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2 **Reverting the mode of action of the mitochondrial F₀F₁-ATPase by**
3 ***Legionella pneumophila* preserves its replication niche**
4

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31 **ABSTRACT**

32 *Legionella pneumophila*, the causative agent of Legionnaires' disease, a severe pneumonia,
33 injects *via* a type-IV-secretion-system (T4SS) more than 300 proteins into macrophages, its
34 main host cell in humans. Certain of these proteins are implicated in reprogramming the
35 metabolism of infected cells by reducing mitochondrial oxidative phosphorylation (OXPHOS)
36 early after infection. Here we show that despite reduced OXPHOS, the mitochondrial
37 membrane potential ($\Delta\psi_m$) is maintained during infection of primary human monocyte-derived
38 macrophages (hMDMs). We reveal that *L. pneumophila* reverses the ATP-synthase activity of
39 the mitochondrial F₀F₁-ATPase to ATP-hydrolase activity in a T4SS-dependent manner, which
40 leads to a conservation of the $\Delta\psi_m$, preserves mitochondrial polarization and prevents
41 macrophage cell death. Analyses of T4SS effectors known to target mitochondrial functions
42 revealed that *LpSpl* is partially involved in conserving the $\Delta\psi_m$, but not LncP and MitF. The
43 inhibition of the *L. pneumophila*-induced "reverse mode" of the F₀F₁-ATPase collapsed the
44 $\Delta\psi_m$ and caused cell death in infected cells. Single-cell analyses suggested that bacterial
45 replication occurs preferentially in hMDMs that conserved the $\Delta\psi_m$ and showed delayed cell
46 death. This direct manipulation of the mode of activity of the F₀F₁-ATPase is a newly identified
47 feature of *L. pneumophila* allowing to delay host cell death and thereby to preserve the bacterial
48 replication niche during infection.

49

50 INTRODUCTION

51 Beyond their essential role in cellular bioenergetics, mitochondria are integrated into diverse
52 signaling pathways in eukaryotic cells and perform various signaling functions, such as immune
53 responses or cell death, as they play crucial roles in the regulation of apoptosis (Bock and Tait,
54 2020). Thus mitochondria are targeted by several intracellular bacteria during infection to
55 modulate their functions to the bacterial advantage (Spier et al., 2019). One of these bacteria is
56 *Legionella pneumophila*, the causative agent of Legionnaires' disease. We have shown
57 previously that this pathogen targets mitochondrial dynamics during infection of primary
58 human monocyte-derived macrophages (hMDMs) by injecting type IV secretion system (T4SS)
59 effectors such as MitF, leading to a fragmented mitochondrial network *via* the recruitment of
60 the host fission protein DNMI1L to the mitochondrial surface (Escoll et al., 2017b). Importantly,
61 *Legionella* induced mitochondrial fragmentation at early time points such as 5 hours post-
62 infection (hpi), when bacterial replication has not started yet, and in the absence of cell death
63 signs. The fragmentation of mitochondrial networks provoked a T4SS-dependent reduction of
64 mitochondrial respiration in *Legionella*-infected macrophages, evidencing a functional
65 connection between mitochondrial dynamics and mitochondrial respiration (Escoll et al.,
66 2017b).

67 Mitochondrial respiration results from coupling the activity of five complexes in the electron
68 transport chain (ETC) at mitochondrial cristae. In this process, the reduced coenzymes NADH
69 and FADH₂ generated at the mitochondrial matrix by the tricarboxylic acid (TCA) cycle are
70 oxidized at Complexes I and II where their electrons are extracted to energize the mitochondrial
71 ETC (Nolfi-Donagan et al., 2020). The sequential transit of these electrons through Complexes
72 I, III and IV allows to pump protons from the matrix to the intermembrane space (IMS) and at
73 Complex IV, diatomic oxygen O₂ serves as the terminal electron acceptor and H₂O is formed.
74 The increased concentration of protons [H⁺] at the IMS, compared to [H⁺] at the matrix,
75 generates the mitochondrial membrane potential ($\Delta\psi_m$). This is necessary to produce ATP
76 by fueling the rotation of Complex V, the mitochondrial F₀F₁-ATPase, in a process termed
77 oxidative phosphorylation, OXPHOS (Nolfi-Donagan et al., 2020). Our previous studies
78 determined that at 5 hpi *L. pneumophila*, by altering mitochondrial dynamics, reduced
79 OXPHOS as well as the cellular ATP content in hMDMs in a T4SS-dependent manner (Escoll
80 et al., 2017b).

81 Why *L. pneumophila* and other species of intracellular bacteria reduce mitochondrial
82 OXPHOS during infection of host cells remains a matter of debate (Escoll and Buchrieser,

83 2018; Russell et al., 2019). As intracellular bacteria can obtain resources only from host cells,
84 it has been suggested that halting mitochondrial OXPHOS during infection might benefit
85 pathogenic bacteria by redirecting cellular resources, such as glycolytic or TCA intermediates,
86 to biosynthetic pathways that might sustain intracellular bacterial replication instead of fueling
87 mitochondria (Escoll and Buchrieser, 2018; Russell et al., 2019). For instance, it has been
88 shown that *Mycobacterium tuberculosis* redirects pyruvate to fatty acid synthesis and
89 *Chlamydia trachomatis* subverts the pentose phosphate pathway to increase the synthesis of
90 nucleotides for its own intracellular growth (Siegl et al., 2014; Singh et al., 2012). On the other
91 hand, upon sensing bacterial lipopolysaccharides, macrophages redirect mitochondrial TCA
92 intermediates, such as citrate or succinate, to drive specific immune functions such as the
93 production of cytokines or the generation of antimicrobial molecules (Escoll and Buchrieser,
94 2019; Russell et al., 2019; O'Neill and Pearce, 2016). Thus, while these metabolic shifts, which
95 are redirecting resources from mitochondria to the cytoplasm should be activated in
96 macrophages to develop their antimicrobial functions, they could also benefit intracellular
97 bacteria, as more resources would be available in the cytoplasm for bacterial growth.
98 Importantly, reduction of OXPHOS may lead to decreased $\Delta\psi_m$ and ATP production at
99 mitochondria, which are events that trigger the activation of cell death programs. How
100 intracellular bacteria withdraw OXPHOS, deal with the subsequent $\Delta\psi_m$ drop and host cell
101 death but manage to preserve their host cell to conserve their replication niche is a question that
102 remains poorly understood.

103 To answer this question, we monitored the evolution of mitochondrial polarization during
104 infection of hMDMs by *L. pneumophila*, and showed that in the absence of OXPHOS,
105 *L. pneumophila* regulates the enzymatic activity of the mitochondrial F_0F_1 -ATPase during
106 infection. This allows maintaining the $\Delta\psi_m$ and delays cell death of infected hMDMs in a T4SS-
107 dependent manner. Our results identified a new virulence mechanism of *L. pneumophila*,
108 namely the manipulation of the mitochondrial F_0F_1 -ATPase to preserve the integrity of infected
109 host cells and thereby the maintenance of the bacterial replication niches.

