

1 **Zebrafish larvae as a powerful model to dissect protective innate immunity in**  
2 **response to *Legionella pneumophila* infection**

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38 **Abstract**

39 The zebrafish has become a powerful model organism to study host-pathogen interactions. Here, we  
40 developed a zebrafish model of *Legionella pneumophila* infection to dissect innate immune  
41 responses. We show that *L. pneumophila* cause zebrafish larvae death in a dose dependent manner,  
42 and that macrophages are the first line of defence, with neutrophils cooperating to clear the  
43 infection. When either macrophages or neutrophils are depleted, the larvae become lethally  
44 sensitive to *L. pneumophila*. As observed in human infections, the adaptor signalling molecule Myd88  
45 is not required to control disease in the larvae. Furthermore, proinflammatory cytokines IL-1 $\beta$  and  
46 TNF $\alpha$  were upregulated during infection, recapitulating key immune responses seen in human  
47 infection. We also uncovered a previously undescribed phenotype in zebrafish larvae, whereby  
48 bloodborne, wild type *L. pneumophila* invade and grow in the larval yolk region but not a T4SS  
49 mutant. Zebrafish larva represent an innovative *L. pneumophila* infection model closely mimicking  
50 important aspects of human infection.

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## 55 INTRODUCTION

56 *Legionella pneumophila*, a gram negative, facultative intracellular bacterium inhabits natural,  
57 freshwater sources<sup>1,2</sup>. As an environmental, aquatic microbe *L. pneumophila* replicates intracellularly  
58 in aquatic protozoa<sup>3</sup>. Most interestingly, in contrast to other intracellular pathogens *L. pneumophila*  
59 is not adapted to a single host, but it exhibits a broad host range including Amoebozoa (amoebae),  
60 Percolozoa (excavates) and Ciliophora (ciliated protozoa)<sup>3,4</sup>. In the environment *L. pneumophila* can  
61 also be found within biofilms where it acquires nutrients from this mixed community, but it can also  
62 survive in a planktonic form for a certain time as well<sup>5</sup>. As fresh water and man-made systems are  
63 connected, *L. pneumophila* can also contaminate artificial water systems. Protected in its protozoan  
64 hosts *L. pneumophila* survives water disinfectants and may gain access to humans *via* aerosols  
65 produced by different man-made structures and devices. The inhalation of *L. pneumophila*  
66 contaminated aerosols can cause a severe pneumonia, the so-called Legionnaires' disease<sup>6</sup>.  
67 However, not every infection leads to disease. Disease outcome is determined by virulence of the  
68 bacterial strain, bacterial burden in the inhaled aerosols and most importantly by the host immune  
69 status. Host factors determining susceptibility include age above 50, smoking and/or having chronic  
70 lung disease, being immunocompromised and genetic factors that alter the immune response<sup>2,7,8</sup>.

71         Once the bacteria reach the lungs of susceptible individuals, they can infect alveolar  
72 macrophages and replicate therein. After being phagocytosed *L. pneumophila* avoids lysosomes and  
73 establishes an endoplasmic reticulum derived vacuole named the *Legionella* containing vacuole (LCV)  
74<sup>9,10</sup>. The LCV, a safe haven for bacterial replication, is established by utilizing the Dot/Icm type IV  
75 secretion system (T4SS) that injects over 350 proteins into the host cell<sup>9-11</sup>. These effector proteins  
76 manipulate a myriad of host pathways to recruit vesicles derived from the endoplasmic reticulum to  
77 the LCV, to supply the bacteria with nutrients, restrain autophagy and suppress apoptosis or to  
78 subvert the host cell immune response<sup>9-11</sup>. A surprising high number of these effectors mimic host  
79 proteins and encode eukaryotic functions helping *L. pneumophila* to subvert numerous host  
80 pathways in remarkable diverse ways<sup>11-13</sup>

81         Intracellular bacterial replication and innate immune responses have been studied *in vitro*  
82 using both murine and human cell lines and *in vivo* using different animal models of *L. pneumophila*  
83 infection. However, results obtained with these models cannot be easily extrapolated to what is  
84 observed in human disease. Studies in invertebrate models, for example in *Galleria mellonella* and  
85 *Caenorhabditis elegans*,<sup>14,15</sup> require further validation in more developed models as their immune  
86 system greatly differs from that of vertebrates. More interestingly, mouse infection fails to recall the  
87 human disease phenotype, as most inbred mice strains are naturally resistant to *L. pneumophila*<sup>16</sup>.  
88 Very early after the discovery of *L. pneumophila* the guinea pig model of Legionnaires' disease was  
89 developed. Guinea pigs are highly susceptible to *L. pneumophila* when infected through injection into

90 the peritoneum <sup>6</sup> or when exposed to *L. pneumophila* containing aerosols <sup>6</sup>. Several studies  
91 thereafter have shown that the guinea pig infection model recalls human disease and allows to study  
92 the immune response to *L. pneumophila* infection <sup>17,18</sup>. However, the guinea pig model is now rarely  
93 used due to the limited availability of specific immunological reagents for these animals and the  
94 demanding laboratory and husbandry requirements to work with guinea pigs.

95                 Since the above-mentioned models, including the widely used murine models,  
96 are limited for studying *L. pneumophila* infection *in vivo* and discrepancies exist between results  
97 obtained in mouse or human cells, the development of new, alternative models for *Legionella*  
98 infection is important. The zebrafish (*Danio rerio*) originally introduced as a model organism in  
99 developmental biology has emerged in recent years as a powerful non-mammalian model to study  
100 nearly every aspect of biology, including immune cell behaviour and host-pathogen interactions <sup>19,20</sup>.  
101 Zebrafish are evolutionary closer to humans than fruit flies and nematodes, easier to manipulate  
102 than mice and their immune system is remarkably similar to the one of mammals, making them an  
103 attractive laboratory model for immunology and infection biology <sup>19,20</sup>. Its popularity is also due to its  
104 small size and the natural translucency of its embryos and larvae, which makes it possible to follow  
105 leukocyte behaviour and infection onset at the level of the whole organism in real-time and high  
106 resolution <sup>21</sup>. Additionally, although adult organisms display a fully developed immune system with  
107 both active innate and adaptive branches, studies can also be conducted at the early stages of life  
108 (embryonic or larvae) when the organism solely relies on innate immunity, allowing to dissect the  
109 mechanisms arising from different immune responses <sup>21-23</sup>. Thus, we sought to examine whether the  
110 zebrafish could be an alternative model for analysing host-pathogen interactions and the innate  
111 immune response to *L. pneumophila* infection.

112                 We show that *L. pneumophila* infection of zebrafish larvae recapitulate human disease onset,  
113 as infected wild-type larvae are generally able to clear the infection, but immunocompromised fish  
114 fail to do so. Both macrophages and neutrophils quickly interact and engulf injected *L. pneumophila*.  
115 Macrophage-depleted larvae show a dramatic increase of bacterial burden concomitant with host  
116 death, pointing to a crucial role of macrophages in controlling the infection. Interestingly, we  
117 discovered a new infection phenotype, as *L. pneumophila* replicates in the larvae yolk region, where  
118 it seems to be able to avoid the immune response of the host.

119

## 120 **RESULTS**

### 121 ***L. pneumophila* infection induces mortality in zebrafish larvae in a dose dependent manner**

122 To analyse whether *L. pneumophila* can cause disease in zebrafish larvae we microinjected larvae 72  
123 hours post fertilisation (hpf) intravenously in the caudal vessels near the cloaca (UGO) (Fig 1A), with  
124 10<sup>3</sup> or 10<sup>4</sup> CFU of wild type (WT) *L. pneumophila* strain Paris expressing GFP (WT-GFP) or the type IV

125 secretion system (T4SS) deficient isogenic mutant expressing GFP ( $\Delta dotA$ -GFP). The infected larvae  
126 were kept at 28°C and were monitored regularly until 72 hours post infection (hpi) to record survival  
127 or death using a stereomicroscope. Larvae infected with doses of up to  $3 \times 10^3$  CFU of WT-GFP  
128 (defined as low dose, LD) all survived (100% survival). In contrast, larvae infected intravenously with  
129 doses of  $10^4$  CFU (defined as high dose, HD) resulted in approximately 30% of death within 72 hpi (Fig  
130 1B). Importantly, all larvae injected with LD or HD of the  $\Delta dotA$ -GFP strain survived for the entire  
131 time of observation (Fig 1B) indicating that the T4SS is important for replication in zebrafish larvae as  
132 it is in other infection models and in humans.

133  
134 We then set up a method to monitor the bacterial burden of the infected zebrafish larvae. The  
135 progression of the infection was followed by analysing the bacterial load at 0, 24, 48 and 72 hpi  
136 comparing three different methods. First, we quantified the pixel counts of GFP fluorescence of live  
137 larvae images (Fig. S1A), secondly, we analysed the number of GFP expressing bacteria present in  
138 lysed infected larvae by FACS (Fig. S1B) and thirdly we plated serial dilutions of homogenates of  
139 euthanized larvae on BCYE medium (Fig S1C). The results obtained with the three methods were  
140 comparable (Fig S1). We choose to routinely monitor the *L. pneumophila* load of zebrafish larvae by  
141 FACS. As shown in Fig. 1C, larvae injected with LD of WT-GFP progressively eliminate the bacteria, by  
142 24 hpi. Similarly, with high doses of  $\Delta dotA$ -GFP were progressively cleared by 24 hpi. In contrast,  
143 some zebrafish larvae injected with HD of WT-GFP were unable to eliminate the bacteria at 72hpi,  
144 and the bacterial burden even increased by 48-72 hpi (Fig 1C). We also monitored infected larvae by  
145 fluorescent microscopy. Immediately upon injection (20 min to 2 hpi), bacteria were detectable as  
146 small foci, probably associated with professional phagocytes (Fig. 1D). By 24 hpi, in both, larvae  
147 injected with LD of WT-GFP as well as larvae injected with HD of the avirulent  $\Delta dotA$ -GFP strain, the  
148 GFP signal declined becoming undetectable by 48 hpi, suggesting that the bacteria were  
149 progressively cleared. Despite showing the same pattern 24 hpi, larvae injected with HD of WT-GFP  
150 displayed a radically different progression of infection at 48 hpi, as bacterial proliferation started in a  
151 fraction of the infected larvae as seen by an increase in GFP signal. Most interestingly, in these  
152 larvae, bacterial proliferation occurred mainly in the yolk region while the bacterial load in the body  
153 decreased simultaneously. These bacterial foci in the yolk increased dramatically over time, causing  
154 death of the infected larvae by 72 hpi (Fig 1D).

