

41 Mitochondria are emblematic structures in cell biology and have traditionally been  
42 considered the powerhouse of the cell, as they play an essential role in cellular bioenergetics.  
43 In this double-membrane organelle, nutrients are oxidized in a process termed oxidative  
44 phosphorylation (OXPHOS), which involves the creation and exploitation of a membrane  
45 potential across the inner mitochondrial membrane, resulting in ATP synthesis (Formosa and  
46 Ryan, 2018). However, mitochondria are currently recognized as multitask organelles in  
47 eukaryotic cell biology as they take part in the regulation of crucial cellular processes.  
48 Mitochondria carry out essential steps of heme, iron-sulfur cluster, and amino acid  
49 biosynthesis as well as fatty acid oxidation (Lill and Freibert, 2020; Schulz, 2013; Spinelli  
50 and Haigis, 2018). They also play an important role in  $\text{Ca}^{2+}$  homeostasis and, as a central  
51 coordination element for  $\text{Ca}^{2+}$ -dependent pathways, mitochondria play a key role in the  
52 regulation of programmed cell death. For these actions, contact sites of mitochondria with the  
53  $\text{Ca}^{2+}$ -storing endoplasmic reticulum (ER) are essential (Giorgi et al., 2012). Furthermore,  
54 mitochondria can release pro-apoptotic proteins such as cytochrome C into the cytosol,  
55 triggering the activation of the intrinsic apoptotic pathway (Obeng, 2021a). Mitochondria are  
56 also engaged in the initiation of immune signaling and take part in innate immune response  
57 by producing mitochondrial reactive oxygen species (mROS), which are secondary  
58 messengers involved in the activation of inflammasomes (Herb and Schramm, 2021; West et  
59 al., 2011b). Like the ER, mitochondria have a stress response mechanism to deal with the  
60 accumulation of unfolded proteins in the matrix (Melber and Haynes, 2018). Along with  
61 these features, the highly dynamic morphology of mitochondria is another characteristic of  
62 this organelle. Mitochondria are able to undergo fission and fusion events depending on the  
63 conditions in the cell. While mitochondrial fission is necessary before cellular division,  
64 fusion events take place when there is a low nutritional supply as it might contribute to  
65 maintain energy levels (Wai and Langer, 2016). Considering their central role in multiple  
66 cellular processes, mitochondria are an attractive target for intracellular pathogens. Multiple  
67 studies have reported how viruses and intracellular bacteria have evolved ways of targeting  
68 mitochondria to influence their intracellular survival, to improve the access to nutrients, to  
69 delay host apoptosis or to evade host immunity (Marchi et al., 2022; Sorouri et al., 2022).  
70 Here we review the different mechanisms that *Legionella pneumophila*, the intracellular

71 bacteria that causes Legionnaires' disease in humans, employs to manipulate mitochondrial  
72 functions during infection of host cells.

73 *Legionella pneumophila* was recognized for the first time during an outbreak of pneumonia in  
74 the summer of 1976 during the 58th annual convention of the American Legion in  
75 Pennsylvania, United States. An unconventional respiratory disease affected 221 participants  
76 and 34 fatal cases were reported (Fraser et al., 1977). In December 1976, Joseph E. McDade  
77 and Charles C. Shepard identified and characterized a rod-shaped gram-negative bacterium as  
78 causative agent of the disease. It was named *Legionella pneumophila* after the American  
79 Legion and the new genus was named *Legionella*, which at that time had only one known  
80 species (Brenner et al., 1979; Fraser et al., 1977; McDade et al., 1977). Today, the genus  
81 *Legionella* comprises at least 65 species but not all of them are equally responsible for  
82 laboratory-confirmed cases of Legionnaires' disease. *L. pneumophila* serogroup (Sg) 1 is the  
83 most relevant, being responsible of 80–90% of the cases in Europe and the United States (Yu  
84 et al., 2002). *L. longbeachae* accounts for approximately 1% of cases worldwide but is on the  
85 rise in Europe and accounts for 50–60% of cases in Australia and New Zealand (Bacigalupe  
86 et al., 2017). Other species and serogroups, such as *L. pneumophila* Sg3 and Sg6 are also  
87 reported to cause disease in some cases as well as species such as, *L. bozemanii* or *L.*  
88 *micdadei*, but they are rare and are isolated mainly from immunocompromised patients  
89 (Beauté, 2017; Currie and Beattie, 2015; Yu et al., 2002). *Legionella* spp. are opportunistic  
90 pathogens for humans as they are naturally present in freshwater environments, as well as in  
91 moist soil and composted material either as free-living biofilm-associated bacteria or  
92 associated with their hosts, primarily aquatic amoebae (Rowbotham, 1980). Human infection  
93 most commonly occurs when susceptible individuals inhale *Legionella*-containing aerosols  
94 generated by contaminated manmade water sources, such as showers, hot tubs, plumbing  
95 networks, and air-conditioning systems (Blatt et al., 1993). Through these routes, *Legionella*  
96 can reach the human lungs, where they can infect alveolar macrophages using the same  
97 mechanisms that they utilize to survive within their amoebal hosts. Indeed, *L. pneumophila*  
98 has the capacity to replicate in numerous phagocytic hosts, ranging from different amoeba  
99 species to mammalian cells (Boamah et al., 2017; Escoll et al., 2013).

100 After uptake by host cells, *L. pneumophila* remodels the phagocytic vesicle into a replicative-  
101 permissive-niche known as the *Legionella*-containing vacuole (LCV). Interestingly, during  
102 the first steps of infection, physical contacts occur between mitochondria and the LCV  
103 (Escoll et al., 2017b; Hoffmann et al., 2014a; Horwitz, 1983). Within this sophisticated

104 intracellular compartment, *L. pneumophila* sequentially evades phagolysosomal degradation,  
105 is protected from intracellular defenses and intercepts nutrients to support growth and  
106 proliferation. *L. pneumophila* employs different secretion systems to deliver virulence-  
107 associated proteins across the LCV membrane that modulate specific host cell functions to  
108 the bacterial advantage. Among them, type 2 and type 4 secretion systems (T2SS and T4SS,  
109 respectively) are present in all *Legionella* species and play essential roles during infection  
110 (Mondino et al., 2020a). The T2SS translocates around 25 effector proteins that are necessary  
111 for environmental persistence and for replication in amoeba, and that play also a role in  
112 replication in macrophages (Cianciotto, 2013, 2009). In contrast, the T4SS translocates more  
113 than 330 effectors which represent about 10% of the bacterial proteome in the host cell and  
114 thereby governs all steps of the intracellular life of *L. pneumophila* in both amoeba and  
115 human macrophages (Ensminger, 2016; Lockwood et al., 2022; Mondino et al., 2020).  
116 Certain of these effectors modulate the cellular metabolism, host cell death and immunity  
117 pathways directly targeting mitochondrial functions (**Table 1**). Despite of their importance  
118 for bacterial virulence, the mechanisms underlying the activity of these effectors on host  
119 mitochondria has not been fully understood yet. Here we present the key findings about the  
120 interactions of *L. pneumophila* with mitochondria during infection, discuss the mechanisms  
121 by which T4SS secreted effectors target mitochondrial functions to subvert infected cells, and  
122 provide a perspective view on the interplay between *Legionella* and mitochondria, the  
123 extraordinary eukaryotic organelles governing cellular bioenergetics, cell-autonomous  
124 immunity and cell death.

## 125 **2. The dynamic contacts between mitochondria and *Legionella*-containing phagosomes**

126 Once *L. pneumophila* is taken up by phagocytic cells, a rapid and extensive communication  
127 between the vacuole and different components of the host membrane system occurs to  
128 convert the phagosome into LCV. This implies a conversion of the plasma membrane of the  
129 phagosome into a smooth ER-like membrane, the interception of vesicles and the escape from  
130 the the endocytic maturation process and the inhibition of the fusion with the lysosome (Ge  
131 and Shao, 2011) These rapid interactions were described as early as 1983 by using electron  
132 microscopy on fixed samples of infected human monocytes and shortly after in  
133 *L. pneumophila*-infected amoeba (Newsome et al., 1985). Indeed, within three minutes after  
134 phagocytosis >95% of vacuoles were surrounded by smooth vesicles apparently fusing with  
135 or budding off from the vacuolar membrane and, 30% of LCVs were also surrounded by  
136 closely associated mitochondria. At 1 hour after infection the proportion of mitochondria

137 associated with the LCV was reported to be 65% (Horwitz, 1983). During recruitment, the  
138 thickness of the LCV membrane, similar to the plasma membrane, was reduced to the  
139 thickness of ER or mitochondrial membranes and the membranes of both organelles were  
140 connected to the LCV by “fine connections or hairs” (Tilney et al., 2001). It was suggested  
141 that this process is T4SS-dependent, as the LCV of *L. pneumophila*  $\Delta dotA$  mutants that do  
142 not have a functional T4SS do not have ER vesicles or mitochondria associated at their  
143 surface and the LCV membrane showed the same thickness as the plasma membrane (Tilney  
144 et al., 2001). These observations generated the understanding that the LCV is rapidly  
145 surrounded by mitochondria, and that these host organelles are recruited by T4SS effectors.  
146 However, new approaches such as time-lapse confocal imaging of living cells of infected  
147 human primary macrophages confirmed that the LCV is indeed rapidly (within 1–6 h post-  
148 infection) surrounded by mitochondria, as reported previously for fixed cells, but it also  
149 revealed that the interactions between mitochondria and the LCV are highly dynamic and  
150 transient. Interestingly, when these mitochondrial contacts were monitored in cells infected  
151 with *L. pneumophila* mutants that lacked a functional T4SS, no difference was observed  
152 compared to cells infected with wild-type bacteria, suggesting that mitochondria are  
153 associated with the LCV independently of T4SS effectors. Mitochondrial-LCV contacts have  
154 been reported for several different amoeba and human cells that can support *L. pneumophila*  
155 infections (Newsome et al., 1985; Abu Kwaik, 1996; Otto et al., 2004; Francione et al., 2009)  
156 except for S2 cells from *Drosophila melanogaster* (Sun et al., 2013). Why such transient  
157 interactions occur during the early hours of infection is still unknown, but as there is no  
158 implication of T4SS effectors this suggests that they might be inherent to phagosome  
159 formation and processing. Indeed, associations of mitochondria and phagosomes were also  
160 observed when macrophages phagocytize lipopolysaccharide (LPS)-coated latex beads (West  
161 et al., 2011a). Moreover, physical interactions of mitochondria with phagosomes have been  
162 observed also for infections with *Chlamydia*, *Shigella* or *Salmonella* (Hall et al., 2013;  
163 Matsumoto et al., 1991; Sirianni et al., 2016). This suggests that these interactions are  
164 infection-dependent and might be a general defence mechanism of the host cell against  
165 pathogens.

166 Although the nature and the biological role of the mitochondria-LCV contacts remain  
167 unclear, the observation of tiny osmiophilic hairs in the contact sites (Tilney et al., 2001) may  
168 suggest a direct physical contact. Generally, inter-organelle microdomains, such as  
169 mitochondria-associated ER membranes (MAMs), play unique regulatory functions and are

170 involved in many biological processes such as lipid synthesis and trafficking, calcium  
171 homeostasis or autophagosome formation (Escoll et al., 2017a; Perrone et al., 2020). Further  
172 experiments are needed to understand whether these contacts play a regulatory role.  
173 Interestingly, we observed that a transfer of MitoTracker dye from mitochondria to the LCV  
174 occurs occasionally during these contacts (Escoll et al., 2017b). As MitoTracker dyes form a  
175 covalent bond with thiols on mitochondrial proteins (Cottet-Rousselle et al., 2011), dye  
176 transfer during contacts might suggest that proteins are transferred from mitochondria to the  
177 LCV. Interestingly, it was reported from proteomic studies of LCVs isolated from  
178 macrophages that, after 1h of infection, about 25-30% of the host proteins identified are  
179 mitochondrial proteins. These are even the most represented proteins of a eukaryotic  
180 organelle within the LCV proteome. A lower but significant proportion of mitochondrial  
181 proteins (19%) was also found in the proteome from LCVs isolated from infected  
182 *D. discoideum* amoeba (Hoffmann et al., 2014b; Naujoks et al., 2016; Schmölders et al.,  
183 2017). To understand whether a functional specificity in the proteins delivered by the  
184 mitochondria exists, we used the dataset published by Hoffmann and colleagues and  
185 performed a functional enrichment analysis for mitochondrial proteins identified in LCVs  
186 isolated from macrophages (Hoffmann et al., 2014a). Our analyses, summarized in **Figure 1**,  
187 show that the proteins identified were not related to a specific mitochondrial function, but we  
188 found an enrichment of nearly all structural proteins as well as of many mitochondrial  
189 functions, such as translation, lipid metabolism, or proteins involved in OXPHOS (**Figure**  
190 **1A**). In line with this, a cell component analysis showed an enrichment of proteins mainly  
191 from the mitochondrial inner membrane and matrix (**Figure 1B**). These analyses might also  
192 suggest that during early steps of infection a cargo-unspecific transfer of proteins occurs  
193 independently of the subcellular localization of the proteins, which might indicate that the  
194 transfer occurs *via* vesicular trafficking. However, we cannot rule out the possibility that  
195 these proteins are from mitochondrial contamination during LCV purification.