110 RESULTS

111 **Despite *L. pneumophila*-induced Reduction of Mitochondrial Respiration, the** 112 **Mitochondrial Membrane Potential Is Maintained**

113 We have previously shown that *L. pneumophila* strain Philadelphia JR32 impairs mitochondrial
114 respiration during infection (Escoll et al., 2017b). Here we analyzed *L. pneumophila* strain Paris
115 (Lpp) to learn whether this is a general characteristic of *L. pneumophila* infection. We infected

116 hMDMs with Lpp WT or a T4SS-deficient mutant ($\Delta dotA$) for 6 hours and analyzed their
117 mitochondrial function compared to uninfected hMDMs by using a cellular *respiratory control*
118 *assay* in living cells (Brand and Nicholls, 2011; Connolly et al., 2018). This assay determines
119 oxygen consumption rate (OCR) in basal conditions and during the sequential addition of
120 mitochondrial respiratory inhibitors. OCR variations observed indicate how mitochondrial
121 respiration is functioning in a cell population (Figure 1A and S1A). Our results showed that
122 basal respiration is significantly reduced ($p < 0.0001$) in WT-infected hMDMs compared to
123 $\Delta dotA$ - and non-infected hMDMs (Figure 1A and 1B). This indicates that O_2 consumption,
124 which is predominantly driven by ATP turnover and the flow of H^+ to the matrix through the
125 mitochondrial F_0F_1 -ATPase, is severely impaired in WT-infected hMDMs. Further analysis of
126 OCR changes upon addition of oligomycin, an inhibitor of the mitochondrial F_0F_1 -ATPase,
127 indicated that the rate of mitochondrial respiration coupled to ATP synthesis is highly reduced
128 in WT-infected hMDMs, compared to $\Delta dotA$ - or non-infected cells. Other respiratory
129 parameters such as proton leak were also reduced in WT-infected macrophages (Figure 1A,
130 S1A and S1B). Subsequent addition of an uncoupler to create a H^+ short-circuit across the inner
131 mitochondrial membrane (IMM), such as FCCP, allowed measuring the maximum respiration
132 rate and the spare respiratory capacity, revealing that both were severely impaired in WT-
133 infected cells compared to $\Delta dotA$ - and non-infected hMDMs (Figure 1A, S1A and S1B).
134 Finally, inhibition of the respiratory complexes I and III with rotenone and antimycin A,
135 respectively, measured O_2 consumption driven by non-mitochondrial processes, such as
136 cytoplasmic NAD(P)H oxidases, which showed similar levels of non-mitochondrial O_2
137 consumption in all infection conditions (Figure 1A, S1A and S1B).

138 Taken together, our results indicated that several mitochondrial respiration parameters were
139 severely altered during infection with Lpp-WT, including respiration coupled to ATP
140 production. Importantly, some of the respiratory parameters measured that are oligomycin-
141 sensitive were reduced in Lpp-WT-infected hMDMs but not in $\Delta dotA$ -infected cells, suggesting
142 that the mitochondrial F_0F_1 -ATPase activity may be altered during *L. pneumophila* infection in
143 a T4SS-dependent manner.

144 The transition of electrons across mitochondrial ETC complexes allows the extrusion of H^+
145 from the matrix to the IMS generating a H^+ circuit where the mitochondrial F_0F_1 -ATPase is the
146 dominant H^+ re-entry site during active ATP synthesis by OXPHOS. In cellular steady-state
147 conditions, extrusion and re-entry H^+ fluxes across mitochondrial membranes are balanced
148 (Brand and Nicholls, 2011). Therefore, any exogenous alteration of ATP turnover and/or F_0F_1 -

149 ATPase activity influences this H⁺ circuit and might be reflected in $\Delta\psi_m$ levels. Thus, we
150 decided to quantify the $\Delta\psi_m$ in infected cells. We developed a miniaturized *high-content* assay
151 based on kinetic measurements of TMRM fluorescence in non-quenching conditions (10nM),
152 where TMRM fluorescence in mitochondria is proportional to the $\Delta\psi_m$ (Connolly et al., 2018;
153 Duchen et al., 2003). This assay allowed to measure changes in the $\Delta\psi_m$ at the single-cell level
154 and in thousands of living cells during the course of infection (Figure 1C). Image analysis
155 showed that the $\Delta\psi_m$ slightly increased in Lpp-WT-, Lpp- $\Delta dotA$ - and non-infected cell
156 populations during the first hours of infection (1-3 hpi), and progressively decreased during the
157 time-course with no differences between the infection conditions (Figure 1D). Single-cell
158 analyses (Figure 1E and S1C) showed that Lpp-WT-, Lpp- $\Delta dotA$ - and non-infected single
159 hMDMs showed a wide range of $\Delta\psi_m$ values at any time-point (Figure S1C) with no significant
160 differences between them at 6 hpi (Figure 1E). Thus, despite a significant reduction of
161 OXPPOS the $\Delta\psi_m$ was maintained in infected cells, suggesting that *L. pneumophila*
162 manipulates the mitochondrial ETC to conserve the $\Delta\psi_m$ of hMDMs in the absence of
163 OXPPOS.

164 ***L. pneumophila* Infection Induces the “Reverse Mode” of the Mitochondrial F₀F₁-ATPase** 165 **in a T4SS-depended Manner**

166 The mitochondrial F₀F₁-ATPase is a fascinating molecular machine that rotates clockwise
167 when it works in the “forward mode”, synthesizing ATP by using the $\Delta\psi_m$ generated by the H⁺
168 circuit (Figure 2A, left). It can also rotate counter-clockwise when it works in the “reverse
169 mode”. In this case, it hydrolyzes ATP to maintain $\Delta\psi_m$ in the absence of OXPPOS (Figure
170 2A, right). As our results showed that *L. pneumophila* highly reduced OXPPOS, likely by an
171 alteration of the F₀F₁-ATPase activity, while the $\Delta\psi_m$ was conserved, we investigated in which
172 activity mode the F₀F₁-ATPase worked during *Legionella* infection. A widely used method to
173 investigate the directionality of the F₀F₁-ATPase in intact cells is to monitor changes in $\Delta\psi_m$
174 after the addition of F₀F₁-ATPase inhibitors, such as oligomycin or DCCD (Connolly et al.,
175 2018; Gandhi et al., 2009). These inhibitors block both modes of function, thus if the F₀F₁-
176 ATPase is working in the “forward mode” the $\Delta\psi_m$ will increase after adding the inhibitor, as
177 the inhibition of the H⁺ flux to the matrix through the ATPase leads to an accumulation of H⁺
178 at the IMS (Figure 2B, left). If $\Delta\psi_m$ decreases after ATPase inhibition, the F₀F₁-ATPase works
179 in the “reverse mode”, since now H⁺ cannot translocate to the IMS to maintain the $\Delta\psi_m$ (Figure
180 2B, right). Here, we used the aforementioned TMRM high-content assay to monitor the $\Delta\psi_m$ in
181 living hMDMs at 6 hpi, when OXPPOS is impaired and $\Delta\psi_m$ is maintained.

182 First, we recorded a baseline and then added medium as a control. As expected, this did not
183 alter $\Delta\psi_m$ in any infection condition (Figure 2C and 2D). However, the addition of FCCP
184 completely depolarized mitochondria, leading to an abrupt drop of $\Delta\psi_m$ in Lpp-WT-, Lpp-
185 $\Delta dotA$ - and non-infected hMDMs (Figure 2C and 2E), demonstrating that this assay can
186 monitor changes in the $\Delta\psi_m$ simultaneously in hundreds of infected cells. To analyze whether
187 the F₀F₁-ATPase worked in the synthase (forward) or the hydrolase (reverse) mode we added
188 oligomycin (Figure 2F) or DCCD (Figure 2G) to the infected cells. The $\Delta\psi_m$ increased in non-
189 infected or $\Delta dotA$ -infected hMDMs, which suggested that the ATPase worked in the “forward
190 mode” in these infection conditions. In contrast, the addition of oligomycin to Lpp-WT-infected
191 hMDMs had no effect on the $\Delta\psi_m$ (Figure 2F), while addition of DCCD decreased the $\Delta\psi_m$
192 (Figure 2G). Thus, our results indicate that the F₀F₁-ATPase worked in the “forward mode” in
193 non-infected or $\Delta dotA$ -infected macrophages, whereas the F₀F₁-ATPase worked in the “reverse
194 mode” during infection of hMDMs with the WT strain. This suggests that the induction of the
195 “reverse mode” depends on the action of T4SS effector(s).