155 Collectively our results indicate that *L. pneumophila* WT, but not the T4SS mutant induces  
156 death of zebrafish larvae. Larvae that were unable to control infection by 72 hpi, showed a unique  
157 phenotype, an increase of the bacterial burden in the yolk region.

158

159 **Bloodstream *L. pneumophila* establishes a proliferative niche in the yolk region causing a persistent**  
160 **infection**

161 To characterise the *L. pneumophila* foci identified in the yolk region of zebrafish larvae, we used high  
162 resolution fluorescent microscopy of HD of WT-GFP bloodstream injected in 72hpf  
163 Tg(*mfap4::mCherryF*) (herein referred as *mfap4:mCherryF*) (red macrophages) or Tg(*Lyz::DsRed*)<sup>nz50</sup>  
164 (herein referred as *lyz:DsRed*) (red neutrophils) or Tg(*kdr1::mCherry*)<sup>is5</sup> (red blood vessels) larvae.  
165 Upon injection of HD of WT-GFP, bacteria were progressively eliminated by the rest of the body and  
166 appeared growing in the yolk region between 48 and 72hpi, with macrophages accumulating there  
167 (Fig. 2A). We observed that *L. pneumophila* foci in the yolk region are highly complex, aggregate-like  
168 structures of long, filamentous bacteria growing in the yolk cell region and not in the visceral organs  
169 of the zebrafish larva. Macrophages were recruited to the yolk region containing *L. pneumophila*,  
170 (Fig. 2B, D Movie S1). Similarly, upon injection of HD of WT-GFP in *lyz:DsRed* larvae (red neutrophils),  
171 neutrophils were recruited to and accumulated around the growing bacterial aggregates, but seem  
172 unable to engulf them (Fig 2E, Movie S2). Moreover, confocal microscopy revealed that *L.*  
173 *pneumophila* exhibits grow in aggregates, and that these growing complex bacterial structures  
174 localize in the yolk and or in the yolk tube (Fig. 2F, Movie S3). Upon injection HD of WT-GFP in  
175 Tg(*kdr1::mCherry*)<sup>is5</sup> (red blood vessels) larvae, we also showed that, the fast growing bacterial  
176 aggregates interact with the blood vessels (Fig 2G, Movie S4). It should be noted that the yolk is the  
177 only food source of the larvae during this developmental stage. The fast proliferation of the bacteria  
178 in the yolk region probably depletes its nutritional content, leading to larvae death (Fig 2, Movie S1).  
179 Strikingly, zebrafish larvae infected with the T4SS deficient  $\Delta dotA$  mutant strain, did neither develop  
180 bacterial colonisation of the yolk nor larval death. This outcome was independent of the used dose,  
181 suggesting that zebrafish susceptibility to *L. pneumophila* infection and yolk penetration depends on  
182 a functional T4SS system.

183 Thus, blood-borne *L. pneumophila* is able to invade the yolk sac of zebrafish larvae, a  
184 previously undescribed phenotype of bacterial infection in this model. Once in the yolk, the bacteria  
185 replicate extensively, forming complex, organized, aggregate-like structures that cannot be removed  
186 by macrophages and neutrophils, thereby avoiding the host's immune control and clearance,  
187 eventually leading to death of the larvae.

188  
189 **Infection of zebrafish larvae with high doses of *L. pneumophila* leads to macrophage and**  
190 **neutrophil death**

191 In human infection, alveolar macrophages are the primary cell type infected by *L. pneumophila*  
192 supporting its intracellular replication. Following infection, neutrophils are recruited to the lung and  
193 are key players for controlling infection as they possess antimicrobial activity and kill *L. pneumophila*

194 <sup>24</sup>. To analyse whether zebrafish infection mirrors human infection we monitored the interaction of  
195 zebrafish macrophages or neutrophils with the bacteria *in vivo*. The transgenic zebrafish larvae  
196 *mfap4:mCherryF* and *lyz:DsRed* were injected with low or high doses of WT-GFP or with high doses of  
197  $\Delta dotA$ -GFP. Infected larvae were monitored using widefield fluorescence microscopy and the  
198 number of leukocytes per larva was assessed by counting fluorescent macrophages and neutrophils  
199 over time until 72hpi. We observed that upon injection of high dose WT-GFP, the macrophage count  
200 decreased dramatically at 24hpi and then remained stable (Fig. 3A, B). Neutrophil counts gave similar  
201 results, as there was a dramatic decrease observed in neutrophil numbers starting at 24hpi, in  
202 particular after injection of high doses of WT bacteria Fig. 3C, D). Interestingly, upon infection with  
203 low doses of WT the neutrophil numbers decreased dramatically only at 24hpi but increased at 48hpi  
204 and 72hpi (Fig. 3D). In contrast macrophage and neutrophil counts remained unaffected upon  
205 injection of equal amounts of the avirulent  $\Delta dotA$  strain, suggesting that phagocyte death is linked to  
206 a functional T4SS system.

207 Taken together, these results show that high dose *L. pneumophila* infection leads to a  
208 decrease in the number of professional phagocytes dependent on the T4SS, similar to what is seen  
209 during human infection by *L. pneumophila* and *Mycobacterium tuberculosis* <sup>24,25</sup>

210

### 211 **Macrophages are the primary cells to phagocytise blood-borne *L. pneumophila* and neutrophils co-** 212 **operate to decrease bacterial load**

213 As macrophages and neutrophils are likely the phagocytes that interact with *L. pneumophila* we  
214 analysed phagocyte-*L. pneumophila* interactions *in vivo* by injecting *mfap4:mCherryF* or *lyz:DsRed*  
215 72hpf larvae with WT-GFP or  $\Delta dotA$ -GFP and recorded phagocyte-*L. pneumophila* interactions using  
216 high resolution confocal microscopy. This showed that upon injection of LD WT-GFP, macrophages  
217 immediately contacted and engulfed blood-borne bacteria, and the initial bacterial load was thereby  
218 unchanged for 8hpi. The GFP signal of the engulfed bacteria was present for a long time in  
219 macrophages, suggesting that live bacteria persist in macrophages *in vivo* over a certain period of  
220 time. However, macrophages were continuously recruited to the site of infection and by 16hpi the  
221 bacteria were mostly undetectable (Fig. 4A top panel, Movie S5). Macrophages that had engulfed a  
222 large amount of *L. pneumophila* stopped moving and rounded-up, suggesting cell death. Similarly,  
223 the inhibition of the migration of phagocytes by *L. pneumophila* has been observed previously during  
224 infection of RAW 264.7 macrophages and the amoeba *Dictyostelium discoideum* and *Acanthamoeba*  
225 *castellanii*, <sup>26,27</sup>. In contrast, zebrafish infected with HD of WT-GFP were not able to restrict the  
226 bacterial growth by 16hpi. HD of *L. pneumophila* formed big aggregates, that were not easily engulfed  
227 and cleared by macrophages (Fig 4A, bottom panel, Movie S5). Remarkably, macrophages were very  
228 efficient in engulfing and rapidly clearing high doses of blood-borne  $\Delta dotA$ -GFP bacteria. By 10hpi

229 most of the bacteria had been engulfed and cleared as suggested by the diffuse GFP staining in  
230 phagocytes (Fig. 4A, bottom panel, Movie S5). However, upon infection with a HD WT-GFP, bacteria  
231 were not completely cleared but persisted, and at 72hpi *L. pneumophila* was found in macrophages,  
232 suggesting that the bacteria are also replicating in macrophages of zebrafish larvae. Indeed, high  
233 resolution confocal microscopy showed that at 72hpi, *L. pneumophila* can also be found inside of  
234 macrophages in replicative vacuoles (Fig. S2).

235

236 The analyses of *L. pneumophila*-neutrophil interactions showed that these engulfed the bacteria  
237 trapped in the mesenchyme around the site of injection, but they were less efficient at clearing  
238 blood-borne bacteria. This is similar to what has been previously observed for infection of zebrafish  
239 larvae with *Escherichia coli* or *Shigella flexneri*<sup>22,28</sup>. Indeed, upon infection with a high dose of WT-  
240 GFP, *L. pneumophila* persisted in neutrophils and massive death of infected neutrophils occurred  
241 (Fig. 4B, second panel, Movies S6). In sharp contrast, neutrophils very efficiently engulfed and  
242 cleared large amounts of  $\Delta dotA$ -GFP aggregated and trapped in the mesenchyme (Fig. 4B, lower  
243 panel, Movie S6) as well as low doses of WT-GFP (Fig 4B upper panel, Movie S6).

244 Altogether this shows that upon bloodstream injection of *L. pneumophila*, macrophages and  
245 neutrophils efficiently cooperate to eliminate the majority of bacteria within 20-24 hpi, with  
246 macrophages playing the primary role. However, *L. pneumophila* is also able to persist and replicate  
247 in macrophages. In contrast, neutrophils interact with *L. pneumophila* by quickly engulfing bacteria  
248 trapped in the mesenchyme near the site of injection but are less efficient in clearing blood-borne  
249 bacteria.