196 In mitochondria, vesicular trafficking and protein delivery to other organelles occur via  
197 mitochondria-derived vesicles (MDVs), firstly described as a quality control mechanism to  
198 transport damaged respiratory chain complexes from mitochondria to the endolysosomal  
199 compartment for degradation (Sugiura et al., 2014). However, there is one example of MDV-  
200 mediated delivery of mitochondrial proteins to bacterial phagosomes reported to happen  
201 during *Staphylococcus aureus* infection (Abuaita et al., 2018). Indeed, *S. aureus* infection  
202 activates the host stress response and stimulates the production of MDVs, which deliver the

203 mitochondrial enzyme superoxide dismutase-2 (Sod2) to bacterial phagosomes. Sod2-  
204 dependent production of mitochondrial peroxide ( $mH_2O_2$ ) in the phagosome contributes to  
205 bacterial killing, as Sod2 depletion compromises  $mH_2O_2$  production and pathogen killing in  
206 the phagosome (Abuaita et al., 2018). Whether similar MDV-based mechanisms occur during  
207 *L. pneumophila* infection remains to be elucidated, however it has been reported that  
208 mitochondrial-derived effector molecules, other than proteins, such as mitochondrial-derived  
209 itaconic acid are delivered to the LCV during infection of macrophages (Naujoks et al.,  
210 2016). Furthermore it was shown that Interferon (IFN) stimulation induces Immune-  
211 Responsive Gene (IRG)1 in the mitochondria of *L. pneumophila*-infected macrophages,  
212 which mediates production of itaconate, a potent antimicrobial. While mitochondria were  
213 dynamically contacting the LCV, mitochondrial-IRG1-derived itaconate was delivered to the  
214 LCV and mediated their antimicrobial effects. This example shows that the dynamic contacts  
215 of mitochondria and the LCV have also functional consequences (Naujoks et al., 2016).

216 Taken together,, although mitochondria-LCV contacts continue to be a mystery, their very  
217 dynamic and T4SS-independent nature and a possible, un-specific transfer of proteins might  
218 reflect the normally occurring inter-organelar interactions between mitochondria and  
219 phagosomes within eukaryotic cells.

### 220 **3. Regulation of mitochondrial functions by *L. pneumophila* with metabolic** 221 **consequences**

222 Intracellular bacterial pathogens such as *L. pneumophila*, can obtain nutrients and energy  
223 needed to support their replication can only from the infected host cell. Due to this metabolic  
224 and bioenergetic dependence intracellular pathogens have evolved different strategies to  
225 reprogram host cell metabolism to their advantage. The specific mechanisms and processes  
226 underlying metabolic changes of host cells upon bacterial infection are only partly  
227 understood. However, it has been shown that mammalian cells infected by different  
228 intracellular bacteria have an altered metabolism that mimics the Warburg effect or aerobic  
229 glycolysis as it is in cancer cells (Eisenreich et al., 2013; Escoll and Buchrieser, 2018). The  
230 Warburg metabolism is an anabolic program that is essential for cell growth and proliferation  
231 and favours glucose intake to fuel glycolysis coupled to fermentation but diminishes ATP  
232 production by mitochondrial OXPHOS (Vander Heiden et al., 2009). As glycolysis also fuels  
233 several biosynthetic pathways, this metabolic switch from an OXPHOS-based metabolism to  
234 aerobic glycolysis might be a faster way to generate biosynthetic intermediates required for  
235 bacterial growth (Eisenreich et al., 2013; Escoll and Buchrieser, 2018). Moreover, during the

236 Warburg effect, mitochondrial activity is repurposed from the bioenergetic role of OXPHOS-  
237 mediated ATP generation to a biosynthetic role where mitochondria are used for amino acid  
238 and fatty acid synthesis. When bioenergetics parameters were measured during  
239 *L. pneumophila* infection, a biphasic alteration of the metabolism of infected macrophages  
240 occurred at very early times (Escoll et al., 2017b). This altered metabolism consisted in a  
241 preliminary phase of increased glycolysis and OXPHOS that peaks at 1 h post infection  
242 followed by a second phase where glycolysis remains high while OXPHOS and  
243 mitochondrial ATP production are dramatically reduced (**Figure 2**). This second phase  
244 mimics a Warburg-type metabolism and lasts at least 5 h after infection, before bacterial  
245 replication takes place and activation of macrophage cell death pathways are triggered (Escoll  
246 et al., 2017b). The analyses of mutants deficient in the T4SS that cannot inject bacterial  
247 effectors and, therefore, cannot replicate within infected cells allowed to elucidate a role of  
248 secreted effectors in this metabolic rewiring. The first phase of increased glycolysis and  
249 OXPHOS occurs in the absence of a functional T4SS (T4SS-independent), whereas the  
250 Warburg-like phase is T4SS-dependent, since OXPHOS is not reduced in T4SS-deficient  
251 mutants. It was reported that several Toll-like receptors (TLR), one of the most studied  
252 families of pathogen recognition receptors that activate innate immunity, are involved in  
253 sensing *L. pneumophila* LPS (Akamine et al., 2005; Grigoryeva and Cianciotto, 2021;  
254 Lachmandas et al., 2016). Specifically, *L. pneumophila* activates TLR2, that leads to  
255 increased glycolysis and OXPHOS in human primary cells (Lachmandas et al., 2016), and  
256 TLR4, that is vital for the macrophage response in the early stages  
257 of *L. pneumophila* infection. Importantly, TLR4 plays a role in the metabolic reprogramming  
258 of macrophages upon LPS activation (Murugina et al., 2020). Taking this in consideration, it  
259 is conceivable that the induction of the first phase (increased glycolysis and increased  
260 OXPHOS) that is T4SS-independent but TLR-dependent is related to a metabolic switch  
261 induced by the infected macrophage in response to *L. pneumophila* LPS. However, the  
262 reduction of OXPHOS observed in the second phase, during the Warburg-like program, is  
263 intimately linked to T4SS effectors, revealing a participation of secreted effector proteins in  
264 the impairment of mitochondrial activity. As *L. pneumophila* T4SS-deficient mutants also  
265 have LPS, and the second phase is settled before bacterial replication within the macrophage  
266 begins, LPS is not responsible for the differences observed between wt and mutant strains in  
267 the second phase. Interestingly, the accumulation of MDV-Sod2-derived mH<sub>2</sub>O<sub>2</sub> discussed  
268 above, requires TLR signalling (Abuaita et al., 2018). Taken together these results support a  
269 model where T4SS-independent increase of OXPHOS at early times of *L. pneumophila*

270 infection (Escoll et al., 2017b), the production of MDVs carrying mROS-generating Sod2  
271 (Abuaita et al., 2018), and the mitochondria-LCV dynamic contacts, might all be governed by  
272 TLR signalling and might be functionally linked to enhance the antimicrobial response in the  
273 infected macrophage.

274 *L. pneumophila* delivers more than 330 effector proteins into its host cells (Ensminger, 2016;  
275 Lockwood et al., 2022; Mondino et al., 2020) and several of them target mitochondrial  
276 dynamics (Abu Khweek et al., 2016; Escoll et al., 2017b). The host mitochondrial network, a  
277 key player in the maintenance of bioenergetics and physiological homeostasis within the cell,  
278 is incessantly dynamic as constant fusion and fission of mitochondria takes place, tightly  
279 governed by a plethora of cytoplasmic and mitochondrial proteins (Tilokani et al., 2018).  
280 While the movement of mitochondria appear to be random, recent studies showed that the  
281 fine-tuned nature of mitochondrial motility and architecture affects and is affected by cellular  
282 bioenergetics, metabolism, survival, stress or disease conditions (Chan, 2020; Wai and  
283 Langer, 2016). An example is mitochondrial fission that leads to changes in mitochondrial  
284 morphology from elongated networks to single rod-shaped mitochondria. It has been shown  
285 that chronic fragmentation of mitochondria leads to a loss of respiratory efficiency, to a loss  
286 of mitochondrial ATP production, and to a metabolic shift of the host cell towards other  
287 sources of energy, such as glycolysis (Escoll et al., 2017b; Yu et al., 2015). This might  
288 explain why triggering mitochondrial fission is common during infection by bacterial  
289 pathogens, as it can effectively reduce OXPHOS and thus induce a Warburg-like metabolism  
290 (Jain et al., 2011; Lum and Morona, 2014). For *L. pneumophila*, two effectors involved in  
291 mitochondrial fission have been reported, MitF and *LpSpl* (**Figure 2**) (Abu Khweek et al.,  
292 2016a, p. 2; Escoll et al., 2017b). MitF or LegG1 (Lpg1976/Lpp1959), is a bacterial activator  
293 of host Ran GTPases that modulates cytoskeleton dynamics and cell migration of host cells  
294 during infection (Rothmeier et al., 2013; Simon et al., 2014). We recently discovered that this  
295 effector is also involved in the activation of mitochondrial fission, as the infection of human  
296 monocyte-derived macrophages (hMDMs) with an  $\Delta mitF$  mutant strain was impaired in the  
297 ability to fragment host mitochondria (Escoll et al., 2017b). Mitochondrial dynamics are  
298 known to be governed by cellular GTPases of the dynamin family (Labbe et al., 2014) and,  
299 among them, the dynamin 1-like protein DNML1 (also known as DRP1) is a critical player in  
300 the process of mitochondrial fission (Chan, 2012). During early infection with  
301 *L. pneumophila* DNML1 aggregates in host mitochondria and promotes mitochondrial  
302 fragmentation in a MitF (and T4SS) dependent manner. This step is key to impair OXPHOS

303 and ATP production in infected human macrophages (**Figure 2**). Homologs of MitF are  
304 present in several *L. pneumophila* strains and other *Legionella* species, suggesting that also  
305 other *L. pneumophila* strains and *Legionella* species might induce mitochondrial  
306 fragmentation by similar mechanisms (Escoll et al., 2017b; Swart et al., 2020). Interestingly,  
307 MitF actions on DNM1L-dependent mitochondrial fission seem to involve cytoskeletal  
308 rearrangements. Indeed, MitF activates Ran and RanBP2 (Rothmeier et al., 2013), and Ran  
309 and RanBP2 are involved in *L. pneumophila* WT-induced mitochondrial fragmentation  
310 (Escoll et al., 2017b). But Ran and RanBP2 have also been shown to activate actin  
311 polymerization and to bind directly to Arp2/3 complex and WASP (Sadhukhan et al., 2014;  
312 Yi et al., 2011). Arp2/3 and WASP are reported to interact with DNM1L in *L. pneumophila*  
313 WT-infected macrophages (Escoll et al., 2017b). As focal assembly of actin through Arp2/3  
314 induces DNM1L-dependent fragmentation (Moore et al., 2016), it is possible that MitF  
315 activation of Ran/RanBP2 induces Arp2/3-WASP-mediated actin polymerization and  
316 DNM1L-dependent fragmentation (Escoll et al., 2017b).