196 **The T4SS Effector *LpSPL* Participates in the Induction of the “Reverse Mode” of the** 197 **Mitochondrial F₀F₁-ATPase During Infection**

198 Among the more than 300 bacterial effectors that *L. pneumophila* injects into host cells through
199 its T4SS (Mondino et al., 2020), three have been shown to target mitochondrial structures or
200 functions. LncP is a T4SS effector targeted to mitochondria that assembles in the inner
201 mitochondrial membrane (IMM) and seems to transport ATP across mitochondrial membranes
202 (Dolezal et al., 2012). The effector *LpSpl* (also known as LegS2) was suggested to target
203 mitochondria (Degtyar et al., 2009), the endoplasmic reticulum (ER, (Rolando et al., 2016), and
204 mitochondrial-associated membranes, MAMs ((Escoll et al., 2017a). *LpSpl* encodes a
205 sphingosine-1 phosphate (S1P) lyase that directly targets the host sphingolipid metabolism and
206 restrains autophagy in infected cells. MitF (LegG1) activates the host small GTPase Ran to
207 promote mitochondrial fragmentation during infection of human macrophages (Escoll et al.,
208 2017b).

209 To learn if any of these effectors is involved in the T4SS-dependent induction of the “reverse
210 mode” of the F₀F₁-ATPase, we infected hMDMs during 6 hours with Lpp-WT or its isogenic
211 mutants lacking the T4SS (Lpp- $\Delta dotA$), lacking the effector LncP (Lpp- $\Delta lncP$), lacking the
212 effector *LpSpl* (Lpp- Δspl), and *L. pneumophila* strain Philadelphia JR32 (JR32-WT) and its
213 isogenic mutants lacking the T4SS (JR32- $\Delta icmT$) or the effector MitF (JR32- $\Delta mitF$). Using the
214 TMRM high-content assay, we measured the $\Delta\psi_m$ after the inhibition of the F₀F₁-ATPase by

215 DCCD (Figure 2H). Our results indicated that, while the F_O-F₁-ATPase worked in the “forward
216 mode” in non-infected hMDMs and during infection with T4SS-deficient mutants (*Lpp-ΔdotA*
217 and *JR32-ΔicmT*), the F_OF₁-ATPase worked in the “reverse mode” during infection with the
218 *Lpp*-WT and *JR32*-WT strains (Figure 2G). Infection with *Lpp-ΔlncP* and *JR32-ΔmitF* were
219 not significantly different compared to the WT strains, suggesting that these effectors are not
220 involved in the induction of the “reverse mode” of the mitochondrial ATPase. However,
221 mitochondria of cells infected with *Lpp-Δspl* showed a significantly higher $\Delta\psi_m$ after DCCD
222 treatment than mitochondria of cells infected with *Lpp*-WT ($p=0.0006$), and a significantly
223 lower $\Delta\psi_m$ after DCCD treatment than mitochondria of cells infected with the *Lpp-ΔdotA* strain
224 ($p=0.0034$). This suggests that *LpSpl* is partially involved in the induction of the “reverse mode”
225 of the F_OF₁-ATPase, however other additional T4SS effector(s) seem to participate in the
226 modulation of the F_OF₁-ATPase activity mode.

227 **Inhibition of *Legionella*-induced “Reverse Mode” Collapses the $\Delta\psi_m$ And Ignites Cell** 228 **Death of Infected Macrophages**

229 To further analyze the importance of the activity mode of the F_OF₁-ATPase during infection,
230 we used BTB06584 (hereafter called BTB), a specific inhibitor of the “reverse mode” of the
231 mitochondrial F_OF₁-ATPase (Ivanov et al., 2014). We used the TMRM high-content assay and
232 added BTB to non-infected, *Lpp*-WT- or *Lpp-ΔdotA*-infected hMDMs at 6 hpi. As shown in
233 Figure 3A, the $\Delta\psi_m$ collapsed specifically and significantly in WT infected cells (Figure 3B and
234 3C) compared to non-infected ($p=0.0022$) and *ΔdotA*-infected cells ($p=0.0238$), further
235 confirming that the F_OF₁-ATPase works in the “reverse mode” during WT infection. Indeed,
236 the addition of BTB to *Lpp*-WT-infected hMDMs led to a significant reduction of the $\Delta\psi_m$
237 ($p<0.0001$) at every time point post-infection (1-10 hpi) and at the single-cell level compared
238 to non-treated *Lpp*-WT-infected cells (Figure 3D), further confirming that conservation of $\Delta\psi_m$
239 during *L. pneumophila* infection is caused by induction of F_O-F₁-ATPase “reverse mode”.

240 As OXPHOS cessation and $\Delta\psi_m$ collapse can trigger cell death, we reasoned that induction
241 of the “reverse mode” of mitochondrial ATPase by *L. pneumophila* to maintain $\Delta\psi_m$ in the
242 absence of OXPHOS might delay cell death of infected cells. To test this hypothesis, we
243 infected hMDMs with *Lpp*-WT and treated them with BTB or left them untreated, and then
244 measured the percentage of living cells among infected cells (Figure 4A). Our results showed
245 that the percentage of living, infected cells significantly decreased after 10 hpi in BTB-treated
246 infected hMDMs compared to non-treated cells. As this reduction in the percentage of living,
247 infected cells upon “reverse mode” inhibition might be caused by increased cell death, we used

248 our high-content assay to measure Annexin-V, a marker of early apoptosis, in a high number
249 of living hMDMs during infection (Figure 4B and 4C). While addition of BTB during 24 hours
250 did not increase the percentage of Annexin-V⁺ cells in non-infected cells, the addition of this
251 “reverse mode” inhibitor to Lpp-WT-infected hMDMs significantly increased the percentage
252 of Annexin-V⁺ cells compared to non-treated cells (p=0.0312). This suggests that the inhibition
253 of the “reverse mode” by BTB leads to a reduction in the percentage of infected cells as
254 increased cell death occurs specifically in infected cells. Single-cell analysis at 12 and 18 hpi
255 (Figure 4D and S2A) also showed higher levels of Annexin-V intensity in BTB-treated Lpp-
256 WT-infected hMDMs compared to non-treated infected cells (p<0.0001). BTB-treated infected
257 cells also showed higher Hoechst nuclear levels compared to non-treated infected cells, a sign
258 of nuclear condensation typical of apoptotic cells (Figure S2B), which further indicates that
259 inhibition of the *Legionella*-induced ATPase “reverse mode” ignites cell death of infected
260 macrophages. Taken together, our results suggest that the *Legionella*-induced “reverse mode”
261 of the mitochondrial F₀F₁-ATPase aids to conserve $\Delta\psi_m$ during infection to delay cell death of
262 infected macrophages.