250

### 251 ***Macrophages are the first line defence restricting L. pneumophila infection***

252 In humans, innate immune responses, based essentially on the activities of professional phagocytes  
253 and pro-inflammatory cytokine induction, are the key players to control and restrict *L. pneumophila*  
254 proliferation. Thus, human disease develops primarily in immunocompromised individuals<sup>10</sup>. To  
255 investigate whether the phagocytes of the innate immune system, macrophages and neutrophils, are  
256 also responsible for controlling *L. pneumophila* infection in zebrafish larvae, we selectively and  
257 transiently depleted macrophages or neutrophils, respectively and infected these  
258 “immunocompromised” larvae with *L. pneumophila*. Depletion of macrophages was achieved by  
259 knocking down the expression of *spi1b*, a transcription factor involved in early myeloid progenitor  
260 formation. A low dose of *spi1b* morpholino was reported to impact macrophages without affecting  
261 neutrophils<sup>29</sup>. We monitored the effect of low doses *spi1b* morpholino injection on macrophage and  
262 neutrophil populations in double transgenic larvae with green neutrophils (*mpx:GFP*) and red



263 macrophages (*mfap4:mCherryF*). The specific depletion of the two cell types was confirmed by  
264 counting macrophages and neutrophils 72hpf (Fig S3A).

265 We then infected macrophage depleted larvae (*spi1b* knockdown) by intravenous injection of  
266 LD or HD of WT-GFP. Independently of the infection dose, a dramatic decrease in survival occurred,  
267 as even injection of low doses of WT-GFP resulted in the death of 30% of the larvae (Fig 5A). When  
268 injecting high doses of WT-GFP nearly all of the infected larvae died by 72hpi, with the earliest  
269 deaths starting 48hpi (Fig 5A). In contrast, *spi1b* knockdown larvae injected with high doses of  $\Delta dotA$ -  
270 GFP did not show impaired survival (Fig 5A). The increased mortality correlated with an increased  
271 bacterial burden in *spi1b* knockdown larvae compared to control larvae as judged from counting  
272 bacteria growing on BCYE agar from homogenates of individual larvae by FACS analyses (Fig 5B).  
273 Intravital imaging of infected *spi1b* knock down larvae also showed that both low and high doses of  
274 WT-GFP failed to be cleared and that the bacteria established a replicative niche in the yolk, where  
275 they proliferated extensively (Fig 5C). This highlights, that macrophages are critical to restrict the  
276 onset of infection and *L. pneumophila* proliferation *in vivo*. Furthermore, these results also suggest  
277 that neutrophils, which are not depleted in *spi1b* knockdown larvae, fail to control *L. pneumophila*  
278 infection in the absence of macrophages.

279 We next analysed the role of neutrophils in controlling the infection. Neutrophil  
280 development was disrupted by knocking down the G-CSF/GCSFR pathway using *csf3R* morpholino,  
281 previously reported to decrease up to 70% of the neutrophils present<sup>30-32</sup>. We then monitored the  
282 efficiency of the *csf3R* morpholino knockdown in double transgenic larvae confirming that 75% of the  
283 neutrophil population was depleted, while macrophage numbers were only slightly decreased (Fig  
284 S3B). When HD  $\Delta dotA$ -GFP was injected, neutrophil-depleted larvae survived, and the bacterial  
285 burden remained unchanged, similar to what we had observed in infections of macrophage-depleted  
286 larvae (Fig. 5D, E). However, when neutrophil-depleted larvae were injected with HD WT-GFP, larvae  
287 survival significantly decreased and bacterial burdens increased at 48hpi (Fig. 5D, E). Neutrophil-  
288 depleted fish larvae showed an intermediate phenotype, displaying less survival and higher bacterial  
289 burden than in WT infected control larvae (Fig. 1A) but more survival and lower bacterial burden  
290 than in macrophage-depleted larvae (Fig. 5D, E). Intravital imaging showed that *csf3R* knockdown  
291 larvae that were unable to control *L. pneumophila* infection showed bacterial proliferation in the yolk  
292 comparable to WT control larvae (Fig 5F).

293 These results show that both neutrophils and macrophages are required for restricting and  
294 controlling *L. pneumophila* infection in the zebrafish model, but macrophages play the key role.  
295 Although neutrophils contributed less to clear the bacteria upon bloodstream injection, neutrophils  
296 might impact the infection outcome through cytokine release that can modulate macrophage  
297 activity.

298 **Key pro-inflammatory cytokines are induced upon *L. pneumophila* infection of zebrafish larvae**

299 Proinflammatory cytokines produced by infected and bystander cells during *L. pneumophila* infection  
300 of humans and mice play crucial roles in orchestrating host defences to control infection<sup>33,34</sup>.  
301 Infected cells produce IL-1 $\alpha$  and IL-1 $\beta$  through a mechanism involving MyD88-dependent  
302 translational bypass. In contrast, bystander cells produce IL-6, TNF- $\alpha$  and IL-12 in an IL-1 receptor (IL-  
303 1R) dependant way<sup>33,35</sup>. To determine the pro-inflammatory responses of zebrafish larvae during *L.*  
304 *pneumophila* infection, we analysed *il1b*, *tnfa*, and *ifng1/2* (orthologues of mammalian *Ifng*) gene  
305 expression levels over time by qRT-PCR on RNA isolated from individual infected larvae. We found  
306 that infection of zebrafish larvae with LD or HD of WT-GFP induced a rapid (by 6hpi) and robust  
307 induction of *il1b* and *tnfa* gene expression. In larvae injected with low doses of WT-GFP the  
308 expression levels started to decrease by 24hpi, and gradually became undetectable at 72hpi. In  
309 contrast, larvae injected with HD of WT-GFP, expression of *il1b* and *tnfa* did not decrease over time  
310 (Fig. S3A and B) and a significant induction of *ifng1* was observed at 48hpi (Fig. S3C) but not of *ifng2*  
311 (Fig. S3D). In parallel, we scored the bacterial burden of the infected larvae before pro-inflammatory  
312 cytokine measurement at each time point under the microscope, which consistently showed that  
313 larvae with increased *il1b* and *tnfa* induction had also high bacterial burdens in the yolk and were not  
314 controlling the infection. These pro-inflammatory responses were T4SS dependent, as zebrafish  
315 larvae infected with HD of  $\Delta dotA$ -GFP did not show significant induction of transcription of *tnfa*, *il1b*  
316 and *ing1/2* (Fig. S3 A-D).

317 Collectively, these results reveal, that key pro-inflammatory cytokines known to orchestrate  
318 the host response during *L. pneumophila* infection in humans are also induced in zebrafish larvae,  
319 and that cytokine gene induction is sustained when uncontrolled *L. pneumophila* proliferation occurs.

320

321 **The immune response of zebrafish larvae to *L. pneumophila* infection is independent of MyD88**  
322 **signalling**

323 In innate immunity, the myeloid differentiation factor 88 (MyD88) plays a pivotal role in immune cell  
324 activation through Toll-like receptors (TLRs). MyD88-deficient mice are highly susceptible to  
325 *L. pneumophila* infection<sup>36-39</sup>, however this is not the case when human macrophages are depleted  
326 of MyD88<sup>40</sup>. Therefore, we sought to analyse which role MyD88 plays in zebrafish larvae during  
327 *L. pneumophila* infection. We injected *myd88*<sup>-/-</sup> and control larvae with LD or HD of WT-GFP, or with  
328 HD of  $\Delta dotA$ -GFP and monitored larvae survival and bacterial burden over time as described in Figure  
329 1. Our results show that susceptibility to infection of *myd88*<sup>-/-</sup> larvae injected with HD of WT-GFP,  
330 was comparable to that of WT larvae (Fig. 6A). Similarly, both control and *myd88*<sup>-/-</sup> larvae injected  
331 with LD WT-GFP or with the avirulent  $\Delta dotA$ -GFP bacteria did not develop an infection, and the

332 bacterial burden decreased over time indicating that bacteria were cleared (Fig. 6A, B). To determine  
333 if pro-inflammatory responses were affected in the absence of MyD88 signalling, we analysed *il1b*  
334 and *tnfa* gene expression levels over time in control and *myd88*<sup>-/-</sup> larvae. Our results showed that  
335 *il1b* and *tnfa* gene expression levels were comparable in control and *myd88*<sup>-/-</sup> infected larvae for all  
336 conditions tested (LD WT-GFP and HD  $\Delta dotA$ -GFP (Fig 6C, D).

337 Taken together, our results suggest that MyD88 signalling is not required for the innate  
338 immune response against *L. pneumophila* infection in the zebrafish larvae, which recapitulates  
339 human infection. However, MyD88 signalling may also be functionally compensated by other  
340 immune signalling pathways.

341

### 342 ***Legionella pneumophila* replication in the yolk of zebrafish larvae is T4SS dependent**

343 Interestingly, replication of *L. pneumophila* mainly took place in the yolk region of infected zebrafish  
344 larvae (Movie S1-4, Fig. 2), dependent on a functioning T4SS as  $\Delta dotA$ -GFP failed to be detected in the  
345 yolk. To investigate whether the secretion mutant would be able to grow in the yolk cell when  
346 reaching it, we injected LD and HD of WT-GFP or  $\Delta dotA$ -GFP directly into the yolk cell cytoplasm of  
347 72hpf *lys:DsRed* zebrafish larvae (Fig. S4A). WT-GFP replicated extensively in the yolk region with low  
348 and high dose infections leading to rapid bacterial proliferation followed by a marked increase of the  
349 bacterial burden and death of the larvae (Fig. 7A, B). Surprisingly,  $\Delta dotA$ -GFP did not replicate in the  
350 yolk even when injected directly but persisted over 72hpi. This result suggests that T4SS system is  
351 not only crucial for crossing the yolk sac syncytium but that its effectors are also necessary to obtain  
352 nutrients from the environment to allow replication. To further analyze this hypothesis, we selected  
353 a mutant in the gene encoding a sphingosine-1 phosphate lyase, (WT,  $\Delta spl$ )<sup>41</sup> as we reasoned that  
354 this enzyme might be implicated in degrading sphingolipids present in the yolk of zebrafish larvae  
355 and thereby might aid *L. pneumophila* to obtain nutrients. Injection of  $\Delta spl$  in the yolk sac region, and  
356 analyses of larvae death as compared to WT or  $\Delta dotA$  showed that survival of zebrafish larvae  
357 injected with the  $\Delta spl$  was slightly higher than with WT injected larvae (Fig. S4B), suggesting that the  
358 T4SS effector *LpSpl* might be implicated in nutrient acquisition in the yolk environment.