317 *LpSpl* (LegS2; Lpp2128 or Lpg2176), a T4SS effector that codes for an eukaryotic  
318 sphingosine 1-P (S1P) lyase and that is involved in the inhibition of starvation-induced  
319 autophagy in infected cells (Rolando et al., 2016), seems also to alter mitochondrial dynamics  
320 (Abu Khweek et al., 2016b). In eukaryotes, the sphingosine 1-phosphate lyase (SPL) enzyme  
321 is responsible for the irreversible degradation of S1P to phosphoethanolamine and  
322 hexadecanal (van Veldhoven and Mannaerts, 1993). S1P is a metabolite involved in the  
323 regulation of many different cell processes like migration, angiogenesis or development.  
324 Therefore, *LpSpl* activity might induce pleiotropic effects in the host cell. Contrary to  
325 eukaryotic SPL that localizes mainly at the ER (Ikeda et al., 2004), *LpSpl* forms patches in  
326 the mitochondrial outer membrane during infection (Degtyar et al., 2009) which might limit  
327 its enzymatic activity to this organelle. Indeed, *LpSpl* shows diverse effects in the  
328 mitochondria and, like MitF, seems involved in the regulation of mitochondrial dynamics, as  
329 infected-BMDM with a mutant of *L. pneumophila*  $\Delta legS2$  (the homologue of *LpSpl* in strain  
330 *L. pneumophila* Philadelphia) lose the ability to fragment the mitochondrial network (Abu  
331 Khweek et al., 2016). The mechanism by which *LpSpl* induces mitochondrial fission and  
332 what are the consequences for mitochondrial bioenergetics or *L. pneumophila* pathogenesis  
333 remains unknown. It was suggested that the atypical morphology of the mitochondria  
334 observed in  $\Delta legS2$ -infected cells was the consequence of the reduced host immune response  
335 provoked by lower NF- $\kappa$ B activation (Abu Khweek et al., 2016). Further studies are needed

336 to understand the role of *LpSpl/LegS2* in the regulation of mitochondrial morphology and  
337 whether this effector cooperates with MitF to induce mitochondrial fragmentation and  
338 OXPHOS reduction and ATP production.

339 One mechanism by which *LpSpl* participates in the modulation of mitochondrial functions  
340 has been reported recently (Escoll et al., 2021) (**Figure 2**). T4SS-dependent fragmentation of  
341 the mitochondrial network triggers a reduction of mitochondrial respiration in  
342 *L. pneumophila*-infected macrophages, which suggests a functional connection between  
343 mitochondrial dynamics and mitochondrial respiration (Escoll et al., 2017b). The molecular  
344 factors determining that fragmented mitochondria are bioenergetically less efficient are in the  
345 internal membrane of the mitochondria (IMM), as mitochondrial cristae represent the IMM  
346 hub where most of the respiratory complexes are embedded and are forming supercomplexes  
347 (Cogliati et al., 2016). Mitochondrial fission induces a remodelling of the mitochondrial  
348 morphology that involves changes in the number or shape of the cristae and directly impacts  
349 the stability and assembly of respiratory complexes and supercomplexes which, consequently  
350 also impacts mitochondrial respiration and OXPHOS (Cogliati et al., 2013; Giacomello et al.,  
351 2020). The electron transport chain (ETC) supercomplexes are composed of five complexes  
352 (CI to CV) whose activity is coupled in the ETC allowing mitochondrial respiration. In this  
353 process, the reduced coenzymes NADH and FADH<sub>2</sub> are oxidized at complexes I and II  
354 where their electrons are extracted to energize the mitochondrial ETC (Nolfi-Donagan et al.,  
355 2020). The sequential transit of these electrons through complexes I, III, and IV (or II, III and  
356 IV) allows to pump protons from the matrix to the intermembrane space (IMS) and at  
357 complex IV, diatomic oxygen O<sub>2</sub> serves as the terminal electron acceptor, and H<sub>2</sub>O is formed  
358 (Nolfi-Donagan et al., 2020). The increased concentration of protons at the intermembrane  
359 space, compared to the matrix, generates a mitochondrial membrane potential ( $\Delta\psi_m$ ). This  
360  $\Delta\psi_m$  allows the utilization of the proton-motive force by the complex V, the F<sub>O</sub>-F<sub>1</sub>-ATPase,  
361 to perform OXPHOS and to generate ATP (Nolfi-Donagan et al., 2020). Therefore,  
362 mitochondrial respiration, OXPHOS and the generation and maintenance of the  $\Delta\psi_m$  are  
363 directly linked. This implies that bacterial-induced OXPHOS reduction may thus trigger  
364 profound consequences for host cells due to the collapse of the  $\Delta\psi_m$  and subsequent death of  
365 infected cells. How *L. pneumophila* and other intracellular bacteria reduce OXPHOS and deal  
366 with the subsequent  $\Delta\psi_m$  drop has recently been started to be understood. *L. pneumophila*-  
367 infected hMDMs showed that despite reduced OXPHOS, the  $\Delta\psi_m$  was maintained during  
368 infection (Escoll et al., 2021) (**Figure 2**). One possibility how this could be achieved lies in

369 the two modes of action of the F<sub>0</sub>F<sub>1</sub>-ATPase. Indeed, the F<sub>0</sub>F<sub>1</sub>-ATPase can rotate clockwise,  
370 it works in the ‘forward mode’ and is synthesizing ATP by using the  $\Delta\psi_m$  generated by the  
371 H<sup>+</sup> circuit but it can also rotate counterclockwise, it works in the ‘reverse mode’ (Campanella  
372 et al., 2009, p. 1) and hydrolyzes ATP to maintain the  $\Delta\psi_m$  in the absence of OXPHOS. It  
373 has been shown that *L. pneumophila* maintains  $\Delta\psi_m$  during infection, in the absence of  
374 OXPHOS, through the induction of the ‘reverse mode’ of the mitochondrial F<sub>0</sub>-F<sub>1</sub>-ATPase  
375 (Escoll et al., 2021). Inhibition of the *L. pneumophila*-induced ‘reverse mode’ of the F<sub>0</sub>-F<sub>1</sub>-  
376 ATPase collapsed the  $\Delta\psi_m$  and caused cell death in infected cells, suggesting that  
377 *L. pneumophila* uses this mechanism to deal with  $\Delta\psi_m$  drops in conditions of highly reduced  
378 OXPHOS, as it is the case at early times post infection (second phase). Induction of the  
379 reverse mode by *L. pneumophila* is T4SS-dependent and involves the above mentioned  
380 effector *LpSpl* (**Figure 2**) (Escoll et al., 2021). Importantly, bacterial replication occurs  
381 preferentially in hMDMs that conserved the  $\Delta\psi_m$  and showed delayed cell death, indicating  
382 that the modulation of the mode of action of the mitochondrial F<sub>0</sub>F<sub>1</sub>-ATPase is a virulence  
383 mechanism that preserves the integrity of infected host cells and thereby maintains replication  
384 niche of *L. pneumophila*. To date, *LpSpl* is the only known bacterial effector that targets the  
385 functions of a component of the ETC, however, it was shown that infection of macrophages  
386 with extracellular *Escherichia coli* produces an alteration of the composition and activity of  
387 ETC complexes and supercomplexes (Garaude et al., 2016; Ramírez-Camacho et al., 2019).  
388 Thus, it is tempting to assume that the configuration and functioning of other components of  
389 the mitochondrial ETC might be altered during infection of macrophages by *L. pneumophila*.

390 The transport of ADP and ATP across the mitochondrial inner membrane is also an essential  
391 activity to maintain OXPHOS, as it provides the substrate for ATP production and allows its  
392 delivery to the cell (Atlante and Valenti, 2021; Klingenberg, 2008). The members of the  
393 adenine nucleotide translocase (ANT) family exchange ATP/ADP across the mitochondrial  
394 inner membrane and, in line with their vital role as gatekeepers of the energy flux, they are  
395 one of the most abundant proteins in the IMM (Klingenberg, 2008). In mammals, four ANT  
396 isoforms with redundant functions that physically interact and cooperate with the OXPHOS  
397 machinery to enhance mitochondrial respiration, as the overexpression of any of the ANT  
398 isoforms increases basal respiration and ATP production, are present (Lu et al., 2017). A  
399 recent study showed that the *L. pneumophila* effector Ceg3 (Lpg0080) is secreted during  
400 infection and specifically blocks ANT-mediated ADP/ATP exchange in host mitochondria  
401 (Fu et al., 2022; Kubori et al., 2022) (**Figure 2**). This effector shows mono-ADP-

402 ribosyltransferase activity and is specifically targeted to mitochondria, where ADP-  
403 ribosylates ANTs on an arginine residue in a conserved motif that is essential for ADP/ATP  
404 transport. Thus, ANTs are ADP-ribosylated in human cells infected with wild-type  
405 *L. pneumophila* but not in cells infected with strains expressing a catalytically inactive form  
406 of Ceg3 or lacking a functional T4SS. This modification specifically blocks transport without  
407 affecting other ANT-related functions, such as the induction of mitophagy (Fu et al., 2022;  
408 Hoshino et al., 2019; Kubori et al., 2022). Interestingly, Lpg0081 is an effector with ADPr  
409 hydrolase activity that reverses the actions of Ceg3 over ANTs (Kubori et al., 2022).  
410 Moreover, over-expression of Ceg3 reduces OXPHOS as a result of the reduction in ADP-  
411 ATP exchange a reduction that was abrogated when Lpg0081 was also overexpressed  
412 (Kubori et al., 2022). It is possible that Ceg3 modulation of OXPHOS is coordinated with  
413 MitF-induced mitochondrial fragmentation and thereby represents a mechanism forcing  
414 infected cells to reduce OXPHOS and to rely on glycolysis to generate ATP and fuel  
415 biosynthetic pathways. Interestingly, besides blocking host ANTs, *L. pneumophila* secretes  
416 LncP (Lpp2981), an effector that is targeted to mitochondria and mimics the ANT family of  
417 transporters. Transport studies using purified LncP reconstituted into liposomes revealed that  
418 LncP mainly catalyzes the exchange of ATP (and GTP) across membranes and is carries out  
419 unidirectional (uniport) transport of ATP (Dolezal et al., 2012). This suggests that  
420 *L. pneumophila* employs its own transporter to transport ATP across the IMM, an activity  
421 that might be coordinated with Ceg3 and Lpg0081. Taken together, during infection,  
422 *L. pneumophila* uses Ceg3 to temporary inhibit the endogenous ANT carrier but substitutes  
423 this function with a bacterial-encoded “ANT-like” carrier, LncP, representing perhaps a fine-  
424 tuning strategy to adapt mitochondrial ATP efflux to the new metabolic conditions occurring  
425 during infection (**Figure 2**).

#### 426 **4. Regulation of mitochondria during *L. pneumophila* infection allows to bypass cell-** 427 **autonomous immunity and to control host cell death**

428 In addition to their role in cellular metabolism, mitochondria are centrally positioned hubs in  
429 the innate immune system and cell death pathways by functioning as signalling platforms of  
430 cell-autonomous defence systems (Tiku et al., 2020; West et al., 2011b). Macrophages are at  
431 the forefront to fight bacterial infection and play a unique role in phagocytosis and pathogen  
432 clearance but they also deploy several cell-autonomous defences to protect themselves from  
433 infection, such as the generation of reactive oxygen species, nitric oxide, anti-microbial  
434 peptides or the establishment of a detrimental nutritive environment (Weiss and Schaible,

435 2015). Interestingly, mitochondria are key structures for triggering and processing many of  
436 these cell-autonomous immune mechanisms.

437 One cell-autonomous defence mechanism employed by macrophages during bacterial  
438 infection is to undergo pyroptosis, a highly macrophage-specific pro-inflammatory cell death  
439 pathway (Casson and Shin, 2013). In contrast to apoptosis, which is a non-inflammatory type  
440 of cell death, when macrophages trigger pyroptotic cell death they generate a potent immune  
441 response while effectively destroying the replication niche of bacterial pathogens, such as  
442 *L. pneumophila*. Pyroptosis is triggered by different signals, some of them are from  
443 mitochondria, which are sensed by the cytoplasmic sensors known as inflammasomes, and  
444 involve the rapid release of cytosolic content, including bacteria, and pro-inflammatory  
445 cytokines, such as IL-1 $\beta$  and IL-18, thus alerting the immune system of an imminent danger  
446 (Bertheloot et al., 2021; Byrne et al., 2013). However, when the bacterial load is low,  
447 macrophages can also mount a rapid autophagic response against bacterial invasion known as  
448 xenophagy, which is also coordinated by the inflammasomes kill intracellular bacteria before  
449 macrophages trigger pyroptosis (Byrne et al., 2013; Wang and Li, 2020). *L. pneumophila*-  
450 infected cells might also trigger classical mitochondrial apoptosis, which is driven by a loss  
451 of the  $\Delta\psi_m$  culminating in this non-inflammatory form of cell death (Speir et al., 2014a).  
452 Thus, intracellular pathogens such as *L. pneumophila* have evolved several mechanisms to  
453 evade cell-autonomous immunity of the infected cells, such as impairing antimicrobial  
454 activity or to efficiently control the different types of cell death, first by likely delaying cell  
455 death to allow bacterial replication and then eventually by facilitating it to favour their  
456 dissemination. We will thus discuss below how *L. pneumophila* subverts mitochondrial  
457 functions linked to cell-autonomous immunity in infected cells, such as the activation of  
458 inflammasomes, the triggering of pyroptosis, the regulation of autophagy and the effective  
459 control of cell death.