263 To simultaneously monitor $\Delta\psi_m$ and early cell death signs in the same infected cell, we
264 multiplexed Annexin-V and TMRM signals in our living cell assay (Figure 4E, S2C and S2D).
265 *L. pneumophila*-infected macrophages where the “reverse mode” activity of the F₀F₁-ATPase
266 was inhibited by BTB suffered a collapsed $\Delta\psi_m$ and showed higher levels of Annexin-V at 12
267 and 18 hpi (Figure 4E and S2D) compared to non-treated cells. Thus both events, collapse of
268 the $\Delta\psi_m$ and triggered cell death, occurred in the same infected cell when the *Legionella*-
269 induced “reverse mode” activity of the F₀F₁-ATPase was inhibited. Furthermore, when the size
270 of the bacterial vacuole was correlated with the $\Delta\psi_m$ and cell death at the single-cell level (12
271 and 18 hpi, Figure 4F and 4G respectively), intracellular bacterial replication occurred
272 preferentially in those infected macrophages with intermediate levels of TMRM (non-collapsed
273 $\Delta\psi_m$) and low Annexin-V levels (yellow color scale, Figure 4F and 4G), further indicating that
274 conservation of $\Delta\psi_m$ and delayed cell death are needed to guarantee the survival of infected
275 macrophages to ensure bacterial replication.

276 DISCUSSION

277 We show here that by inducing the “reverse mode” of the mitochondrial F₀F₁-ATPase,
278 *L. pneumophila* circumvents the collapse of $\Delta\psi_m$ and cell death caused by OXPHOS cessation
279 in infected cells. This mechanism, which partially involves the T4SS effector *LpSpl*, maintains
280 the $\Delta\psi_m$ and delays host cell death during infection, thus preserving bacterial replication niches

281 in conditions where mitochondrial respiration is abruptly reduced. Indeed, not only
282 *L. pneumophila*, but also several other intracellular bacterial pathogens, such as
283 *Mycobacterium tuberculosis* or *Chlamydia pneumoniae*, reduce mitochondrial OXPHOS
284 during infection (Siegl et al., 2014; Singh et al., 2012)(Escoll and Buchrieser, 2019). OXPHOS
285 reduction allows the pathogen to redirect cellular resources from mitochondria to the cytoplasm,
286 which enhances glycolysis and biosynthetic pathways that can provide intracellular bacteria
287 with resources needed for bacterial growth (Escoll and Buchrieser, 2018; Russell et al., 2019).
288 In contrast, OXPHOS cessation in macrophages also enhances the biosynthetic pathways
289 leading to the synthesis of cytokines and antimicrobial compounds (O’Neill and Pearce, 2016;
290 Russell et al., 2019). Furthermore, OXPHOS reduction may trigger profound consequences for
291 host cells, such as the collapse of $\Delta\psi_m$ that may lead to subsequent cell death of infected cells.
292 For macrophages, cell death is considered as a defense mechanism against infection (Chow et
293 al., 2016). Indeed, pyroptosis of infected macrophages permits the spread of inflammatory
294 mediators such as IL-1 β . Thus, for intracellular bacteria, many of which infect macrophages
295 (Mitchell et al., 2016), the death of their host cell is an obstacle as their cellular replication
296 niche is destroyed. Therefore, while bacterial-induced reduction of OXPHOS might be
297 beneficial for intracellular bacteria to obtain host cell resources, they need to counterbalance
298 the consequences of OXPHOS cessation, i.e. the collapse of the mitochondrial $\Delta\psi_m$ and
299 subsequent cell death, to preserve their replication niches.

300 We have previously shown that the *L. pneumophila* T4SS effector MitF is implicated in
301 fragmenting the mitochondrial networks of infected macrophages. These changes in the
302 mitochondrial dynamics have a profound impact on OXPHOS that was severely impaired and
303 accompanied by increased glycolysis in *Legionella*-infected cells (Escoll et al., 2017b). Here
304 we show that, despite the impairment of mitochondrial respiration in infected cells,
305 *L. pneumophila* conserves the $\Delta\psi_m$ of host cells by inducing the “reverse mode” of the F_oF₁-
306 ATPase by a mechanism that is T4SS-dependent and partially mediated by the T4SS effector
307 *LpSpl*. When translocated into human cells, the S1P-lyase activity of *L. pneumophila* *LpSpl*
308 reduces S1P levels in infected cells and restrains autophagy, likely because S1P is involved in
309 the initiation of autophagosome formation at MAMs (Rolando et al., 2016).

310 How *LpSpl* may regulate the activity of the F_oF₁-ATPase is an interesting question. Indeed,
311 phosphorylated lipids are critical regulators of mitochondrial functions and S1P is a potent lipid
312 mediator that regulates various physiological processes as well as diverse mitochondrial
313 functions such as mitochondrial respiration, ETC functioning or mitochondrial-dependent cell
314 death (Hernández-Corbacho et al., 2017; Nielson and Rutter, 2018). Furthermore it was

315 reported that S1P interaction with Prohibitin 2 (PHB2) regulates ETC functioning and
316 mitochondrial respiration (Strub et al., 2011) and that a link between PHB2, ETC functioning
317 and the activation of “mitoflashes” (Jian et al., 2017) exists, which are dynamic and transient
318 uncouplings of mitochondrial respiration from ATP production that are partially dependent on
319 the “reverse mode” of the F_oF₁-ATPase (Wei-LaPierre and Dirksen, 2019). Thus, it is possible
320 that *LpSpl* modulates mitochondrial S1P levels helping the induction of the “reverse mode” of
321 the mitochondrial F_oF₁-ATPase by involving PHB2, ETC complex assembly or the generation
322 of mitoflashes, a fascinating possibility that we will further investigate.

323 The regulation of host cell death by intracellular bacteria is widely studied (Rudel et al.,
324 2010). For *L. pneumophila*, T4SS effectors activating and inhibiting cell death of infected cells
325 have been described (Speir et al., 2014), suggesting that a very delicate interplay of positive
326 and negative signals governs the fate of infected macrophages. Here we have shown that
327 bacterial replication occurs preferentially in those infected macrophages that are able to
328 conserve the $\Delta\psi_m$ and delay cell death, a condition that is difficult to achieve in the absence of
329 mitochondrial respiration. Thus, the manipulation of the activity of the mitochondrial F_oF₁-
330 ATPase by *L. pneumophila*, which allows this pathogen to use the ATP hydrolase activity to
331 pump H⁺ to the IMS to maintain the $\Delta\psi_m$ in infected cells, is a novel virulence strategy that
332 might contribute to the fine-tuning of the timing of host cell death during bacterial infection.

333 **MATERIALS and METHODS**

334 **Human Primary Cell Cultures**

335 Human blood was collected from healthy volunteers under the ethical rules established by the
336 French National Blood Service (EFS). Peripheral blood mononuclear cells (PBMCs) were
337 isolated by Ficoll-Hypaque density-gradient separation (Lympholyte-H; Cedarlane
338 Laboratories) at room temperature. PBMCs were incubated with anti-human CD14 antibodies
339 coupled to magnetic beads (Miltenyi Biotec) and subjected to magnetic separation using LS
340 columns (Miltenyi Biotec). Positive selected CD14⁺ cells were counted and CD14 expression
341 was analysed by flow cytometry, repeatedly showing a purity > 90%. CD14 cells were plated
342 in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated fetal
343 bovine serum (FBS, Biowest) in 6 well multi-dish Nunc UpCell Surface cell culture plates or
344 10 cm Nunc UpCell Surface cell culture dishes (Thermo Fisher) and differentiated to human
345 monocyte-derived macrophages (hMDMs) by incubation with 100 ng/ml of recombinant
346 human macrophage colony-stimulating factor (rhMCSF, Miltenyi Biotec) for 6 days at 37°C
347 with 5% CO₂ in a humidified atmosphere. At day 3, additional rhMCSF (50 ng/ml) was added.