359 Interestingly, the first isolation of *L. pneumophila* was achieved by inoculating the yolk region  
360 of embryonated eggs probably due to the richness in nutrients provided by the yolk<sup>6</sup>. Later yolk sacs  
361 of embryonated hen's eggs were used to produce polyvalent antigens for the diagnosis of  
362 *L. pneumophila*<sup>42</sup>. Thus, we decided to analyse *L. pneumophila* WT and  $\Delta dotA$  phenotypes in the yolk  
363 sac of embryonated chicken eggs (ECE). We inoculated ECE directly in the yolk region with WT and  
364 with the  $\Delta dotA$  strain at a concentration of 9.2 log<sub>10</sub> CFU/mL and 9.1 log<sub>10</sub> CFU/mL, respectively and  
365 assessed mortality of the embryos daily. The total mortality during the 6-day observation period in  
366 WT-GFP infected eggs was significantly higher (88.9%) than in the  $\Delta dotA$ -infected eggs (14.3%;

367  $p=0.010$ ) or PBS inoculated control eggs (28.6%;  $p=0.010$  and  $p=0.021$ , respectively), which were not  
368 significantly different from each other ( $p=0.253$ ) (Fig. S4C). The highest mortality was observed at 2  
369 days post infection in WT inoculated eggs with 55.6% mortality *versus* 0% in  $\Delta dotA$  or 28.6%  
370 mortality in PBS inoculated eggs. Quantification of *L. pneumophila* in the yolk sac region at the day of  
371 mortality or at day 6 post infection revealed that the number of bacteria in the yolk sac of WT-  
372 infected ECE, was significantly higher than that in the yolk sac of those infected with the  $\Delta dotA$  strain  
373 ( $7.8 \log_{10}$  CFU/mL and  $5.9 \log_{10}$  CFU/mL, respectively,  $p=0.0127$ ) (Fig. S4D). Controls inoculated with  
374 PBS ( $n=2$ ) showed no *L. pneumophila* growth. Thus, like in zebrafish larvae only the WT strain is able  
375 to replicate in the yolk region and of inducing mortality in the embryos, while the T4SS mutant strain  
376 persists but is not able to replicate and does not induce high embryo mortality. This result further  
377 supports the finding that the T4SS system is crucial for obtaining nutrients when lipids are the major  
378 energy source available.

379 We next monitored neutrophil behaviour in the yolk-injected *lyz:DsRed* larvae in which  
380 neutrophils are labelled red. This showed that replication of WT-GFP in the yolk coincides with  
381 neutrophil death (Fig. 7C and D). The yolk cell is a single large cell where leukocytes were described  
382 to be unable to enter<sup>43</sup>, but interestingly, macrophages and neutrophils were highly recruited to the  
383 yolk of WT-GFP infected larvae (Figure 2B-E), suggesting that *L. pneumophila* is sensed by the  
384 immune system even when replicating in the yolk, and could induce neutrophil death “at distance”. It  
385 is likely neutrophils can partly counteract *L. pneumophila* growth in the yolk by degranulating “at  
386 distance”, as previously shown in a zebrafish notochord infection model using non-pathogenic *E. coli*  
387<sup>32</sup>.

388 Our results suggest that the *L. pneumophila* T4SS plays a crucial role for the bacteria to pass  
389 from the blood circulation into the yolk and that T4SS effectors play an important role to obtain  
390 nutrients for bacterial proliferation.

## 391 392 **DISCUSSION**

393 In this study, we developed a zebrafish larva infection model for *L. pneumophila* and have analysed  
394 host pathogen interactions and the innate immune response of the host. We have found that a  
395 successful infection of zebrafish larvae by *L. pneumophila* depends on the infection site, the infection  
396 dose, the T4SS Dot/Icm and the host innate immune response, in particular macrophages and  
397 neutrophils. Wild type zebrafish larvae are susceptible to infection in a dose dependent manner, as  
398 larvae infected with a highly concentrated bacterial inoculum displayed bacterial dissemination and  
399 replication, concomitant with host death. However, as only about 30% of the larvae displayed this  
400 phenotype, the innate host defence of the larvae against *L. pneumophila* infection is relatively  
401 efficient. Thus, similar to what is observed in *L. pneumophila* infection of immune competent

402 individuals, the development of Legionnaire's disease is determined not only by the infection dose  
403 but also by the capacity of the host immune system to quickly and efficiently respond to infection.

404 Only blood borne bacteria are able to proliferate and induce mortality in zebrafish larvae.  
405 Once in the blood circulation, bacteria are actively engulfed and eliminated by both macrophages  
406 and neutrophils. However, some bacteria resist intracellular killing and replicate extensively inside  
407 macrophages (Fig. S2), get released into the blood flow and circulate in the zebrafish larvae. Some  
408 reach the yolk sac syncytium and T4SS competent *L. pneumophila* are able to cross this barrier and  
409 enter the yolk sac region. Once in the yolk, *L. pneumophila* gains a significant advantage in the  
410 pathogen-host arms race and establishes a replicative niche where it proliferates extensively. Indeed,  
411 in the yolk sac region *L. pneumophila* is protected from the host immune system as professional  
412 phagocytes are unable to enter in the yolk. Proliferation of the bacteria leads to host death, likely  
413 due to exhaustion of the nutrients present in the yolk, which are key in supporting the larvae  
414 development and due to the physical compression of the visceral developing organs, in particular the  
415 gastro-intestinal tract, exerted by the growing bacterial aggregate. Interestingly, we have also  
416 observed that in few cases the infected larvae were able to extrude the bacterial aggregates growing  
417 in the yolk and survived. This host defence mechanism has also been reported in a caudal fin model  
418 of *Mycobacterium marinum* infection, where infected zebrafish larvae extruded the bacteria-  
419 containing granuloma <sup>44</sup>.

420 To our knowledge, the establishment of a replicative niche in the yolk upon injection in the  
421 bloodstream is unique to *L. pneumophila*. Most interestingly, direct yolk sac injection revealed that  
422 only the WT strain but not the T4SS knockout strain is able to replicate and establish a persistent  
423 infection, irrespective of the dose injected. This result points towards the involvement of the T4SS  
424 system and its secreted effectors in infection, replication and nutrient uptake in the yolk  
425 environment. Further analyses of this phenotype in embryonated chicken eggs, a commonly used  
426 model for antigen preparation, showed again, that only WT *L. pneumophila* are able to replicate in  
427 the yolk sac region, confirming the importance of the T4SS in nutrient uptake in addition to its known  
428 role in infection (Fig. S4A, B, C). *L. pneumophila* is known to mainly use amino acids as carbon and  
429 energy sources for growth <sup>45</sup> and secreted T4SS effectors have been shown to aid in amino acid  
430 uptake <sup>46</sup>, however, fatty acids, glucose and/or glycerol also serve as carbon sources during the later  
431 stages of the life cycle of *L. pneumophila* <sup>47,48</sup>, but no effectors connected to the uptake of these  
432 nutrients have been identified yet. The yolk cell is composed of a complex and dynamic mixture of  
433 different lipids on which the zebrafish larvae rely on for nutrition throughout development in the  
434 early larva phase. Cholesterol and phosphatidylcholine are the main constituents until 120hpf, with  
435 triacylglycerol, phosphatidylinositol, phosphatidylethanolamine, diacyl-glycerol, cholesteryl esters  
436 and sphingomyelins also present in significant concentrations <sup>49</sup>. *L. pneumophila* is known to secrete

437 several effectors with lipolytic activity through its T4SS which could be important for growth in a lipid  
438 rich environment like the yolk (Hiller et al., 2018). In a first attempt to identify one of these effectors  
439 we analysed the growth of a *L. pneumophila* mutant in a gene encoding a sphingosine-1 phosphate  
440 lyase (*LpSpl*)<sup>41</sup> compared to the WT strain after direct injection in the zebrafish larvae yolk sac.  
441 Indeed, a small difference in larvae mortality was observed for the  $\Delta spl$  strain, suggesting that *LpSpl*  
442 is one of several effectors that might participate in nutrient acquisition from lipids (Fig. S4B).  
443 However, further analyses are needed to identify all effectors implicated in this phenotype.

444 Studies of *Legionella* infection in humans, guinea pigs and mouse lungs have shown that  
445 *L. pneumophila* interacts closely with neutrophils and mononuclear phagocytes<sup>50,51</sup>. Professional  
446 phagocytes are the main replication niche for *L. pneumophila* with monocytes and macrophages, in  
447 particular alveolar macrophages, representing the main cells for replication in the lungs<sup>52-55</sup>. *In vivo*  
448 studies in mice have shown that upon lung infection with *L. pneumophila* neutrophils, cDCs,  
449 monocytes, and monocyte-like cells are rapidly recruited to the infection site, but although all these  
450 cells seem to engulf the bacteria, *L. pneumophila* appears to be able to translocate effectors only into  
451 neutrophils and alveolar macrophages. In zebrafish macrophages appear during the first days of  
452 development, followed by neutrophils a day later forming together an efficient immune system that  
453 protects the developing fish<sup>23,56-58</sup>. Therefore, the zebrafish larva offers a unique possibility to  
454 interrogate the role of innate immune responses to infection<sup>21</sup>. Indeed, macrophage depleted larvae  
455 showed a dramatically increased susceptibility to *L. pneumophila* infection as nearly 100% of larvae  
456 inoculated with HD of WT and 30% of larvae inoculated with LD of *L. pneumophila* died from the  
457 infection. Hence, macrophages are the first line of infection control against *L. pneumophila* and are  
458 essential for restricting and controlling blood-borne infections, similar to what was observed for  
459 *Burkholderia cenocepacia* or *Staphylococcus aureus* infection<sup>59,60</sup>. In contrast, when neutrophils were  
460 depleted, the innate immune response was impaired to a lesser extent, suggesting that neutrophils  
461 are required to ensure an effective innate immune response and, that macrophages alone are not  
462 able to contain high burdens of *L. pneumophila* infection (Fig. 5).