#### 460 **4.1 Reactive oxygen species (ROS), mitochondrial ROS (mROS) and inflammasome-** 461 **mediated pyroptosis**

462 Innate immune detection of intracellular bacteria mobilizes a myriad of toxic anti-microbial  
463 effectors to combat the pathogen and destroy it within the phagosomes. One of these defenses  
464 is the induction of large amounts of reactive oxygen species (ROS). Although ROS are  
465 primarily produced by the NADPH oxidase system in phagocytes, mitochondrial oxidative  
466 metabolism is also a source of cellular ROS. The production of mROS has been considered a  
467 deleterious consequence of the malfunctioning of the mitochondrial ETC. Now it is thought

468 that mROS play an important role as a secondary messenger in the cell and regulate several  
469 defence mechanisms contributing to bacterial clearance (**Figure 3**) (Geng et al., 2015; West  
470 et al., 2011a).

471 The production of mROS in mitochondria depends on several factors, including premature  
472 leakage of electrons from complexes I, II, and III, increased  $\Delta\psi_m$  or the energetic status of the  
473 mitochondria (Antonucci et al., 2021; Escoll et al., 2019). Upon sensing LPS, complex I is  
474 one of the major sites of production of mROS in macrophages. TLR signaling by LPS  
475 decreases OXPHOS-dependent ATP production, increases oxidation of succinate by  
476 Complex II, decreases the forward transport at Complex I, and increases the  $\Delta\psi_m$ , which all  
477 together trigger the reverse electron transport and mROS production at Complex I (Mills et  
478 al., 2016). In addition, blocking the reverse electron transport with Complex I inhibitors  
479 reduces LPS-induced mROS and IL1 $\beta$  production (Kelly et al., 2015; Mills et al., 2016),  
480 demonstrating that Complex I functions are necessary to regulate inflammation. The  
481 mechanisms underlying TLR-induced production of mROS have been started to be  
482 uncovered. TLR1, TLR2 and/or TLR4 activation leads to the mitochondrial recruitment to  
483 phagosomes, mediated by the assembly of a complex of the ubiquitin ligase TRAF6 and the  
484 mitochondrial complex I-assembly factor ECSIT, which results in the increase of both,  
485 cellular ROS and mROS production (West et al., 2011a). Consistent with a role of ROS in  
486 bactericidal responses, ECSIT- or TRAF6-deficient macrophages exhibit decreased levels of  
487 TLR-induced cellular ROS and are impaired in their ability to kill *Salmonella Typhimurium*  
488 (West et al., 2011a). Additional components upstream are Mst1 and Mst2 kinases, which  
489 participate in activating the GTPase Rac to promote TLR-triggered assembly of the TRAF6-  
490 ECSIT complex (Geng et al., 2015). Reducing macrophage mROS by expressing catalase in  
491 mitochondria results in defective bacterial killing (West et al., 2011a). *L. pneumophila*  
492 infection inhibits cellular ROS and mROS in cultured cells, as well as dramatically  
493 suppresses mROS in the lungs of infected mice (**Figure 3**) (Harada et al., 2007; Kajiwara et  
494 al., 2018). Additionally, metformin, a drug that inhibits mitochondrial ETC complex I,  
495 induces mROS production and AMPK activation, resulting in protection against  
496 *L. pneumophila* infection both *in vivo* and *in vitro*. Interestingly, the protective role of  
497 metformin was partially mediated by mROS and was independent of AMPK related-effects  
498 (Kajiwara et al., 2018), suggesting that mROS have also an anti-bacterial role during  
499 *L. pneumophila* infection and the bacterium has mechanisms to downregulate its production.

500 Although these results clearly pointed to a role of mROS in the anti-bacterial response of  
501 macrophages, to date it is still unknown whether the direct transfer of mROS from  
502 mitochondria to phagosomes kills phagocytized bacteria, or whether mROS generated in the  
503 mitochondria and delivered into the cytosol have direct anti-bacterial effects. As the levels of  
504 cellular ROS produced by the NADPH-oxidase in the cytoplasm of phagocytes are expected  
505 to exceed ETC-derived mROS (West et al., 2011b), it cannot be ruled out that the bactericidal  
506 activity triggered by mROS is related to its role in innate immunity as an activator of  
507 inflammasome signalling (discussed below). Moreover, as discussed above, TLR-dependent  
508 MDV transfer and accumulation of mitochondrial Sod2 into phagosomes produces ROS in  
509 the phagosome and contributes to anti-microbial toxicity, showing an additional mechanisms  
510 by which mitochondrial-derived ROS kills bacteria. However, ETC-derived mROS is not  
511 involved as the mitochondrial ROS-generating enzyme Sod2 is targeted to phagosomes *via*  
512 MDVs (Abuaita et al., 2018), but as ROS are volatile molecules that can cross membranes  
513 (Fisher, 2009), the possibility that mitochondria-phagosome contacts facilitate the direct  
514 transfer of mROS from mitochondria to the phagosome to kill bacteria exists. Thus, a  
515 remaining question is to understand whether the main contribution of mROS to bactericidal  
516 activity is through the generation and diffusion of mROS that directly kill bacteria, through  
517 the activation of the inflammasome, or through the delivery of mitochondrial Sod2 to  
518 phagosomes.

519 As introduced before, ROS and mROS are activators of inflammasomes, which are cytosolic  
520 multiprotein oligomers responsible for the activation of inflammatory responses in  
521 macrophages. Macrophages are equipped with an arsenal of pattern recognition receptors  
522 (PRRs) sensing microbial- or danger-associated molecular patterns (MAMPs/DAMPs) and  
523 elicit rapid innate immunity responses to protect the organism (Amarante-Mendes et al.,  
524 2018). In addition to TLRs that are located at the surface of mammalian cells and recognize  
525 microbial ligands such as LPS, the Nod-like receptors (NLRs) are other important types of  
526 PRRs that detect bacterial molecules in the cytoplasm (Akira et al., 2006). NLRs are  
527 fundamental components of the signaling platforms known as inflammasomes. TLRs and  
528 NLRs are thought to activate distinct signaling pathways in response to *L. pneumophila* and  
529 promote diverse outcomes to defend the cell against infection (Speir et al., 2016). NLRs can  
530 activate inflammasomes after direct recognition of PAMPs/DAMPs or downstream of TLR  
531 signaling pathways (Motta et al., 2015). Upon activation, NLRs recruit apoptosis-associated  
532 speck-like protein containing a CARD (ASC) and/or pro-caspase-1, which activate caspase-1

533 and promotes pyroptosis progress *via* the secretion of mature IL-1 $\beta$ /IL-18 and gasdermin D  
534 activation (**Figure 3**) (Motta et al., 2015). ROS and mROS released during infection serves as  
535 an important NLRP3-inflammasome activating signal, as they promote mitochondrial damage  
536 and liberation of mitochondrial DAMPs, which lead to macrophage pyroptosis (Weinberg et  
537 al., 2015). Thus, the presence of mitochondrial elements in the cytoplasm, such as mROS,  
538 oxidized mitochondrial DNA or the presence of cardiolipin from the mitochondrial inner  
539 membrane, is sensed as DAMPs and induce the inflammatory response via the NLRP3  
540 inflammasome (Weinberg et al., 2015). Therefore, besides its potential role as antimicrobial  
541 toxic effector, mROS play an important role as signaling molecule as they are direct  
542 activators of the NLRP3 inflammasome to trigger pyroptosis (**Figure 3**) (Heid et al., 2013;  
543 Weinberg et al., 2015).

544 *L. pneumophila* and other intracellular bacteria induce pyroptosis in macrophages through the  
545 NLRC4 inflammasome, by the direct recognition of bacterial PAMPs in the cytoplasm, such  
546 as flagellin (Byrne et al., 2013a; Ren et al., 2006). Interestingly, studies in *Pseudomonas*  
547 *aeruginosa* infected BMDMs showed that mitochondrial dysfunction is also essential for  
548 proper NLRC4 inflammasome activation, as inhibiting mROS or degrading intracellular  
549 mitochondrial DNA abrogated NLRC4 inflammasome activation (Jabir et al., 2015). Thus, to  
550 avoid inflammasome-mediated pyroptosis, pathogens control mROS levels by sustaining  
551 optimal mitochondrial activity. Indeed, removal of damaged mitochondria by autophagy of  
552 mitochondria (mitophagy) significantly attenuated NLRC4 inflammasome activation (Jabir et  
553 al., 2015), indicating that *P. aeruginosa* activates mitophagy to selectively target  
554 dysfunctional mitochondria and avoid pyroptosis. Although a similar strategy was also  
555 observed for other intracellular bacteria, such as *L. monocytogenes* (Zhang et al., 2019), no  
556 bacterial effector specifically regulating mitophagy during infection has been identified. As  
557 commented before, *L. pneumophila* infection inhibited mROS and ROS *in vitro*, as well as  
558 dramatically suppressed mROS *in vivo* (Harada et al., 2007; Kajiwara et al., 2018). The  
559 consequences of *L. pneumophila*-induced regulation of mROS remain a mystery, however  
560 these actions over ROS and mROS could reflect a reduced mitochondrial damage during  
561 infection and might thus also contribute to inhibition of inflammasome activation.

562 Certain bacteria, such as *Salmonella enterica* or *E. coli*, influence the levels and activity of  
563 different ETC complexes during infection (Garaude et al., 2016). However, to date there are  
564 no studies on the function of ETC complexes during *L. pneumophila* infection and no effector  
565 directly targeting ETC complexes has been identified. The T4SS effector MitF secreted by

566 *L. pneumophila* induces fragmentation of mitochondrial networks, leading to a decrease in  
567 OXPHOS (Escoll et al., 2017b). As mitochondrial fission induces an alteration of the  
568 structure of mitochondria this should lead also to a reconfiguration of the ETC complexes  
569 during infection. Furthermore, *L. pneumophila* modulation of the mitochondrial dynamics  
570 might lead not only to decreased OXPHOS, but also to altered mROS levels (**Figure 3**). The  
571 activity of Ceg3 and LncP effectors on mitochondrial ATP/ADP fluxes might also impact  
572 mROS production (Dolezal et al., 2012; Fu et al., 2022; Kubori et al., 2022). Moreover,  
573 *L. pneumophila* maintains the  $\Delta\psi_m$  in the infected macrophage cell, in the absence of  
574 OXPHOS, through the induction of the ‘reverse mode’ of the mitochondrial  $F_0F_1$ -ATPase  
575 (Escoll et al., 2021). Thus, *Legionella*-induced low OXPHOS might be sensed by the cell as  
576 adequate mitochondrial functioning as long as mitochondrial integrity and danger signals are  
577 controlled, which would be related to the maintenance of the  $\Delta\psi_m$  (Escoll et al., 2021) or the  
578 inhibition of mROS production (Harada et al., 2007; Kajiwara et al., 2018). Therefore,  
579 modulation of mitochondrial OXPHOS levels, ATPase functioning and mROS production by  
580 *L. pneumophila* might have additional benefits than bioenergetics, as these regulations might  
581 be intimately linked to the regulation of cell-death in infected macrophages (**Figure 3**).

#### 582 **4.2 Modulation of autophagy at mitochondrial-associated ER contacts by** 583 ***L. pneumophila***

584 Autophagy, a fundamental and evolutionary conserved eukaryotic process responsible for  
585 maintaining cellular homeostasis and controlling the quality and quantity of the organelles  
586 and the cytoplasmic biomass is a cell-autonomous immune response partially linked to  
587 mitochondria (Wang and Li, 2020). Double-membrane vesicles called “autophagosomes”  
588 engulf nonfunctional or damaged cellular components and deliver them to lysosomes for its  
589 degradation (Mizushima, 2011). However, the autophagic machinery enables also the  
590 detection and elimination of intracellular pathogens through lysosomal degradation, in a  
591 process known as xenophagy (Wang and Li, 2020). Bacterial engulfment by macrophages  
592 generally drives a pronounced autophagic response that targets the bacterial phagosome  
593 (xenophagy) as well as mitochondria damaged by infection (mitophagy). Mitophagy limits  
594 the production of mROS and mitochondrial DAMPs, thus inhibiting the activation of NLRP3  
595 inflammasomes and pyroptosis (Galluzzi et al., 2017; Marchi et al., 2022). Autophagy is,  
596 therefore, a cell-autonomous immune process fighting bacterial infection. It is essential to  
597 restrict intracellular growth of many bacteria, contributes to antibacterial immunity and also  
598 coordinates the inflammatory immune response of the macrophage. For example, for

599 *L. pneumophila* it has been shown that early decisions in the infected macrophage cell  
600 determine whether autophagy or pyroptosis are triggered (Byrne et al., 2013b).