348 After 6 days differentiation, UpCell plates were placed at 20°C during 10 minutes and hMDMs
349 were gently detached, counted and plated in RPMI 1640 10% FBS in 384-well plates (Greiner
350 Bio-One).

351 **Bacterial strains and mutant construction**

352 *L. pneumophila* strain Paris or JR32 and their derivatives were grown for 3 days on N-(2-
353 acetamido)-2-amino-ethanesulfonic acid (ACES)-buffered charcoal-yeast (BCYE) extract
354 agar, at 37°C. For eGFP-expressing strains harbouring pNT28 plasmid (Tiaden et al., 2007),
355 chloramphenicol (Cam; 5 µg/mL) was added. Knock-out mutant strains of *L. pneumophila*
356 genes coding for the T4SS effectors *LpSpl* and *MitF/LegG1* were previously described (Escoll
357 et al., 2017b; Rolando et al., 2016; Rothmeier et al., 2013). The knock-out mutant strain of the
358 *L. pneumophila* gene coding for the effector *LncP* was constructed as previously described
359 (Brüggemann et al., 2006; Rolando et al., 2016). In brief, the gene of interest was inactivated
360 by introduction of an apramycin resistance (*apraR*) cassette into the chromosomal gene by 3-
361 steps PCR. The g primers used for the *lncP* (lpp2981) knock out mutant are: *LncP_F*:
362 ACCCTGGTTCATGGTAACAATGG; *LncP_Inv_R*:
363 GAGCGGATCGGGGATTGTCTTATCAGGCGAATGGTGTGAAAGG; *LncP_Inv_F*:
364 GCTGATGGAGCTGCACATGAAACGTCATGGTCGTGCTGGTTG; *LncP_R*:
365 AATCAGATGGGTAAGCCGATTGG. To amplify the apramycin cassette, the primers
366 *Apra_F*: TTCATGTGCAGCTCCATCAGC and *Apra_R*: AAGACAATCCCCGATCCGCTC
367 were used.

368 **Infection of hMDMs and automatic confocal imaging**

369 hMDMs were infected with *L. pneumophila* grown for three days on BCYE agar plates.
370 Bacteria were dissolved in 1X PBS (Life Technologies), the optical density (OD) was adjusted
371 to OD₆₀₀ of 2.5 (2.2×10^9 bacteria/mL) and the bacteria were then further diluted in serum-free
372 XVIVO-15 medium (Lonza) prior to infection to obtain the respective multiplicity of infection
373 (MOI). hMDMs were washed twice with serum-free XVIVO-15 medium and then infected
374 (MOI = 10) with 25 µL of bacteria in 384-well plates (Greiner Bio-One). The infection was
375 synchronized by centrifugation (200 g for 5 min) and the infected cells were incubated at 37°C
376 for 5 min in a water bath and then for 25 min at 37°C/5%CO₂. After three intensive washes
377 with serum-free XVIVO-15 medium, the infection proceeded in serum-free XVIVO-15
378 medium for the respective time points. 30 min prior imaging, 25 µL of culture medium were
379 removed and replaced by 25 µL of 2X mix of dyes, to a final concentration of 200 ng/mL of
380 Hoechst H33342 (nuclear staining; Life Technologies), 10 nM of TMRM (mitochondrial

381 membrane potential; Life Technologies), and/or 1/100 Annexin-V-Alexa Fluor 647 (early
382 apoptosis; Life Technologies). If chemical inhibitors of the Electron Transport Chain (ETC)
383 were used in the experiments, they were added to hMDMs at the indicated times points at the
384 following concentrations: 5 μ M Oligomycin (Enzo), 100 μ M DCCD
385 (Dicyclohexylcarbodiimide, Sigma), 10 μ M FCCP (Tocris), 50 μ M BTB06584 (Sigma). Image
386 acquisitions of multiple fields (9 to 25) per well were performed on an automated confocal
387 microscope (OPERA Phenix, Perkin Elmer) using 60X objective, excitation lasers at 405, 488,
388 561 and 640 nm, and emission filters at 450, 540, 600 and 690 nm, respectively.

389 **Metabolic Extracellular Flux Analysis**

390 hMDMs (50,000) were plated in XF-96-cell culture plates (Seahorse Bioscience). For OCR
391 measurements, XF Assay Medium (Seahorse Bioscience) supplemented with 1 mM pyruvate
392 and 10 mM glucose was used, and OCR was measured in a XF-96 Flux Analyzer (Seahorse
393 Bioscience). For the mitochondrial respiratory control assay, hMDMs were infected at MOI =
394 10 and at 6 hpi., different drugs were injected (Mitostress kit, Seahorse Bioscience) while OCR
395 was monitored. Specifically, Olygomycin was injected through the port A, then FCCP was
396 injected through the port B, and finally Rotenone + Antimycin A were injected through the port
397 C, to reach each of the drugs a final concentration in the well of 0.5 μ M.

398 **Automatic High-Content Analyses (HCA)**

399 All analyses were performed with Harmony software v.4.9 (Perkin Elmer) using in-house
400 developed scripts (available upon request). For the HCA of the mitochondrial membrane
401 potential ($\Delta\psi_m$), the Hoechst signal was used to segment nuclei in the 405/450 channel
402 (excitation/emission), Hoechst background signal in the cytoplasm was used to segment the
403 cytoplasm region in the 405/450 channel, *L. pneumophila* was identified by measuring the GFP
404 signal in the 488/540 channel, and TMRM (10 nM) signal in the 561/600 channel was used to
405 measure $\Delta\psi_m$ by calculating SD/Mean TMRM intensity values in each infected and non-
406 infected cell. For the HCA of cell death, the Hoechst signal was used to segment nuclei in the
407 405/450 channel, Hoechst background signal in the cytoplasm was used to segment the
408 cytoplasm region, and the identification of *L. pneumophila* was performed using the GFP signal
409 in the 488/540 channel. Then, Annexin-V-AlexaFluor 647 signal was measured in the 640/690
410 channel and the Hoechst signal intensity was measured in the 405/450 channel for each infected
411 or non-infected cell. For the HCA analyses combining $\Delta\psi_m$ and cell death, both HCA strategies
412 aforementioned were merged, using high Hoechst signal in the 405/450 channel to segment
413 nuclei, low Hoechst signal in the 405/450 channel to segment cytoplasm, GFP signal in the

414 488/540 channel to identify bacteria, TMRM signal in the 561/600 channel to measure $\Delta\psi_m$
415 (SD/Mean) and Annexin-V-AlexaFluor 647 signal in the 640/690 channel to measure cell
416 death.