463 Human innate immune signalling relies strongly on activation of Toll-like receptors (TLRs) and  
464 respective adaptor molecules, all of which are highly conserved in the zebrafish<sup>61,62</sup>. One of these  
465 adaptors is MyD88, known as a central player in interleukin 1 receptor (IL-1R) and TLR signalling in  
466 humans and mammalian models<sup>63</sup>. MyD88 signalling is crucial for mice to combat *L. pneumophila*  
467 infection, as it triggers the early secretion of inflammatory cytokines, neutrophil recruitment, and the  
468 host immune response to the infection. Consequently, mice that lack MyD88 are highly susceptible  
469 to infection<sup>35-38</sup>. However, in MyD88 depleted human macrophages *L. pneumophila* replication is not  
470 different to replication in WT cells<sup>40</sup> Here we show, that *L. pneumophila* infected *myd88*<sup>-/-</sup> zebrafish  
471 larvae have the same replication phenotype as WT larvae. Thus, Myd88 signalling does not play a key

472 role or may be redundant in the control of the innate immune response to *L. pneumophila* in  
473 zebrafish larvae, indicating that zebrafish mirrors human infection better than the mouse model. In  
474 the mouse model infected macrophages are incapable of producing cytokines, such as tumor  
475 necrosis factor (TNF) and interleukin-12 (IL-12), which are necessary to control infection. In contrast,  
476 infection of zebrafish larvae with WT *L. pneumophila* induced a rapid (by 6hpi) and robust induction  
477 of *il1b* and *tnfa* gene expression. However, it is thought that IL-1 released initially by *L. pneumophila*-  
478 infected macrophages drives the production of critical cytokines by bystander cells<sup>33</sup>. Infection of  
479 zebrafish larvae with HD of WT *L. pneumophila* induced a rapid (by 6hpi) and robust induction of *il1b*  
480 and *tnfa* gene expression whereas WT LD infection leads only to a short induction of *il1b* transcript  
481 levels at 6hpi before declining to CTRL levels at later time points, suggesting that a short boost of IL-  
482 1 $\beta$  is sufficient to control LD of *L. pneumophila*. However, for a high load of *L. pneumophila* even a  
483 high and long-term induction of IL-1 $\beta$  is not allowing to control the infection, suggesting that the self-  
484 regulation of the immune response may be abrogated leading to a constant activation of IL-1 $\beta$   
485 expression. Moreover, gene expression analyses also confirms that Myd88 has no influence on the  
486 control of the infection, as no difference in the transcript levels of *il1b*, *tnfa*, *ifng1* or *ifng2* was  
487 observed further suggesting that activation of the IL1R and certain TLR pathways are not crucial for  
488 *L. pneumophila* clearance in zebrafish larvae. One may even hypothesise that IL-1 $\beta$  release could be  
489 beneficial for *L. pneumophila* replication, as it was shown that IL-1 $\beta$  also may indicate an activation of  
490 the metabolic state of the bystander cells as it was shown that IL-1 $\beta$  induces a shift towards more  
491 metabolically active cells and increased cellular glucose uptake<sup>64</sup>, which could aid *L. pneumophila*  
492 replication.

493 In conclusion, we have set up a new infection model for *L. pneumophila* that mimics human  
494 infection better than the mouse model. The unique advantages of the zebrafish provide now exciting  
495 possibilities to further explore different aspects of the relationship between, *L. pneumophila* and its  
496 host: the dynamics of bacterial dissemination, the interactions of the bacteria with macrophages and  
497 neutrophils, as well as the host immune response by intravital imaging.

498

## 499 **EXPERIMENTAL PROCEDURES**

500 **Ethics Statement.** Animal experiments were performed according to European Union guidelines for  
501 handling of laboratory animals

502 ([http://ec.europa.eu/environment/chemicals/lab\\_animals/home\\_en.htm](http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm)) and were approved by  
503 the Institut Pasteur Animal Care and Use Committee. and the French Ministry of Research  
504 (APAFIS#31827). The inoculation of embryonated chicken eggs is a standard procedure in diagnostics  
505 for the multiplication and antigen production of *Legionella* and is not covered by the national law for  
506 animal experiments in France (Décret n° 2013-118 du 1er février 2013).

507 **Zebrafish care and maintenance.** Wild-type AB fish, initially obtained from the Zebrafish  
508 International Resource Center (Eugene, OR), Tg(*Lyz::DsRed*)<sup>nz50 65</sup>, Tg(*mfap4::mCherryF*) (ump6Tg)<sup>32</sup>  
509 Tg(*mpx:GFP*)<sup>i114 66</sup>, Tg(*kdr1::mCherry*)<sup>is5 67</sup> and *myd88*<sup>hu3568</sup> mutant line (obtained from the Hubrecht  
510 Laboratory and the Sanger Institute Zebrafish Mutation Resource)<sup>68</sup>, were raised in our facility. Eggs  
511 were obtained by natural spawning, bleached according to standard protocols, and kept in Petri  
512 dishes containing Volvic source water and, from 24 hours post fertilization (hpf) onwards 0.003% 1-  
513 phenyl-2-thiourea (PTU) (Sigma-Aldrich) was added to prevent pigmentation. Embryos were reared  
514 at 28°C or 24°C according to the desired speed of development; infected larvae were kept at 28°C.  
515 Timings in the text refer to the developmental stage at the reference temperature of 28.5°C. Larvae  
516 were anesthetized with 200µg/ml tricaine (Sigma-Aldrich) during the injection procedure as well as  
517 during *in vivo* imaging and processing for bacterial burden evaluation or cytokine expression studies.

518  
519 **Bacterial strains and growth conditions.** *Legionella pneumophila* strain Paris carrying the pNT28  
520 plasmid encoding for green fluorescent protein (constitutive GFP)<sup>69</sup>, wild-type (WT-GFP) or  $\Delta dotA$ -  
521 GFP were plated from -80°C glycerol stocks on N-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-  
522 buffered charcoal yeast-extract (BCYE) medium supplemented with 10 µg/ml of chloramphenicol and  
523 cultured for 3 days at 37°C. Suspensions were prepared by resuspending bacteria in sterile 1x  
524 Phosphate Buffered Saline (PBS) and adjusting the OD 600 according to the desired bacterial  
525 concentrations for injection.

526  
527 **Morpholino injections.** Morpholino antisense oligonucleotides (Gene Tools LLC, Philomath, OR, USA)  
528 were injected at the one to two cell stage as described<sup>70</sup> A low dose (4ng) of *spi1b* (previously named  
529 *pu1*) translation blocking morpholino (GATATACTGATACTCCATTGGTGGT)<sup>71</sup> blocks macrophage  
530 development only, but can also block neutrophil development when it is injected at a higher dose  
531 (20ng in 2nl). The *csf3r* translation blocking morpholino (GAACTGGCGGATCTGTAAAGACAAA) (4ng)<sup>30</sup>  
532 was injected to block neutrophil development. Control morphants were injected with 4ng control  
533 morpholino, with no known target (GAAAGCATGGCATCTGGATCATCGA).

534  
535 **Zebrafish infections.** The volume of injected suspension was deduced from the diameter of the drop  
536 obtained after mock microinjection, as described in<sup>70</sup>. Bacteria were recovered by centrifugation,  
537 washed, resuspended at the desired concentration in PBS. 72h post-fertilization (hpf) anesthetized  
538 zebrafish larvae were microinjected iv or in the yolk with 0.5-1nl of bacterial suspension at the  
539 desired dose (~10<sup>3</sup> bacteria/nl for Low Dose (LD) and ~10<sup>4</sup> bacteria/nl for High Dose (HD) as  
540 described<sup>22,28</sup>. Infected larvae were transferred into individual wells (containing 1ml of Volvic water +



541 0.003% PTU in 24-well culture plates), incubated at 28°C and regularly observed under a  
542 stereomicroscope.

543

544 **Evaluation of the bacterial burden in infected larvae.** Infected zebrafish larvae were collected at 0,  
545 24, 48 and 72hpi and lysed for analysing the bacterial burden by FACS. Each larva was placed in a 1.5  
546 ml Eppendorf tube and anesthetized with tricaine (200µg/ml), washed with 1ml of sterile water and  
547 placed in 150 µl of sterile water. Larvae were then homogenized using a pestle motor mixer (Argos).  
548 Each sample was transferred to an individual well of a 96 well plate, counted on a MACSQuant VYB  
549 FACS (Miltenyi Biotec) and data analysed using FlowJo version 7.6.5. For CFU enumeration, serial  
550 dilutions were plated on BCYE agar plates supplemented with Chloramphenicol and the *Legionella*  
551 Selective Supplement GVPN (Sigma). Plates were incubated for 4-5 days at 37°C and colonies with  
552 the appropriate morphology and colour were scored using the G-Box imaging system (Syngene) and  
553 colonies enumerated using the Gene Tools software (Syngene).