601 Intracellular pathogens subvert the host autophagy machinery to block xenophagy as it is  
602 allowing host cells to resist infection (Escoll et al., 2016; Zheng et al., 2022). However,  
603 pathogens might also exploit other forms of autophagy, such as mitophagy, to remove  
604 damaged mitochondria, and thus reduce host cell death to maintain their replicative niche  
605 (Jabir et al., 2015; Zhang et al., 2019) . Moreover, autophagy and pyroptosis seem to have  
606 different possible outputs in single bacterial-infected macrophages, as autophagy inhibits  
607 inflammasome activation in macrophages and *vice versa* during *L. pneumophila* infection,  
608 suggesting a model where pyroptosis is triggered if the autophagic capacity of macrophages  
609 to remove bacteria is exceeded (Byrne et al., 2013b).

610 *L. pneumophila* possesses different effectors modulating the autophagic machinery, such as  
611 RavZ, LegA9, *LpSpl*, Lpg1137/Ceg20 or Lpg2411 (Abu Khweek et al., 2016a; Arasaki et al.,  
612 2017; Choy et al., 2012; Rolando et al., 2016), each having a distinct role in modulating  
613 autophagy. Interestingly, two of them *LpSpl* and Lpg1137, hamper autophagosome formation  
614 presumably by targeting the ER-mitochondria communication at MAMs, which are specific  
615 membranous microdomains at the ER-mitochondria interface. MAMs are inter-organellar  
616 sites of coordination, representing key hubs where many cellular processes are regulated  
617 (Perrone et al., 2020). MAMs participate in the coordination of autophagosome formation,  
618 lipid synthesis and trafficking, calcium homeostasis, mitochondrial morphology,  
619 inflammasome activation and apoptosis (Missiroli et al., 2018; Vance, 2014). Therefore,  
620 MAMs are attractive targets for intracellular pathogens and their effectors to shut down  
621 MAM communication which has pleiotropic effects on the host cell (Escoll et al., 2017a;  
622 Missiroli et al., 2018). One example is *LpSpl*, a *L. pneumophila* T4SS secreted effector  
623 detected at ER and mitochondrial membranes (Degtyar et al., 2009; Rolando et al., 2016),  
624 indicating that *LpSpl* is probably localized at MAMs, where it modulates sphingolipid levels  
625 thereby limiting autophagosome formation and counteracting the antibacterial autophagy  
626 response of the infected host cell (Rolando et al., 2016). Additionally, as discussed above,  
627 *LpSpl* is involved in the regulation of mitochondrial dynamics (Abu Khweek et al., 2016a),  
628 consistent with the fact that mitochondrial fission also takes place at MAMs (Friedman et al.,  
629 2011). Another *L. pneumophila* effector, Lpg1137 (Ceg20), accumulates at MAMs to  
630 promote the degradation of the scaffold protein syntaxin 17 (STX17) and disrupts ER–  
631 mitochondria communication, ultimately inhibiting autophagy to support pathogen survival

632 (Arasaki et al., 2017). These results suggests that although *L. pneumophila* possesses  
633 different effectors participating in the modulation of the fine balance of autophagy levels,  
634 hijacking MAM communications might be a key target of *L. pneumophila*, allowing it  
635 subvert the intricate autophagy process of the host cell to survive and preserve its niche to  
636 proliferate.

### 637 **4.3 Modulation of the mitochondrial-apoptotic pathway during *L. pneumophila*** 638 **infection**

639 Programmed cell death through apoptosis is also critical for the response of immune system  
640 cells to *L. pneumophila* infection. Apoptosis is a well-orchestrated suicide program that  
641 culminates in a non-inflammatory dismantling of the cell (Bertheloot et al., 2021; Obeng,  
642 2021). It can be considered as a “silent” way of reducing the bacterial load by removing their  
643 replicative niche and triggering bacterial clearance in a cell autonomous manner (Youle and  
644 Strasser, 2008). Apoptosis can be triggered either by an intrinsic or an extrinsic pathway  
645 (Lossi, 2022; Obeng, 2021). Although both converge in the activation of the executers,  
646 caspase-3 and -7, they function differently. The extrinsic pathway transmits external  
647 apoptotic stimuli to the cell death machinery though the stimulation of death receptors, such  
648 as TNFRs, FasR or TRAILRs (Lossi, 2022). The intrinsic pathway is initialized in the  
649 mitochondria and its activation depends on an intricate balance of pro- and anti-apoptotic  
650 factors of the Bcl-2 protein family that control the activity of the sentinel cell death  
651 regulators, Bax, and Bak (Edlich, 2018). When pro-apoptotic factors overcome the inhibitory  
652 effects of the anti-apoptotic ones, Bax and Bak oligomerize in the mitochondrial outer  
653 membrane and contribute to the mitochondrial membrane permeabilization and release of  
654 cytochrome C, which nucleates apoptosome formation. The apoptosome leads to the cleavage  
655 and activation of caspases-3 and -7, initiating the organized dismantling of the cell (Peña-  
656 Blanco and García-Sáez, 2018). Thus, apoptotic, programmed cell death might play two  
657 defensive roles during infection. One eliminates pathogens at the early stage of infection  
658 without emitting alarm signals, the other one induces dendritic cells (DCs) to engulf apoptotic  
659 bodies containing infected microbes, which allows extracellular antigens to access MHC-II  
660 molecules and subsequently induces a protective immune response (Elliott and Ravichandran,  
661 2010).

662 As *L. pneumophila* is an intracellular pathogen that reside within macrophages, the apoptotic  
663 pathway can be considered a double-edged sword, that can be detrimental or beneficial  
664 depending on the course of the infection. At early stages of infection, *L. pneumophila* inhibits

665 host cell death and promotes survival pathways to maintain its replicative niche (Behar et al.,  
666 2010; Lamkanfi and Dixit, 2010). However, at later phases of infection, the activation of the  
667 apoptotic pathway allows *L. pneumophila* to efficiently exit the host cell and spread to the  
668 neighbouring cells. Thus, *L. pneumophila* has evolved sophisticated strategies to subvert the  
669 host cell death machinery and, as mitochondria play a central role in the induction of cell  
670 death pathways, several T4SS effectors target mitochondrial functions. This is particularly  
671 intriguing as *L. pneumophila* evolved with amoeba, its natural host, and there is no evidence  
672 for the presence of apoptotic programmed cell death or caspases in amoebae, although  
673 caspase-like proteins (metacaspases or paracaspases) have been discovered (Gao and Kwaik,  
674 2000). Interestingly, *L. pneumophila*-infected amoeba undergo necrosis mediated by pore-  
675 forming activity (Gao and Kwaik, 2000), but apoptotic programmed cell death was not  
676 reported to be triggered by *L. pneumophila* infection in amoeba (Gao and Kwaik, 2000;  
677 Hägele et al., 1998). How *L. pneumophila* evolved mechanisms to hijack the apoptotic  
678 pathway of macrophages is thus still an enigma.

679 In macrophages *L. pneumophila* actively delays apoptosis to complete its life cycle, as  
680 macrophages show increasing apoptotic phenotypes, such as nuclear condensation and  
681 phosphatidylserine exposure, which are caspase-3 dependent, only at the late stages of  
682 infection (Abu-Zant et al., 2007a; Gao and Abu Kwaik, 1999) (**Figure 4**). During the early  
683 stage, approximately until 18h post-infection, *L. pneumophila*-infected hMDMs exhibit a  
684 remarkable, T4SS-dependent resistance to the induction of apoptosis (Abu-Zant et al., 2007a,  
685 2005). Paradoxically, in a wide variety of host cells such as human and murine macrophage  
686 or monocyte cell lines *L. pneumophila* infection triggers activation of the executioner  
687 caspase-3 at early stages, also in a T4SS-dependent manner, and this activation is even more  
688 robust during exponential bacterial replication (Abu-Zant et al., 2005; Molmeret et al., 2004).

689 How *L. pneumophila* evades apoptosis, since *L. pneumophila* can trigger caspase3 activation  
690 during macrophage invasion without inducing immediate cell death, remains unresolved. This  
691 might due to a pro/anti-apoptotic balance moving towards cell survival, as a loss of anti-  
692 apoptotic BCL-2 family members, BCL-XL and MCL-1, induces cell death of infected  
693 macrophages and promotes *L. pneumophila* clearance in lungs (Speir et al., 2016). Several  
694 T4SS effectors are involved in triggering sustained NF- $\kappa$ B signalling. Early post-infection,  
695 *L. pneumophila* induces the transcriptional upregulation of several anti-apoptotic BCL-2  
696 family members *via* the NF $\kappa$ B transcription factor in a T4SS-dependent manner. In  
697 agreement, pharmacological inhibition of NF $\kappa$ B induces apoptosis and impairs

698 *L. pneumophila* growth (Abu-Zant et al., 2007b). This strong anti-apoptotic signaling cascade  
699 seems to render infected cells refractory to potent, external apoptotic stimuli and might  
700 compensate the activation of Caspase-3 and other pro-apoptotic factors such as BNIP3 that  
701 are activated during *L. pneumophila* infection, (Abu-Zant et al., 2007a; Losick and Isberg,  
702 2006).

703 *L. pneumophila* relies also on additional mechanisms to sustain macrophage viability (**Figure**  
704 **4**). The *L. pneumophila* effector SidF binds and inhibits BNIP3 and Bcl-rambo, two pro-  
705 apoptotic members of the BCL-2 family, which are primarily located in the outer membrane  
706 of mitochondria (Banga et al., 2007). Both BNIP3 and Bcl-rambo are putative pro-apoptotic  
707 proteins and their activity can be blocked by the anti-apoptotic protein BCL-2 (Kataoka et al.,  
708 2001; Zhang and Ney, 2009). Moreover, BNIP3 and Bcl-rambo can target mitochondria  
709 directly and induce cell death in a BCL-2-independent manner by interacting with ANT, the  
710 ADP/ATP carrier of mitochondrial membranes, to induce loss of mitochondrial membrane  
711 potential and caspase activation (Kim et al., 2012). Consistent with this, loss of SidF was  
712 reported to result in increased apoptotic cell death of *L. pneumophila* infected macrophages.  
713 This finding raised the possibility that BNIP3 and Bcl-rambo are key host factors controlled  
714 by *L. pneumophila* to regulate host cell death (Banga et al., 2007). However, a more recent  
715 study using single-cell analysis of *L. pneumophila*-infected macrophages demonstrated that  
716 *L. pneumophila* can replicate and induce normal macrophage killing rates in the absence of  
717 SidF, as well as BNIP3 and Bcl-rambo. In line with this, the inhibition of SidF, BNIP3, or  
718 Bcl-rambo did not affect *L. pneumophila* lung infections in mice (Speir et al., 2017). These  
719 results suggest that *L. pneumophila* can evade apoptosis independently of SidF and highlights  
720 that host cell death signalling following *L. pneumophila* infection might depend on the  
721 expression levels of different cell death components in the infected cell at the single-cell  
722 level. However, a recent study reported that different clinical isolates of *L. pneumophila* Sg1  
723 induce diverse levels of caspase-3 activation and cell death, which highlights that host cell  
724 death susceptibility to infection depends also on specific bacterial determinants  
725 (Guillemot et al., 2022).

726 Indeed, additional effectors have been reported to be involved in the regulation of  
727 mitochondrial-derived apoptosis. SdhA was identified through a genetic screen of  
728 *L. pneumophila* mutants with intracellular growth defects. This protein shows no  
729 recognizable domain, is constitutively expressed, and is translocated into the host cell by the  
730 T4SS throughout the intracellular growth cycle. SdhA has pleiotropic functions, such as

731 maintaining the integrity of the LCV or inhibiting host pyroptosis and it is thought to be  
732 involved in protecting *L. pneumophila* against stress conditions. Infection of macrophages  
733 with *sdhA*-deficient *L. pneumophila* results in mitochondrial disruption, membrane  
734 permeability, caspase-3 activation, and caspase-1- and caspase-11-dependent pyroptosis,  
735 suggesting that SdhA likely suppresses mitochondrial- as well as non-mitochondrial-  
736 mediated apoptosis (Laguna et al., 2006).