417 **Whole genome sequencing for mutant validation**

418 Chromosomal DNA was extracted from BCYE-grown *L. pneumophila* using the DNeasy Blood
419 and Tissue Kit (Qiagen). The Illumina NGS libraries were prepared using the Nextera DNA
420 Flex Library Prep following the manufacturer's instructions (Illumina Inc.). High-throughput
421 sequencing was performed with a MiSeq Illumina sequencer (2×300 bp, Illumina Inc.) by the
422 Biomics Pole (Institut Pasteur). For the analysis, we first removed adapters from Illumina
423 sequencing reads using Cutadapt software version 1.15 (Martin, 2011) and we used Sickle
424 (<https://github.com/najoshi/sickle>) with a quality threshold of 20 (Phred score) to trim bad
425 quality extremities. Reads were assembled using Spades (Nurk et al., 2013) and different K-
426 mer values. The region corresponding to the gene of interest was identified by blastn, extracted,
427 and compared to the homologous region in the *L. pneumophila* strain Paris WT genome and to
428 the antibiotic cassette sequence using blastn. The results are visually inspected with ACT
429 (Artemis Comparison Tool) (Carver et al., 2005). In addition, we searched the entire genome
430 whether off-target mutations had occurred, using Bowtie 2 (Langmead and Salzberg, 2012) to
431 perform a mapping against the genome sequence of *L. pneumophila* strain Paris
432 (NC_006368.1). SNPs and small indels were searched for with freebayes SNP caller (Garrison
433 and Marth, 2012), mutations and small indels were visualized in the Artemis genome viewer
434 (Carver et al., 2005) to analyze them (new amino acid, synonymous mutation, frameshifts, etc).
435 We used Samtools to find regions with no coverage (or close to zero) (Li et al., 2009). Regions
436 or positions with such anomalies were visualized and compared with the corresponding region
437 of the assembly. This confirmed that no off-target mutations impacting the phenotype of the
438 mutant had occurred.

439 **Statistical analyses**

440 The two-sample Student's t-test (Mann-Whitney *U* test, non-assumption of Gaussian
441 distributions) was used in all data sets unless stated otherwise. Data analysis was performed
442 using Prism v9 (Graphpad Software).

443 **AUTHOR CONTRIBUTIONS**

444 PE conceived the study; PE, LP, MD and SS prepared blood-derived human cells and/or
445 performed experiments; TS constructed bacterial mutants; PE, LP and CR analyzed data; PE
446 and CB provided funding; CB provided critical advice; PE and CB wrote the manuscript.

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583

584

585 FIGURE LEGENDS

586 **Figure 1. Despite a reduction of oxidative phosphorylation (OXPHOS), hMDMs maintain**
587 **their $\Delta\psi_m$ during infection by *L. pneumophila*.** (A) hMDMs were infected with
588 *L. pneumophila* strain Paris (Lpp) wild-type (WT), a T4SS-deficient $\Delta dotA$ mutant, or left
589 uninfected (Non-infected). At 6 hours post-infection (hpi), a cellular respiratory control assay
590 was performed by measuring oxygen consumption rate (OCR) during the sequential addition
591 of mitochondrial respiratory inhibitors (see also Figure S1A). (B) Basal respiration of hMDMs
592 in the same conditions as in (A), at 6 hpi. (C) hMDMs were infected as in (A) with GFP-
593 expressing bacteria (green), nuclei of host cells were stained with Hoechst (Nuc, blue) and $\Delta\psi_m$
594 was monitored from 1 to 12 hpi using TMRM dye in non-quenching conditions (10 nM).
595 Representative confocal microscope images of non-infected and infected cells at 3, 6, 9 and 12
596 hpi are shown. Intracellular bacterial replication can be observed in Lpp-WT infected hMDMs
597 at 12 hpi. Bar: 20 μm . (D) Quantification of TMRM intensity at 1-10 hpi (expressed as
598 SD/Mean) in the assays described in (C). Data from 4 independent experiments with a total of
599 10 replicates (E) Single-cell analysis at 6 hpi of the assays described in (C). Single-cell data
600 from a representative experiment (full time-course in Figure S1C) **p-value < 0.01; ****p-
601 value < 0.00001; ns = non-significant (Mann-Whitney *U* test).

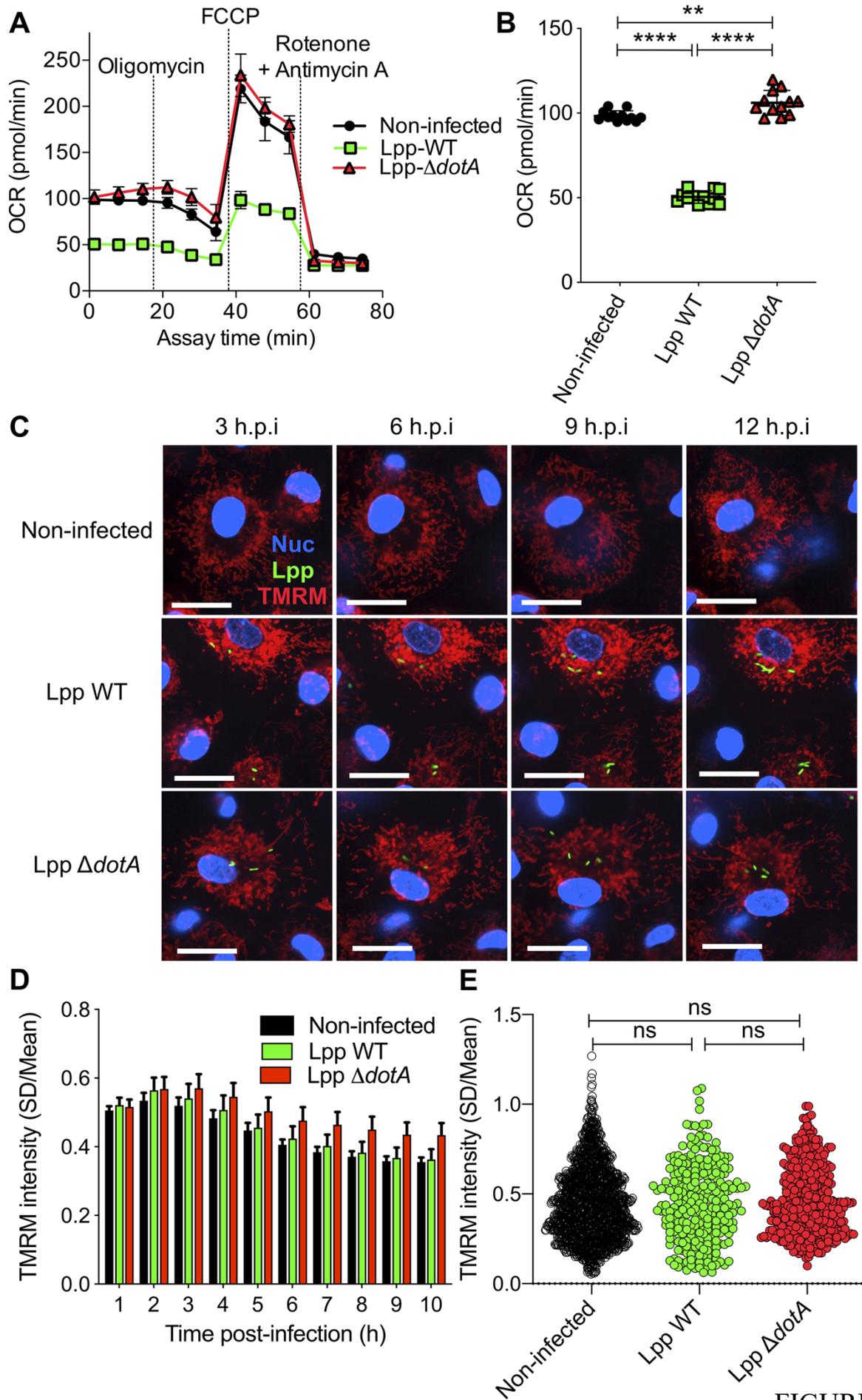
602 **Figure 2. The mitochondrial F_0F_1 -ATPase works in the “reverse mode” during infection**
603 **of hMDMs by *L. pneumophila*.** (A) In the “forward mode” of the mitochondrial ATPase, the
604 $\Delta\psi_m$ generated by the Electron Transport Chain is used by the F_0F_1 -ATPase to synthesize ATP.
605 The “reverse mode” of the F_0F_1 -ATPase leads to ATP hydrolysis to pump H^+ to the
606 intermembrane space (IMS). IMM: inner mitochondrial membrane. (B) When the F_0F_1 -ATPase
607 is inhibited by Oligomycin or DCCD, an increase in $\Delta\psi_m$ indicates that the ATPase was
608 working in the “forward mode” (H^+ accumulate in the IMS), while a decrease in $\Delta\psi_m$ indicates
609 functioning in the “reverse mode” (H^+ cannot be translocated to the IMS by the F_0F_1 -ATPase
610 to sustain the $\Delta\psi_m$). (C) hMDMs were infected with GFP-expressing bacteria (green), or left
611 uninfected (Non-infected). At 5.5 hpi cells were labeled with Hoechst to identify the cell
612 nucleus (Nuc, blue) and TMRM (red) to quantify $\Delta\psi_m$. At 6 hpi, addition of medium (no
613 changes) or FCCP (complete depolarization) was used as controls. Representative confocal
614 images of Lpp-WT-infected hMDMs (6 hpi) at 5 min before the addition of medium (top) or
615 FCCP (bottom), and at 5, 25 and 50 min after addition of medium or FCCP. Bar: 20 μm . (D)
616 Quantification of (C) before (baseline) and after the addition of medium. Each dot represents
617 mean \pm SD of 3 independent experiments with a total of 8 replicates. (E) Same as (D) but FCCP