554

555 **Dissociation of zebrafish larvae for FACS analysis of macrophages.** Three to five  
556 Tg(*mfap4::mCherryF*) larvae were pooled in single 1.5 ml Eppendorf tubes and anesthetized with  
557 tricaine. The supernatant was discarded, and the larvae were resuspended in 1ml of 1x trypsin-EDTA  
558 solution (SIGMA) and incubated in a dry heat block at 30°C for 10 - 20 min. Every 2 minutes, the  
559 suspensions were homogenised by pipetting, until full homogenisation was reached. CaCl<sub>2</sub> (final  
560 concentration of 2µM) and foetal bovine serum (final concentration of 10%) were added to each  
561 tube and samples were kept on ice. Lysates were filtered using 40 µm strainers, washed with 20 ml  
562 ice cold 1X PBS and centrifuged 5 min at 1500 g, 4°C. Remaining pellets were resuspended in 250 µl  
563 1X PBS and analysed with a MACSQuant VYB FACS (Miltenyi Biotec).

564

565 **Live imaging, image processing and analysis.** Quantification of total neutrophils and/or macrophages  
566 on living transgenic reporter larvae was performed upon infection as previously described<sup>28</sup>. Briefly,  
567 bright field, DsRed and GFP images of whole living anesthetized larvae were taken using a Leica  
568 Macrofluor™ Z16 APOA (zoom 16:1) equipped with a Leica PlanApo 2.0X lens, and a Photometrics®  
569 CoolSNAP™ HQ2 camera. Images were captured using Metavue software 7.5.6.0 (MDS Analytical  
570 Technologies). Then larvae were washed and transferred in a new 24 wells plate filled with 1ml of  
571 fresh water per well, incubated at 28°C and imaged again under the same conditions the day after.  
572 Pictures were analysed, and Tg(*lyzC::DsRed*) neutrophils or Tg(*mfap4::mCherryF*) macrophages  
573 manually counted using the ImageJ software (V 1.52a). Counts shown in figures are numbers of cells  
574 per image.

575 The bacterial burden was measured by counting the total number of pixels corresponding to  
576 the GFP channel (Metavue software 7.5.6.0). Briefly, images corresponding to the GFP channel were  
577 adjusted to a fixed threshold that allowed to abrogate the background of the autofluorescence of the  
578 yolk. The same threshold was used for all images of one experiment. Histogram in the Analyze menu  
579 was used to obtain the number of black and white pixels. As shown in figure S1A, number of white  
580 pixels corresponding to *L. pneumophila* are plotted using GraphPad Prism® software.

581 High resolution confocal live imaging of injected larvae was performed as previously  
582 described<sup>72</sup>. Briefly, injected larvae were positioned in lateral or ventral position in 35 mm glass-  
583 bottom-Dishes (Ibidi Cat#: 81158). Larvae were immobilized using a 1% low-melting-point agarose  
584 (Promega; Cat#: V2111) solution and covered with Volvic water containing tricaine. A Leica SP8  
585 confocal microscope equipped with two PMT and Hybrid detector, a 20X IMM objective (HC PL APO  
586 CS2 20X/0.75), a X–Y motorized stage and with the LAS-X software was used to live image injected  
587 larvae. To generate images of the whole larvae, a mosaic of confocal z-stack of images was taken  
588 with the 20X objective using the Tile Scan tool of the LAS-X software and was stitched together using  
589 the Mosaic Merge tool of the LAS-X software. All samples were acquired using the same settings,  
590 allowing comparisons of independent experiments. After acquisition, larvae were washed and  
591 transferred in a new 24-well plate filled with 1 ml of fresh water per well, incubated at 28°C and  
592 imaged again under the same conditions over time. A Leica SPE inverted confocal microscope and a  
593 40x oil immersion objective (ACS APO 40 × 1.15 UV) was also used to live image larvae  
594 infected with *L. pneumophila*  $\Delta$ dotA-GFP (Figure 4).

595 The 4D files generated by the time-lapse acquisitions were processed, cropped, analysed,  
596 and annotated using the LAS-X and LAS-AF Leica software. Acquired Z-stacks were projected using  
597 maximum intensity projection and exported as AVI files. Frames were captured from the AVI files and  
598 handled with Photoshop software to mount figures. AVI files were also cropped and annotated with  
599 ImageJ software. Files generated with the LAS-X software were also processed and analysed with the  
600 Imaris software version9.5 (Bitplane, OXFORD Instruments) for 3D reconstruction, surfacing and  
601 volume rendering.

602

603 ***qRT-PCR to measure gene expression of cytokine encoding genes***. RNA was extracted from  
604 individual larvae using the RNeasy® Mini Kit (Qiagen). cDNA was obtained using M-MLV H- reverse-  
605 transcriptase (Promega) with a dT<sub>17</sub> primer. Quantitative PCR was performed on an ABI7300  
606 thermocycler (Applied Biosystems) using Takyon™ ROX SYBR® 2X MasterMix (Eurogentec) in a final  
607 volume of 10 µl. Primers used: *ef1a* (*housekeeping gene used for normalization*):  
608 GCTGATCGTTGGAGTCAACA and ACAGACTTGACCTCAGTGGT; *il1b*: GAGACAGACGGTGCTGTTA and

609 GTAAGACGGCACTGAATCCA; *tnfa*: TTCACGCTCCATAAGACCCA and CAGAGTTGTATCCACCTGTTA; *ifng*-  
610 1-1: ACCAGCTGAATTCTAAGCCAA and TTTTCGCCTTGACTGAGTGAA; *ifng*-2: GAATCTTGAGGAAAGTG  
611 AGCA and TCGTTTTCTTGATCGCCCA

612

613 **Statistical analysis.** Normal distributions were analysed with the Kolmogorov– Smirnov and the  
614 Shapiro–Wilk tests. To evaluate difference between means of normally distributed data (for  
615 neutrophil and macrophage numbers), an analysis of variance followed by Bonferroni’s multiple  
616 comparison tests was used. For bacterial burdens (CFU/FACS counts), values were Log<sub>10</sub>  
617 transformed. Values of FACS and CFU counts did not pass the normality test, data were analysed  
618 following the Mann-Whitney test. For cytokine expression and bacterial burdens, non-Gaussian data  
619 were analysed with the Kruskal– Wallis test followed by Dunn’s multiple comparison test.  $P < 0.05$   
620 was considered statistically significant (symbols: \*\*\*\*  $P < 0.0001$ ; \*\*\*  $P < 0.001$ ; \*\*  $P < 0.01$ ; \*  $P <$   
621  $0.05$ ). Survival data were plotted using the Kaplan–Meier estimator and log-rank (Mantel–Cox) tests  
622 were performed to assess differences between groups. Statistical analyses were performed using  
623 GraphPad Prism® software. Statistical analyses for *in ovo* experiments, were performed using  
624 GraphPrism version 7. Comparison of survival curves between different infection groups was carried  
625 out with the Log-rank (Mantel-Cox) test. Comparisons of the means of *L. pneumophila* CFU counts  
626 between groups were performed by the Mann–Whitney test. A p-value under 0.05 was considered  
627 statistically significant.

628

629 **Inoculation and quantification of *L. pneumophila* strains in *in ovo* experiments.** Fertilized chicken  
630 eggs purchased from a local producer (Saint-Maurice-sur-Dargoire, Rhône, France) were incubated at  
631 35°C in an egg incubator (Maino, Italy) to maintain normal embryonic development. Eggs were  
632 pathogen and antibiotic free. On day 0, 23 embryonated chicken eggs (ECE) were inoculated at 8  
633 days of embryonation (DOE) with either *L. pneumophila* WT (n=9), *L. pneumophila*  $\Delta dotA$  (n=7) or  
634 sterile PBS as control (n=7). *L. pneumophila* concentration in WT and  $\Delta dotA$  suspensions before ECE  
635 injection was quantified at 9.2 log<sub>10</sub> CFU/mL and 9.1 log<sub>10</sub> CFU/mL, respectively. *L. pneumophila*  
636 concentration in the yolk sac of ECE directly after injection were estimated, considering both the  
637 measured inoculum counts and the yolk sac volumes (median (interquartile range) [IQR] volume, 30  
638 [28.7-31.2] mL), at 7.4 and 7.3 log<sub>10</sub> CFU/mL in the WT and  $\Delta dotA$  groups, respectively. Two-day  
639 cultures of Lpp-WT and Lpp- $\Delta dotA$  on BCYE at 36°C were suspended in PBS at a DO = 2.5 McFarland  
640 (9 log<sub>10</sub> CFU/mL) and 0.5 mL of suspensions or PBS as negative control were inoculated in the yolk sac  
641 of ECE. After inoculation, ECE were candled every 24 hours to assess embryo viability until day-6 post  
642 infection. Embryos that died the day after inoculation (n=2, corresponding to one WT-infected and  
643 one  $\Delta dotA$ -infected embryo) were discarded for *L. pneumophila* quantification as death was probably

644 due to bad inoculation. Dead embryos were stored at 4°C overnight prior to harvesting the yolk sacs.  
645 Remaining live embryos at 6-days post injection were euthanized by refrigeration overnight and the  
646 yolk sacs were collected. After measuring their volume, yolk sacs were crushed using gentleMACS™  
647 Octo Dissociator (Miltenyi Biotec, Germany) and 100 µL of serial dilutions at 10<sup>-2</sup>, 10<sup>-4</sup> and 10<sup>-6</sup> were  
648 automatically plated using easySpiral® automatic plater (Interscience, France) in triplicates on BCYE  
649 agar. *L. pneumophila* were quantified after 5 days-incubation using Scan® 1200 Automatic HD colony  
650 counter (Interscience, France).

651

#### 652 **Author contributions**

653 FV, LB, DS, VL, MI and ECG performed the experiments, FV, SJ, LB, ECG and CB designed the  
654 experiments, FV, LB, ECG analyzed the experiments, VL performed IMARIS analysis of the raw  
655 confocal high resolution acquisition data, FV, ECG and CB wrote the article, ECG and CB supervised  
656 the work and acquired funds.