737 Host ATP/ADP carriers, such as ANTs, are thought to play a critical role in apoptosis by their  
738 ability to compromise the bioenergetics of mitochondria, resulting in Cytochrome C release  
739 and apoptosome formation (Zhivotovsky et al., 2009). Indeed, ANTs are essential  
740 components of the mitochondrial permeability transition pore (mPTP) and its formation  
741 triggers cell death by caspase-dependent or -independent mechanisms (Belzacq et al., 2002;  
742 Kinnally et al., 2011) (**Figure 5**). Therefore, as the T4SS effector SidF binds and inhibit  
743 BNIP3-ANT interaction, *L. pneumophila* might reduce apoptosis also by blocking mPTP  
744 opening. Additional inhibition of host ANT by Ceg3-mediated mono-ADP-ribosylation could  
745 also participate in blocking mPTP opening (**Figure 5**).

746 The effector LncP might be critical in this process, as it is an ATP/ADP carrier targeted to the  
747 inner mitochondrial membrane (Dolezal et al., 2012). Although loss of LncP does not  
748 abrogate intracellular *L. pneumophila* growth, and it does not complement yeast mutants  
749 lacking endogenous carrier proteins, overexpression of LncP is toxic for yeast grown in  
750 nutrient-limiting media. Interestingly, the yeast mutant lacking the carrier protein Aac was  
751 resistant to this LncP-induced inhibition of cell viability as both transport ATP, and most  
752 likely the combined export of ATP from the matrix by LncP and Aac1 leads to a growth  
753 defect (Dolezal et al., 2012). Thus, it is possible that LncP induces cell death on its own  
754 under certain conditions (**Figure 5**), particularly in conditions of low ATP/ADP transport.  
755 This is particularly interesting, as the effectors Ceg3 and Lpg0081 specifically modulate  
756 endogenous ATP/ADP carrier activity during infection (Fu et al., 2022; Kubori et al., 2022).  
757 Further studies are needed to address the role of LncP and its possible coordination with  
758 Ceg3-mediated inhibition of endogenous ATP/ADP carriers in the induction of apoptosis.

759 Besides macrophages, *L. pneumophila* can also infect other immune cell types, such as  
760 dendritic cells (DCs). However, murine DCs are poor hosts for as they are more sensitive to  
761 *L. pneumophila*-induced cell death (Nogueira et al., 2009). Compared to macrophages  
762 infected at the same conditions, apoptotic cell death occurs much earlier and is induced by the  
763 caspase-3 mediated canonical intrinsic pathway in DCs infected with *L. pneumophila*.

764 Although activation of caspase-3 does not trigger immediate apoptosis or disrupts bacterial  
765 replication in DCs, *L. pneumophila*-induced cell death of DCs is completely abrogated in the  
766 absence of caspase-3 or Bax/Bak and is also reduced upon expression of the anti-apoptotic  
767 protein BCL-2. Furthermore, different to macrophages, caspase-3 and, particularly, Bax/Bak-  
768 deficient DCs, can sustain *L. pneumophila* growth over several days, demonstrating that  
769 mitochondrial-dependent apoptosis is the primary mechanism of cell death of DCs caused by  
770 *L. pneumophila* infection (Nogueira et al., 2009). This suggest that unlike *L. pneumophila*-  
771 infected macrophages, murine DCs fail to upregulate anti-apoptotic factors (Abu-Zant et al.,  
772 2007b). Why DCs undergo rapid apoptosis upon *L. pneumophila* infection is still a matter of  
773 debate. It is hypothesized that this response may be designed to interfere with the migration  
774 of infected DCs through the lymphatic system, to prevent DCs from serving as “Trojan  
775 Horses” transporting pathogenic microbes from peripheral sites to central organs (Nogueira et  
776 al., 2009). However, it is also possible that *L. pneumophila* actively induces apoptosis in DCs  
777 to prevent their maturation and, hence, activation of adaptive immune responses in the  
778 lymphatic system and therefore avoids antigen processing and presentation, DC migration to  
779 lymph nodes and subsequent T-cell co-stimulation (Mellman and Steinman, 2001).

780 *L. pneumophila* can still induce cell death normally in the absence of a functional  
781 mitochondrial permeability transition pore complex (Speir et al., 2017), which confirms that  
782 at late stages, macrophage cell death includes signals operating independently of the intrinsic  
783 mitochondrial apoptotic pathway. In line with this, a strong correlation between caspase-3  
784 dependent apoptosis and TNF- $\alpha$  levels in infected macrophages was observed, despite the  
785 variability seen among the *L. pneumophila* isolates (Guillemot et al., 2022). Interestingly,  
786 inhibiting TNF- $\alpha$  by using anti-TNF- $\alpha$  antibodies dramatically reduces apoptosis of the  
787 different isolates, highlighting the major role of the extrinsic-apoptotic pathway in promoting  
788 caspase-3-mediated cell death during *L. pneumophila* infection and the involvement of TNF-  
789  $\alpha$ , which is a cytokine produced by macrophages themselves during infection.

790 At least five effectors have been identified that induce caspase-3 activation when expressed  
791 in HEK293 cell line, VipD, *LpSpl/LegS2*, Lem12, Ceg18 and Lpg0716 (Zhu et al., 2013)  
792 (**Figure 4**). At least three of them, (*LpSpl/LegS2*, Lem12 and Ceg18) are targeted to  
793 mitochondria and a fourth, VipD, a phospholipase which hydrolyses phosphoethanolamine  
794 and phosphocholine from the mitochondrial membrane, was shown to cause destabilization of  
795 the mitochondrial membrane and the release of Cytochrome C (Zhu et al., 2013). However,  
796 the exact roles of these effectors during infection and their influence in triggering apoptosis

797 remain elusive, as the co-deletion of these five effectors in *L. pneumophila* made DCs  
798 partially less sensitive to cell death but it did not impair caspase-3 activation in the U937  
799 human macrophage cell line nor did they influence bacterial infection or intracellular  
800 replication in murine primary macrophages (Zhu et al., 2013). In contrast, *LpSpl/LegS2* over  
801 expression on HEK293 cells rendered different results in two studies measuring cell death  
802 (Escoll et al., 2021; Zhu et al., 2013). When early apoptosis was measured in single,  
803 transfected cells upon over expression of *LpSpl* (*lpp2128*), transfected HEK293 cells were  
804 more resistant to Staurosporin-induced cell death, in line with the role of *LpSpl* inducing the  
805 reverse mode of the ATPase to delay cell death (Escoll et al., 2021). Thus, *LpSpl* might  
806 protect cells from externally induced apoptosis. However, other study showed that over  
807 expression of *LegS2* (*lpg2176*) in HEK293 cells increased Caspase-3 cleavage, a measure of  
808 late apoptosis (Zhu et al., 2013). As *LpSpl/LegS2* encodes a S1P lyase, and S1P and ceramide  
809 compose a rheostat regulating autophagy and cell death (Bedia et al., 2011; Iessi et al., 2020),  
810 these results might indicate that overexpression of the bacterial effector in human cell lines  
811 dysregulates this rheostat and leads to cell death. Overexpression of the effector in  
812 transfection experiments most likely induces higher levels of the bacterial protein in the  
813 cytoplasm than those occurring during infection, which might strongly dysregulate S1P levels  
814 and hence the rheostat. Thus, as transfection may lack the fine tuning of *LpSpl/LegS2*  
815 expression levels at mitochondria and the ER occurring during infection, excessive SPL  
816 activity and abnormal ceramide accumulation might increase cell death of transfected cells.  
817 Probably, those transfected cells that survived transfection as *LpSpl* is highly toxic for  
818 eukaryotic cells expressed the bacterial protein at lower levels. Measuring *LpSpl* effects  
819 specifically and uniquely in transfected cells by using quantitative microscopy might thus  
820 uncover phenotypes, such as protection from externally-induced apoptosis, that are otherwise  
821 hidden in bulk analyses of *e.g.* western blots of Caspase-3 cleavage. Further investigations  
822 are needed to understand the exact role of *LpSpl/LegS2* on cell death during infection and  
823 how bacterial manipulation of sphingosine metabolism in the infected cell impacts the  
824 S1P/ceramide rheostat.

825 Taken together, it seems that the infected cell defends itself by inducing cell death, while  
826 *L. pneumophila* delay it by the action of multiple T4SS factors. The bacterial-induced  
827 modulations intersect with several signalling hubs where host mitochondria play key roles,  
828 such as inflammasome-dependent pyroptosis and the intrinsic-mitochondrial-apoptosis  
829 pathway. These leads to an intricate network of signalling cross-talks within the infected cells

830 that complicates the study of mitochondrial-dependent cell death pathways during  
831 *L. pneumophila* infection. The application of single-cell approaches might help to dissect this  
832 complicated network of functional interactions regulating cell death pathways.

### 833 **5. Conclusions**

834 Since the very early times of research on *L. pneumophila*-host interactions, the relationship of  
835 the bacterium with host organelles, and specifically with mitochondria, has been studied.  
836 Early observations of mitochondrial recruitment to the bacterial vacuole, together with recent  
837 live imaging in single cells, have led to a model where *L. pneumophila*-mitochondria contacts  
838 are highly dynamic, probably unspecific, independent of T4SS effectors, and where a transfer  
839 of proteins between both structures might occur, probably repositioning the nature of  
840 mitochondria-phagosome contacts within the dynamics of vesicular trafficking. Although it  
841 has been suggested that these contacts serve different roles, such as the release of anti-  
842 microbial mROS or itaconate to the phagosome, further investigations are needed to  
843 understand the role of such dynamic contacts.

844 As mitochondria are the powerhouses of the cell, any changes in mitochondrial functions  
845 have an important impact in cellular bioenergetics. Indeed, *L. pneumophila* manipulates *via*  
846 its T4SS effectors OXPHOS functioning and ATP/ADP transport across mitochondrial  
847 membranes, the mitochondrial F<sub>0</sub>F<sub>1</sub>-ATPase, the maintenance of  $\Delta\psi_m$  and the delay of host  
848 cell death thus modifying mitochondrial functions seem to have cellular implications beyond  
849 metabolism. A model could be that reducing OXPHOS promotes a glycolytic metabolism,  
850 and as glycolysis is intimately linked to biosynthetic pathways, this metabolic switch might  
851 provide *L. pneumophila* with the amino acids and lipids for intracellular growth. OXPHOS  
852 reduction collapses the  $\Delta\psi_m$  and promotes cell death of the infected cell, therefore the  
853 induction of the ‘reverse mode’ of the F<sub>0</sub>F<sub>1</sub>-ATPase by *L. pneumophila* at the expenses of  
854 glycolytic ATP, plays a role in maintaining the  $\Delta\psi_m$  independently of OXPHOS. This also  
855 avoids the collapse of mitochondrial polarization and evading the early death of the infected  
856 cell. As mitochondria are signalling hubs in the cell and guardians of cellular homeostasis  
857 mitochondria might also be sensors of danger-associated metabolic modifications (DAMMs)  
858 triggered during infection (Dramé et al., 2020), and *L. pneumophila* and other intracellular  
859 bacteria hijack these mechanisms to avoid premature cell death of the infected macrophage  
860 cell.

861 The ability of cells to trigger cell-autonomous immune responses to infection seems  
862 intimately linked to mitochondrial functioning, thereby constituting a key component of  
863 intracellular homeostasis during infection (West et al., 2011b). Central molecules regulating  
864 cell-autonomous immune responses are mROS, which are generated at the mitochondrial  
865 ETC when the electron flux is altered, and which can trigger pyroptosis *via* inflammasome  
866 activation or be direct anti-microbials delivered to the bacterial phagosome (Herb and  
867 Schramm, 2021; Li and Chang, 2021). In this regard, the interplay between autophagy and  
868 pyroptosis during *Legionella* infection has elegantly shown the kind of fates an infected  
869 macrophage can undergo and *Legionella* might modulate (Byrne et al., 2013b). Indeed,  
870 *Legionella* modulates autophagy and pyroptosis *via* different T4SS effectors. MAMs, the  
871 membrane contacts between the ER and mitochondria where several of these processes such  
872 as the mitochondrial fission machinery, the formation of autophagosomes, the activation of  
873 the NLRP3-inflammasome, and the location of several apoptosis regulators, such as BCL-2  
874 and BCL-XL are regulated, might be targeted by additional *L. pneumophila* effectors that  
875 need to be identified.