618 was added. **(F)** Same as (D) but oligomycin was added. **(G)** Same as (D) but DCCD was added.
619 **(H)** Same as (C) but infection was performed with Lpp-WT, Lpp- $\Delta dotA$, Lpp- $\Delta incP$, Lpp- Δspl ,
620 *L. pneumophila* strain Philadelphia JR32 (JR32)-WT, JR32- $\Delta icmT$ or JR32- $\Delta mitF$. TMRM
621 values (SD/Mean) at 50 min after DCCD addition are shown. Data from a minimum of 3
622 experiments per strain with 10 or more replicates per strain **p-value < 0.01; ***p-value <
623 0.001; ****p-value < 0.00001; ns = non-significant (Mann-Whitney *U* test)

624 **Figure 3. Inhibition of the “reverse mode” of mitochondrial F₀F₁ ATPase reduces the $\Delta\psi_m$**
625 **of *L. pneumophila*-infected hMDMs. (A)** hMDMs were infected with GFP-expressing
626 bacteria (green), Lpp-WT or Lpp- $\Delta dotA$, or left uninfected (Non-infected). At 5.5 hpi cells were
627 labeled with Hoechst to identify cell nucleus (Nuc, blue) and TMRM (red) to quantify $\Delta\psi_m$. At
628 6 hpi, BTB06584 (BTB, 50 μ M), a specific inhibitor of the “reverse mode” of the ATPase, was
629 added and $\Delta\psi_m$ monitored. Representative confocal microscopy images of non-infected (top)
630 and Lpp-WT-infected (bottom) hMDMs (6 hpi) at 5 min before the addition and at 5, 25 and
631 50 min after the addition of BTB. Bar: 20 μ m. **(B)** Quantification of (C) before (baseline) and
632 after the addition of BTB. Each dot represents the mean \pm SD of 3 independent experiments
633 with a total of 6 replicates. **(C)** Same infection conditions than (A) but TMRM values
634 (SD/Mean) at 50 min after BTB addition are shown. Data from 3 experiments with a total of 6
635 replicates (3 replicates for Lpp- $\Delta dotA$) **(D)** Single-cell analysis of $\Delta\psi_m$ in Lpp-WT-infected
636 hMDMs treated with BTB (50 μ M) or left untreated (non-treated). Single-cell data from one
637 representative experiment *p-value < 0.05; **p-value < 0.01; ns = non-significant (Mann-
638 Whitney *U* test)

639 **Figure 4. Inhibition of F₀-F₁ ATPase “reverse mode” increases cell death in**
640 ***L. pneumophila*-infected hMDMs. (A)** hMDMs were infected with Lpp-WT-GFP and were
641 non-treated or treated with 50 μ M BTB. The presence of GFP-expressing bacteria in each cell
642 was monitored and the number of infected cells in the whole population was graphed as
643 percentage of infected cells. Data from 3 independent experiments with a total of 7 replicates
644 per condition and time-point **(B)** hMDMs were infected with Lpp-WT-GFP (green), the nuclei
645 of host cells were stained with Hoechst (Nuc, blue) and Annexin-V Alexa Fluor 647 was added
646 to the cell culture to monitor early cell death (Annexin, yellow) from 1 to 18 hpi in non-treated
647 or BTB-treated hMDMs. Representative confocal images of non-treated and Lpp-WT-GFP-
648 infected cells at 6, 12 and 18 hpi are shown. Intracellular bacterial replication can be observed
649 in non-treated Lpp-WT infected hMDMs at 12 and 18 hpi. Bar: 20 μ m. **(C)** hMDMs stained as

650 in (B) were infected with Lpp-WT-GFP or left uninfected (Non-infected), and then were treated
651 or not with BTB (50 μ M). Percentage of Annexin-V⁺ cells at 24 hpi is shown. Data from 3
652 independent experiments with a total of 7 replicates per condition **(D)** Single-cell analysis (12
653 hpi) of Annexin-V intensity of the assays described in (B). Single-cell data from one
654 representative experiment (18 hpi shown in Figure S2A) **(E)** hMDMs were infected with Lpp-
655 WT-GFP, nuclei of host cells were stained with Hoechst, and TMRM and Annexin-V Alexa
656 Fluor 647 were added to the cells to simultaneously monitor (1-18 hpi) $\Delta\psi_m$ and early cell death,
657 respectively, in non-treated or BTB-treated hMDMs (representative multi-field confocal
658 images in Figure S2C). Single-cell analyses (12 hpi) of $\Delta\psi_m$ (TMRM SD/Mean) and cell death
659 (Annexin-V intensity) in more than 1600 cells per condition are shown. Single-cell data from
660 one representative experiment; Green dots: Non-treated Lpp-WT-infected single cells. Orange
661 dots: BTB-treated Lpp-WT-infected single cells. **(F)** Same infection conditions as in (E) but
662 vacuole size was monitored in each Lpp-WT-infected single cell. Single-cell analyses (12 hpi)
663 of $\Delta\psi_m$ (TMRM SD/Mean), vacuole size (μm^2), and cell death (Annexin-V intensity) in more
664 than 3800 cells are shown. Single-cell data from one representative experiment; Color scale
665 (yellow) represents Annexin V intensity per cell (AU). **(G)** Same as in (F) at 18 hpi. *p-value
666 < 0.05; **p-value < 0.01; ***p-value < 0.001; ****p-value < 0.00001; ns = non-significant
667 (Mann-Whitney *U* test)