657

#### 658 **Competing Interest**

659 The authors declare there are no competing interests.

660

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670

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- 854



855 **FIGURES LEGENDS**

856

857 **Figure 1. Zebrafish larvae are susceptible to intravenous *L. pneumophila* infection in a dose**

858 **dependend manner. A)** Scheme of the experimental set up of bacterial infection using zebrafish. A

859 72hpf zebrafish larva is shown. Bacteria are injected in the bloodstream (iv) via the caudal vein

860 (green arrow). **B)** Survival curves (established from three independent experiments) of zebrafish

861 larvae injected with WT-GFP Low Dose (WT LD) (blue curve, n=60) or High Dose (HD) (red curve,

862 n=60), or with  $\Delta dotA$ -GFP Low Dose ( $\Delta dotA$  LD) (green curve, n=12) or High Dose ( $\Delta dotA$  HD) (green

863 curve, n=36), and incubated at 28°C. Non-injected fish (CTRL, black curve; n= 24). Three independent

864 experiments. **C)** Bacterial burden quantification by enumerating live bacteria in homogenates from

865 individual larvae infected with WT-GFP Low Dose (blue symbols) or High Dose (red symbols), or with

866  $\Delta dotA$ -GFP High Dose (green symbols) measured by FACS immediately after *L. pneumophila* injection

867 and 24h, 48h and 72h post *L. pneumophila* injection. n=10 larvae for each condition. **D)**

868 Representative images of *L. pneumophila* dissemination, determined by live imaging using a

869 fluorescence stereomicroscope, of zebrafish AB larvae infected with a LD or a HD of WT-GFP, or a HD

870 of  $\Delta dotA$ -GFP. The same infected larvae were live imaged 4h, 24h, 48h, and 72h post injection of the

871 different *L. pneumophila* strains. GFP fluorescence of the injected bacteria is shown.

872

873 **Figure 2. Bloodstream *L. pneumophila* establish a proliferative niche in the yolk causing a persistent**

874 **local infection.** Characterization of the *L. pneumophila* foci growing in the yolk region of zebrafish

875 larvae. Maximum intensity projection of confocal acquisition using high resolution fluorescent

876 microscope. **A)** 72hpf *mfap4*: mCherry larva (red macrophages) injected in the bloodstream with HD

877 of WT-GFP and followed over time with confocal fluorescent microscopy. **B)** Imaris 3D reconstruction

878 and volume rendering of the *L. pneumophila* growth in the yolk of the same infected larva at 72hpi,

879 shown laterally. Inset shows the maximum intensity projection of the *L. pneumophila* foci in the same

880 larva mounted ventrally. **C)** Scheme of 72hpf larva indicating with green dots the yolk sustaining

881 *L. pneumophila* growing. **D)** Imaris 3D reconstruction and volume rendering of the *L. pneumophila*

882 growth (GFP labelling) in the yolk of the same infected larva at 72hpi, showed ventrally. **E)** Imaris 3D

883 reconstruction and volume rendering of the *L. pneumophila* growth in the yolk of *lyz*:DsRed (red

884 neutrophils) infected larva at 72hpi, showed laterally. **F)** Imaris 3D reconstruction and volume

885 rendering of the *L. pneumophila* growth (GFP labelling) in the yolk of wild type AB infected larva at

886 72hpi, showed laterally. Overlay of GFP and mCherry, or DsRed fluorescence is shown (2B, E, G), and

887 BF is shown to help to visualize the yolk region and host anatomy (2A, D, F). See also related Movies

888 S1-S4.

889 **Figure 3. *L. pneumophila* high dose infection results in (systemic) macrophage and neutrophil**  
890 **death. A)** Representative images of *L. pneumophila* dissemination, determined by live imaging using  
891 a fluorescence stereomicroscope of zebrafish Tg(*mfap4::mCherryF*) larvae infected with a Low Dose  
892 or a HD of WT-GFP, or a HD of  $\Delta dotA$ -GFP. The same infected larvae were live imaged 4h, 24h, 48h,  
893 and 72h post *L. pneumophila* injection. Overlay of GFP and mCherry fluorescence is shown.  
894 **B)** Macrophage counts in uninfected larvae (black symbols) or upon Low Dose (blue symbols) or High  
895 Dose of WT-GFP (red symbols), or High Dose (green symbols) of  $\Delta dotA$ -GFP injections. Macrophages  
896 were counted manually from images taken on live infected larvae, using ImageJ software, and results  
897 were plotted using GraphPad Prism® software. Mean±SEM are also shown (horizontal bars). Data  
898 plotted are from two pooled independent experiments (n=12 larvae scored for each condition).  
899 **C)** Representative images of *L. pneumophila* dissemination, determined by live imaging using a  
900 fluorescence stereomicroscope, of zebrafish Tg(*LysC::DsRed*)<sup>nz50</sup> larvae infected with a Low Dose or a  
901 High Dose of WT-GFP or a High Dose of  $\Delta dotA$ -GFP. The same infected larvae were live imaged 4h,  
902 24h, 48h, and 72h post *L. pneumophila* injection. Overlay of GFP and DsRed fluorescence is shown.  
903 **D)** Neutrophil counts in uninfected (CTRL, black symbols) or upon Low Dose or High Dose of WT-GFP  
904 (blue or red symbols), or High Dose of  $\Delta dotA$ -GFP (green symbols) injections. Data plotted in the  
905 same way as for macrophage counts, are from two pooled independent experiments (n=10 larvae  
906 scored for each condition).

907

908 **Figure 4. Live imaging of macrophage and neutrophil interaction with *L. pneumophila***

909 Frames extracted from maximum intensity projection of *in vivo* time-lapse confocal fluorescent  
910 microscopy of 72hpf Tg(*mfap4::mCherryF*) larvae injected in the bloodstream (iv) with a LD, HD (of WT-  
911 GFP or a HD of  $\Delta dotA$ -GFP (upper panel) or Tg(*LysC::DsRed*)<sup>nz50</sup> in the bloodstream (iv) with a LD, HD of  
912 WT-GFP or a HD of  $\Delta dotA$ -GFP (lower panel) to follow macrophage and neutrophil interaction with *L.*  
913 *pneumophila* respectively. Images were taken from time lapse at different time points (0hpi, 2hpi,  
914 4hpi, 8hpi and 16hpi). Overlay of green (*L. pneumophila*) and red (leucocytes) fluorescence of the  
915 caudal area of the larvae (region boxed in the scheme on the right of the panel) is shown. Scale bar:  
916 50µm. See also related Movies S5, S6.

917

918 **Figure 5. Macrophages are crucial to restrict *Legionella pneumophila* dissemination**

919 **A)** Survival curves of CTRL morphant zebrafish larvae injected with a Low Dose (LD) (blue dashed  
920 curve, n=34 larvae) or a High Dose (HD) (red dashed curve, n=34) of WT-GFP, or with a HD (green  
921 dashed curve, n=24) of  $\Delta dotA$ -GFP, and *spi1b* morphant zebrafish larvae injected with a LD (blue  
922 curve, n=48) or a HD (red curve, n=48) of WT-GFP, or with a High Dose (HD) (green curve, n=48) of  
923  $\Delta dotA$ -GFP. Non-injected CTRL morphant fish (black dashed curve, n=48), and *spi1b* morphant fish