876 The investigation of the relationship between *L. pneumophila* and mitochondria has moved  
877 forward, however, several intriguing questions remain to be answered. Are *L. pneumophila*-  
878 mitochondria contacts providing an advantage to the pathogen? Why OXPHOS is regulated  
879 in a biphasic mode during *L. pneumophila* infection, with T4SS-independent and -dependent  
880 phases, and what is the impact of TLR signaling on these phases? Is the glycolytic switch  
881 beneficial for the pathogen? What is the role of the *L. pneumophila*-induced modulation of  
882 ATP/ADP transport in the regulation of OXPHOS and/or mitochondrial F<sub>0</sub>F<sub>1</sub>-ATPase  
883 functioning? Is *L. pneumophila* -induced regulation of mROS avoiding inflammasome  
884 activation and pyroptosis? How *L. pneumophila* T4SS effectors regulating caspase-dependent  
885 apoptosis evolved if the natural host, amoeba, lack caspases? Answering these and other  
886 questions will be a passionate journey to understand mitochondrial biology during  
887 *L. pneumophila* infection.

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1458 TABLES

1459 Table 1. List of *L. pneumophila* effectors described to target mitochondria

1460

<i>L. pneumophila</i> effector	Effector activity	Mitochondrial target	Mitochondrial outcome	Cellular outcome	Refs
Lpg1976 / Lpp1959 (LegG1/MitF)	Ran GTPase activator	DRP1 activation	Mitochondrial fragmentation	Metabolic rewiring	(Escoll et al., 2017b)
Lpg2176 / Lpp2128 (LegS2, LpSpl)	Sphingosine-1P lyase	-	Mitochondrial fragmentation	Metabolic rewiring	(Abu Khweek et al., 2016a)
		F <sub>0</sub> F <sub>1</sub> -ATPase reverse mode	Maintenance of $\Delta\psi_m$	Inhibition of cell death	(Escoll et al., 2021)
		MAMs ?	-	Blockage of autophagy	(Rolando et al., 2016)
		-	Increased caspase 3	Induction of cell death	(Zhu et al., 2013)
Lpg0080 (Ceg3)	mono-ADP-ribosyltransferase	ANT carrier inhibition	Alteration of ATP/ADP efflux	Metabolic rewiring	(Fu et al., 2022; Hoshino et al., 2019)
Lpg0081	ADP-ribosylhydrolases	ANT carrier activation	Alteration of ATP/ADP efflux	Metabolic rewiring	(Kubori et al., 2022)
Lpp2981 (LncP)	ANT transporter	-	Alteration of ATP/ADP efflux	Metabolic rewiring?	(Dolezal et al., 2012)
Lpg0376 (SdhA)	-	-	Mitochondrial integrity	Inhibition of cell death	(Laguna et al., 2006)
Lpg2584 (SidF)	Protein-protein interaction	BNIP3 and Bcl2RAMBO inhibition	Reduced proapoptotic signals	Inhibition of cell death	(Speir et al., 2017)
			ANT-mediated apoptotic blockage		
Lpg2831 (VipD)	Phospholipase A2	-	Increased caspase 3	Induction of cell death	(Zhu et al., 2013)
Lpg1625 (Lem12)	-	-	Increased caspase 3	Induction of cell death	(Zhu et al., 2013)
Lpg0898 (Ceg18)	-	-	Increased caspase 3	Induction of cell death	(Zhu et al., 2013)
Lpg0716	-	-	Increased caspase 3	Induction of cell death	(Zhu et al., 2013)
Lpg1137 (Ceg20)	Serine protease	STX17 inhibition	-	Blockage of autophagy	(Arasaki et al., 2017)

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1466 **FIGURE LEGENDS**

1467 **Figure 1. Functional analysis of the mitochondrial proteins identified in the proteome of**  
1468 **the *Legionella*-containing vacuoles isolated from RAW264.7 macrophages.** Gene  
1469 Ontology enrichment analyses of biological processes (A) and cellular components (B).  
1470 Functional categories were assigned using DAVID (Dennis et al., 2003), for the 499  
1471 mitochondrial proteins (47%) obtained from the purified LCV protein dataset of infected  
1472 macrophages from Hoffmann *et al.*, 2014. GO terms shown in graphs represent the top 20  
1473 with the highest significant p-value ( $\leq 0.001$ ). Redundant GO terms were removed using  
1474 REVIGO (Supek et al., 2011). Enrichment fold change is defined as Observed/Expected.

1475

1476 **Figure 2. Regulation of mitochondrial functions by *Legionella* with metabolic**  
1477 **consequences.** Once *L. pneumophila* is phagocytosed, the recognition of specific ligands by  
1478 pattern recognition receptors, such as TLR, triggers a rapid cell autonomous innate immune  
1479 response leading to increased OXPHOS and glycolysis. During infection, *L. pneumophila*  
1480 induces mitochondrial fission, in a T4SS-dependent manner. At least two effectors are  
1481 involved: MitF an activator of Ran GTPase that induces the DRP1-mediated fragmentation of  
1482 mitochondria and *LpSpl* (*LegS2*), sphingosine-1 phosphate lyase, which induces fission by  
1483 unknown mechanisms. The fission process leads to reduced OXPHOS, which together with  
1484 high glycolysis, represents an optimal metabolic program (Warburg-like) to promote  
1485 biosynthetic pathways and sustain intracellular growth. To deal with the consequent drop of  
1486  $\Delta\psi_m$  as result of reduced OXPHOS, *L. pneumophila*-induces the ‘reverse mode’ of the  $F_0F_1$ -  
1487 ATPase to pump protons through the inner mitochondrial membrane (IMM) in a T4SS-  
1488 dependent process, partially mediated by *LpSpl*. *L. pneumophila* also adapts ATP/ADP efflux  
1489 during infection by modulating the activity of host ANTs through the effectors Ceg3 and  
1490 *Lpg0081*, that show opposing enzymatic activities as ADP ribosyltransferase and ADP  
1491 ribosylhydrolase, respectively. They might coordinate this function together with *LncP*, a  
1492 *L. pneumophila* effector with ANT-like carrier functions.

1493 **Figure 3. Role of mitochondrial ROS as signaling molecule in inflammasome-mediated**  
1494 **pyroptosis and its modulation during *L. pneumophila* infection.** TLR recognition of  
1495 *L. pneumophila* leads to production of cellular ROS and mROS, which play essential roles as  
1496 signaling molecules during inflammasome activation. NLRs are important innate immune  
1497 receptors that upon the recognition of DAMPs/PAMPs nucleate and form the inflammasome,

1498 inducing a proinflammatory cell death termed pyroptosis. mROS are essential for NLR  
1499 signaling as: (1) mROS (and cellular ROS) released during infection promotes mitochondrial  
1500 damage and the release of mitochondrial DAMPs, which are signals for NLRP3-  
1501 inflammasome activation, and (2) mROS is required for proper NLRC4-dependent  
1502 inflammasome activation upon recognition of bacterial PAMPs. *L. pneumophila* reduces  
1503 mROS during infection, thus we hypothesize that *L. pneumophila*-induced modulation of  
1504 mitochondrial dynamics might lead not only to decreased OXPHOS, but also to reduced  
1505 mROS, which would reduce xenophagy and subvert inflammasome activation. In addition,  
1506 other *L. pneumophila* effectors seem to maintain stable mitochondrial “danger parameters” in  
1507 infected macrophages, such as  $\Delta\psi_m$  and ATP efflux, through the induction of the ‘reverse  
1508 mode’ of the mitochondrial F<sub>0</sub>F<sub>1</sub>-ATPase and the direct regulation of ANTs, respectively. All  
1509 together this might allow mitochondrial functioning and macrophage survival, even at low  
1510 OXPHOS levels.

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1512 **Figure 4. Modulation of the intrinsic apoptotic pathway by *L. pneumophila***

1513 The activation of the mitochondrial intrinsic pathway depends on a fine balance of pro- and  
1514 anti-apoptotic factors of the BCL-2 protein family. After pathogen recognition, the innate  
1515 immune pathways lead to a pro-apoptotic signal, which overcomes the anti-apoptotic one and  
1516 promotes the oligomerization of Bax and Bak. This provokes mitochondrial membrane  
1517 permeabilization and Cytochrome C (CytC) release, initiating a signaling cascade that results  
1518 in cleavage and activation of caspase-3 and the onset of apoptosis. During the early steps of  
1519 infection, *L. pneumophila* preferentially promotes anti-apoptotic signals by secreting T4SS-  
1520 dependent factors to preserve its niche, such as SidF, which binds to and inhibits the  
1521 functions of the pro-apoptotic proteins BNIP3 and Bcl-rambo. In contrast, at late phases of  
1522 infection *L. pneumophila* favors the pro-apoptotic signals to efficiently exit the host cell. Five  
1523 T4SS effectors show activated caspase-3 during infection VipD, *LpSpl/LegS2*, Lem12,  
1524 Ceg18 and Lpg0716. These factors might co-ordinately act with other cell death signals, such  
1525 as active TNF- $\alpha$ , to induce caspase-3 activation and cell death at late infection.

1526

1527 **Figure 5. Modulation of cell death by *L. pneumophila* by interfering with mitochondrial**  
1528 **permeability transition pore (mPTP) formation.** ANTs are essential components of  
1529 mPTPs and its formation triggers cell death by caspase-dependent or -independent  
1530 mechanisms. *L. pneumophila*-induced innate immune responses increase pro-apoptotic

1531 signals, such as BNIP3, which binds to ANTs, and induces apoptosis *via* mPTP. During early  
1532 infection *L. pneumophila* secretes T4SS effectors with potential anti-apoptotic roles, such as  
1533 SidF, that binds and inhibits BNIP3-ANT interaction. Host ANT-inhibition by favoring a  
1534 Ceg3-mediated mono-ADP-ribosylation over Lpg0081-mediated ADPr hydrolysis might also  
1535 participate in blocking mPTP. A pro-apoptotic role for the *L. pneumophila* effector LncP has  
1536 been proposed, as it induces cell death under nutrient-limiting conditions during late stages of  
1537 the infection.