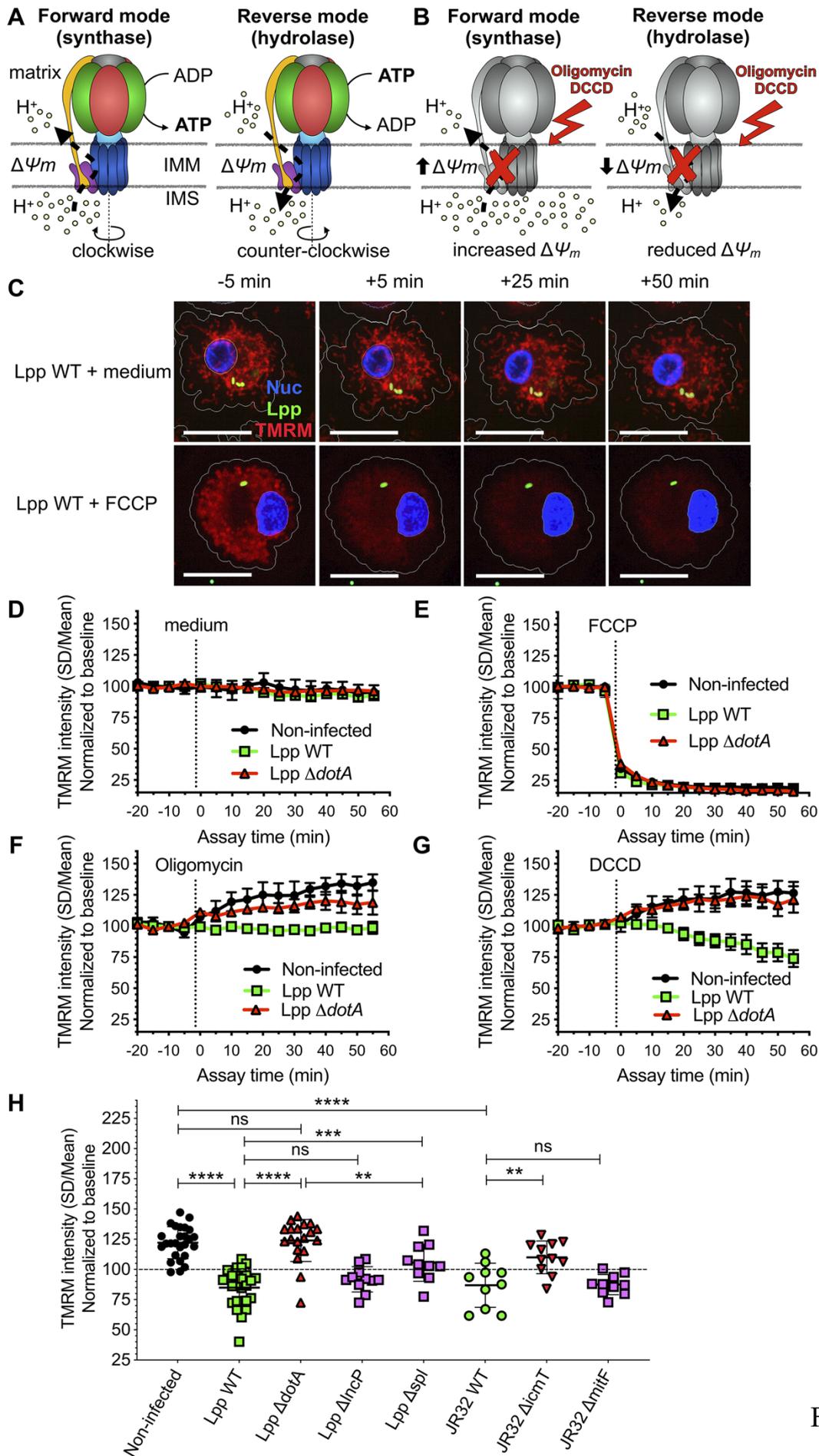
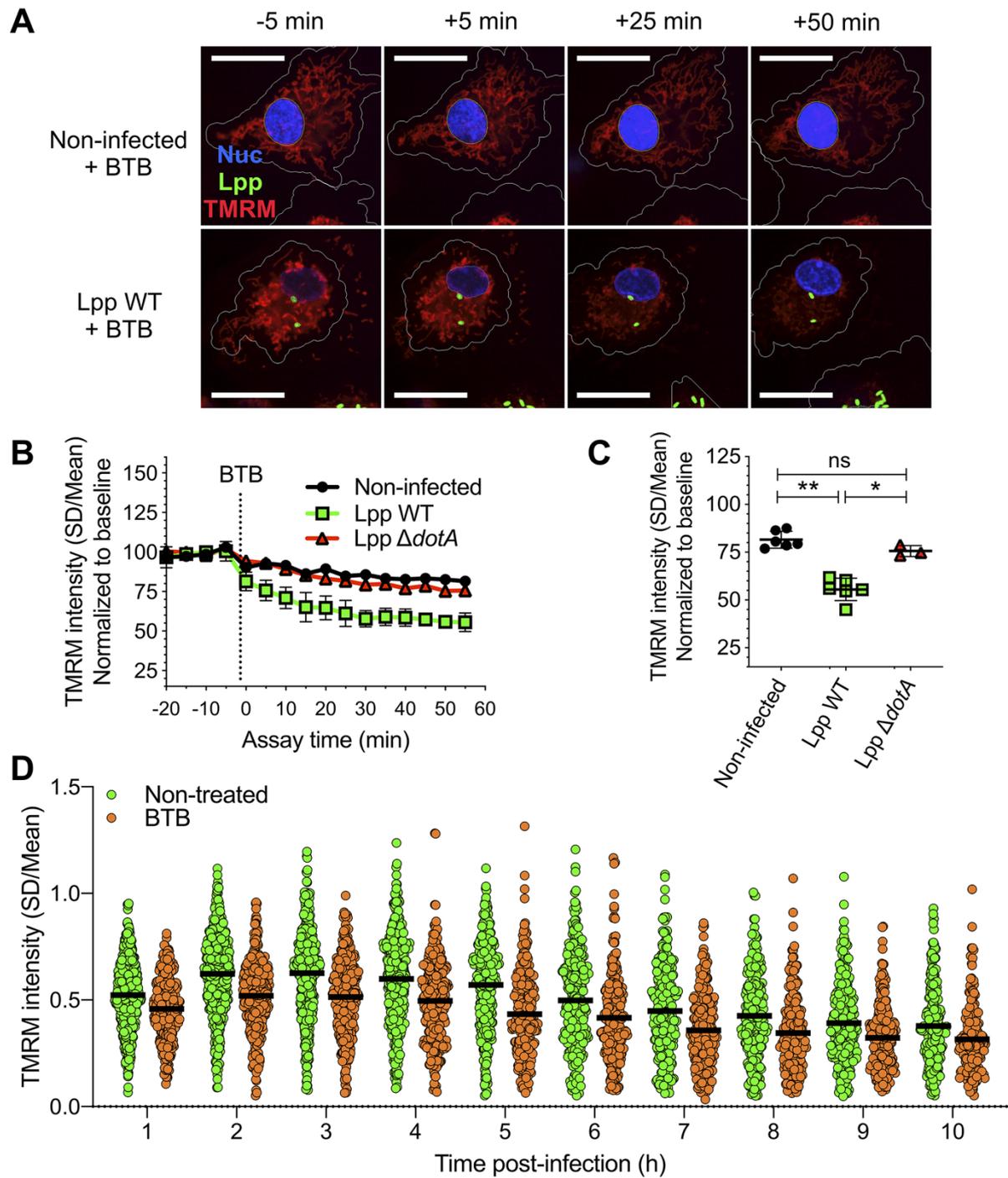


FIGURE 2



670

FIGURE 3