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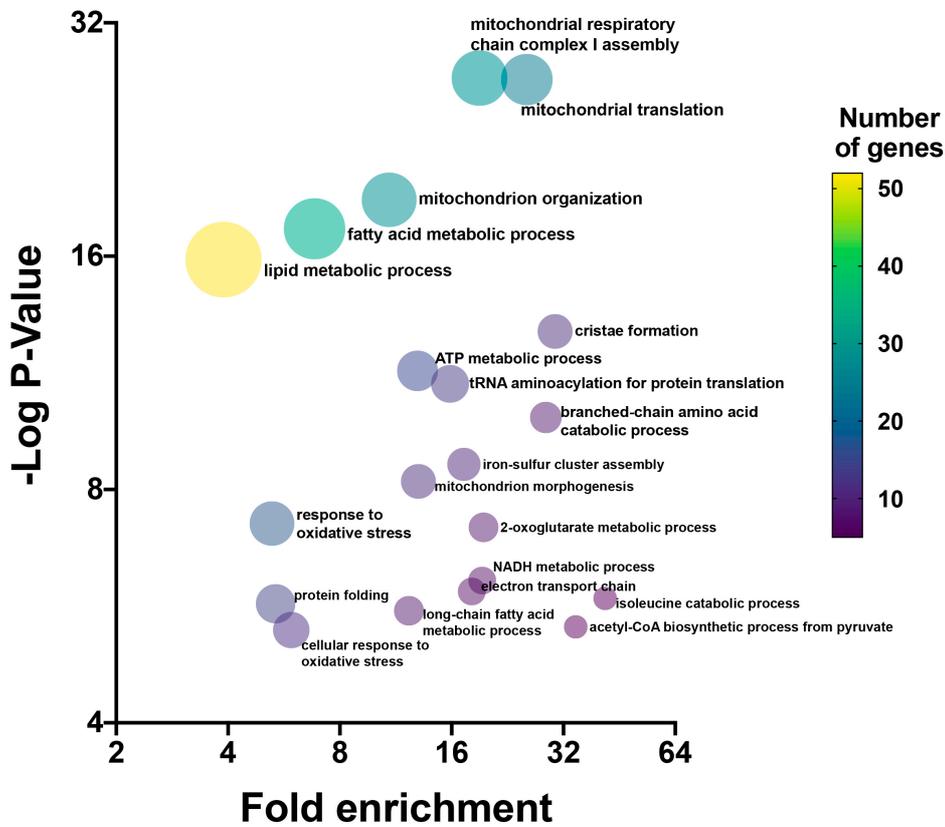
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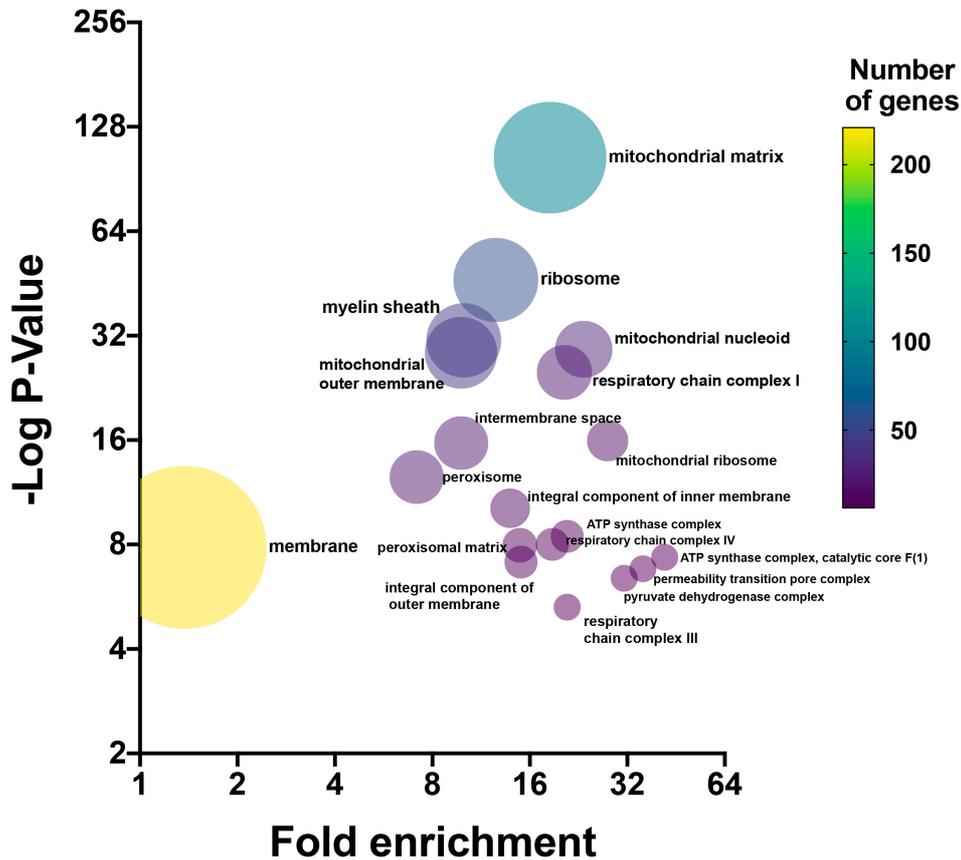
1544 FIGURES

1545 FIGURE 1

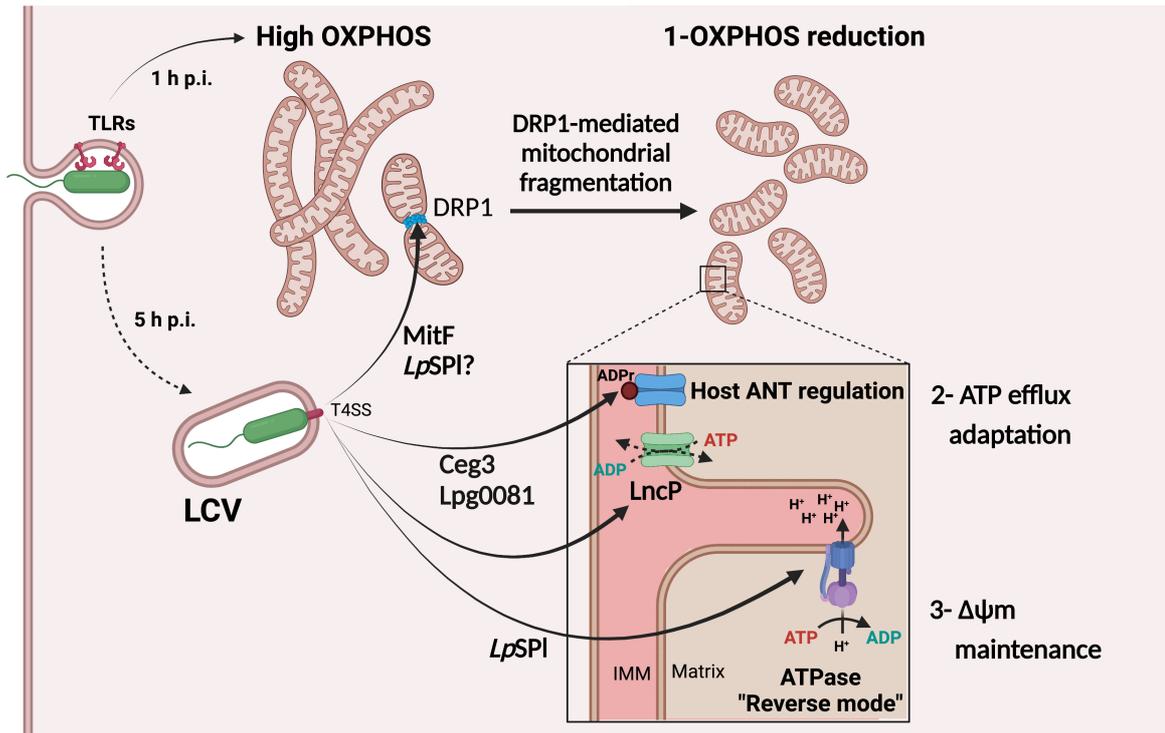
1546 A



1558 B



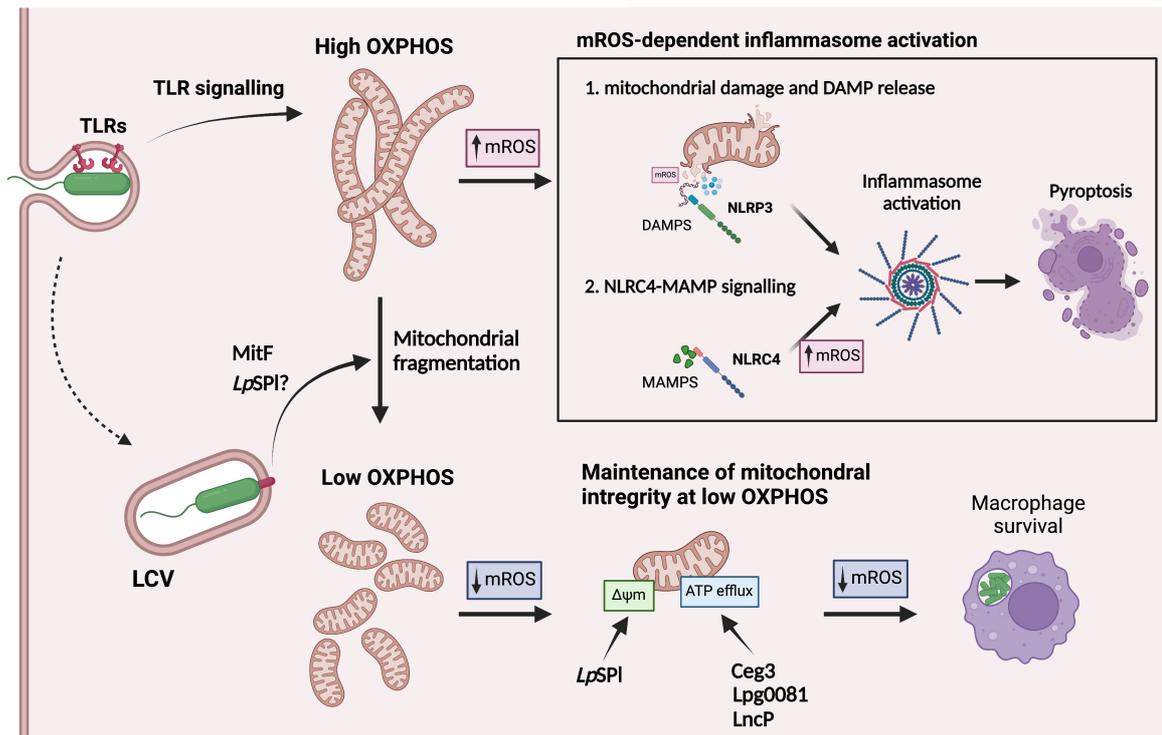
1570 **FIGURE 2**



1571

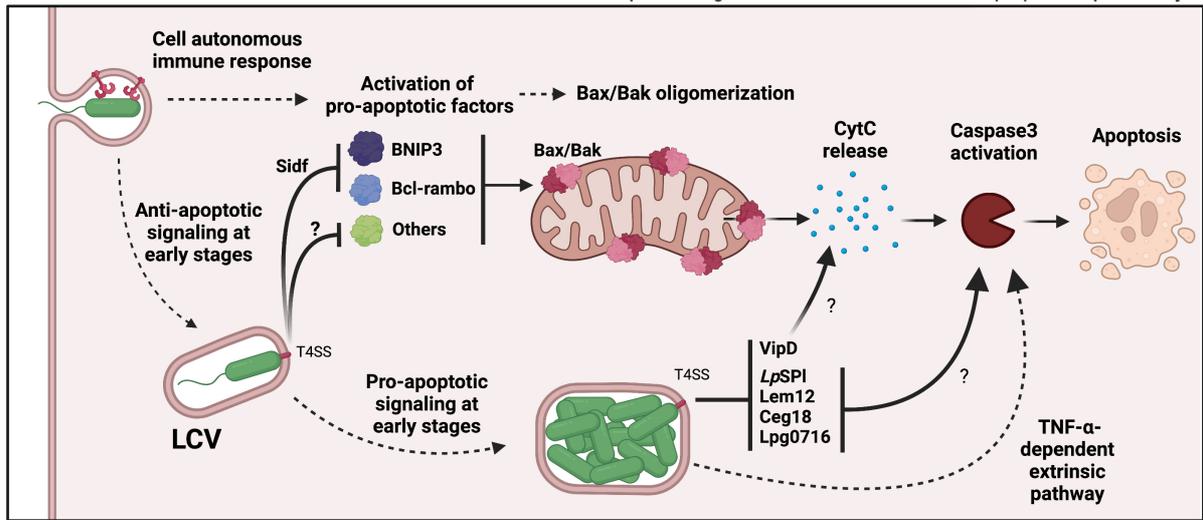
1572

1572 **FIGURE 3**  
1573



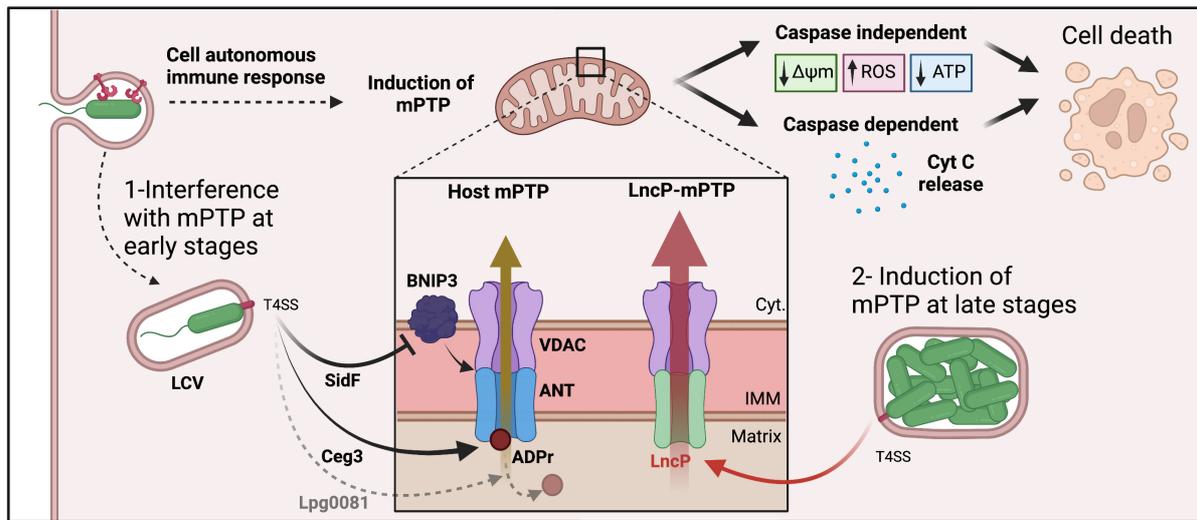
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1576 **FIGURE 4**  
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1580 **FIGURE 5**  
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