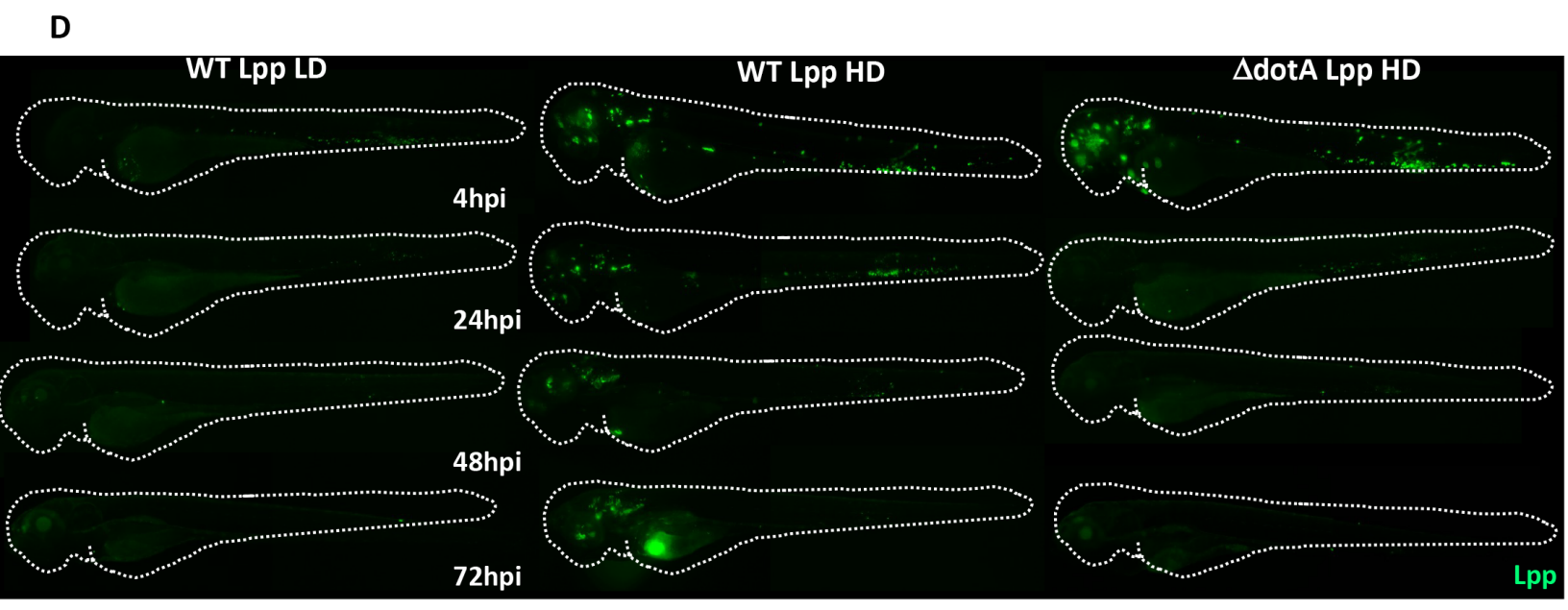
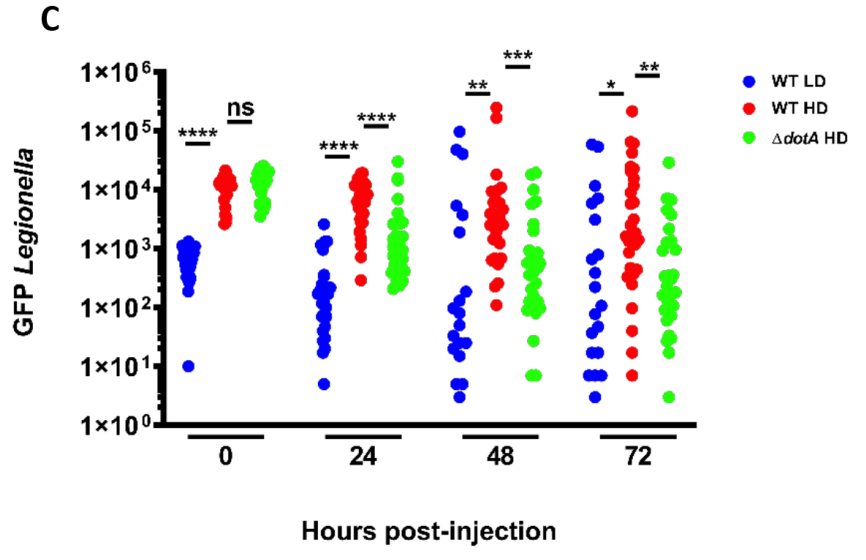
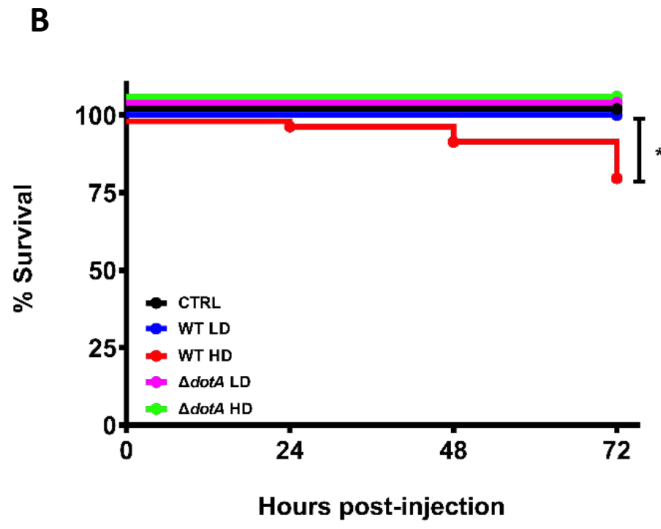
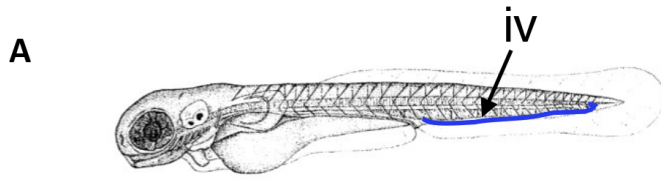
924 (black curves, n=48) were used as control. Infected and control larvae were incubated at 28°C. Data  
925 plotted are from two pooled independent experiments. **B) and E)** Bacterial burden quantification by  
926 enumerating live bacteria in homogenates from individual larvae infected with LD of WT-GFP (blue  
927 symbols) or HD (red symbols), or with LD of  $\Delta dotA$ -GFP (magenta symbols) or HD (green symbols),  
928 measured by plating onto BCYE agar plates supplemented with Chloramphenicol and the *Legionella*  
929 Selective Supplement GVPN immediately after *L. pneumophila* injection and 24h, 48h and 48h post *L.*  
930 *pneumophila* injection. n=10 larvae for each condition. **D)** Survival curves of CTRL morphant zebrafish  
931 larvae injected with a LD (blue dashed curve, n=36) or a HD (red dashed curve, n=36) of WT-GFP, or  
932 with a HD (green dashed curve, n=24) of  $\Delta dotA$ -GFP, and *csf3r* morphant zebrafish larvae injected  
933 with a LD (blue curve, n=24) or a HD (red curve, n=36) of WT-GFP, or with a HD (green curve, n=36) of  
934  $\Delta dotA$ -GFP. Non-injected CTRL morphant fish (black dashed curve, n=48), and *csf3r* morphant fish  
935 (black curve, n=36) were used as control. Data plotted are from two pooled independent  
936 experiments. **C) and F)** Representative images of *L. pneumophila* dissemination, determined by live  
937 imaging using a fluorescence stereomicroscope, of Tg(*mfap4::mCherryF*) *spe1b* morphant larvae (**C**)  
938 and of Tg(*LysC::DsRed*)<sup>nz50</sup> (**F**) *csf3r* morphant larvae non infected, or infected with a LD or a HD of  
939 WT-GFP, or a HD of  $\Delta dotA$ -GFP. The same infected larvae were live imaged 4h, 24h, 48h, and 72h  
940 post *L. pneumophila* injection. Overlay of GFP and mCherry fluorescence is shown.

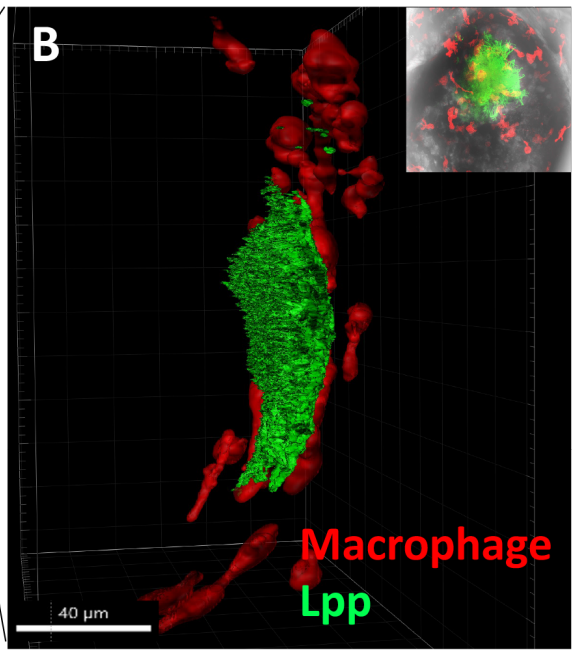
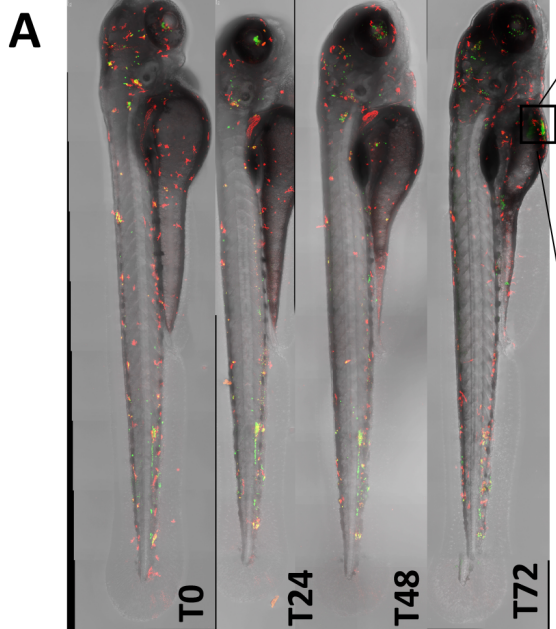
941  
942 **Figure 6. Zebrafish larva Immunity to *L. pneumophila* is independent from signalling through**  
943 **MyD88 or compensated by other signalling pathways. A)** Survival curves of CTRL zebrafish larvae  
944 injected with WT-GFP Low Dose (LD) (blue dashed curve) or High Dose (HD) (red dashed curve), or  
945 with  $\Delta dotA$ -GFP HD (green dashed curve), and *myd88*<sup>hu3568</sup> mutant zebrafish larvae injected with WT-  
946 GFP LD (blue curve) or HD (red curve), or with  $\Delta dotA$ -GFP HD (green curve). Non-injected CTRL  
947 larvae (black dashed curves), and *myd88*<sup>hu3568</sup> mutant larvae (black curves) were used as control.  
948 Infected and control larvae (n= 72 fish for *myd88*<sup>hu3568</sup> mutant conditions and n= 57 fish for CTRL  
949 conditions) were incubated at 28°C. Data plotted are from 3 pooled independent experiments. **B)**  
950 Bacterial Burden of *myd88*<sup>hu3568</sup> mutant zebrafish larvae are the same as what is observed for control  
951 larvae. Bacterial burden quantification by enumerating live bacteria in homogenates from individual  
952 larvae infected with WT-GFP LD (blue symbols) or HD (red symbols), or with  $\Delta dotA$ -GFP HD (green  
953 symbols) were measured by plating onto BCYE agar plates supplemented with Chloramphenicol and  
954 the *L. pneumophila* Selective Supplement GVPN immediately after *Legionella* injection and 24h, 48h  
955 and 48h post *Legionella* injection. n=15 larvae for each condition. **C-D)** Cytokine (*il1b*, *tnfa*) induction  
956 was measured from individual *myd88*<sup>hu3568</sup> mutant larvae injected with a HD (red curves) of WT-GFP  
957 and non-injected fish as control (CTRL, black curves). The same colours are used in individual CTRL  
958 zebrafish with dashed curves. Data plotted are from one experiment (n=5 larvae for each condition);

959 individual values are shown, and curves correspond to the medians. There is no statistically  
960 significant difference between CTRL and *myd88*<sup>hu3568</sup> mutant curves over time for all the conditions  
961 analysed.

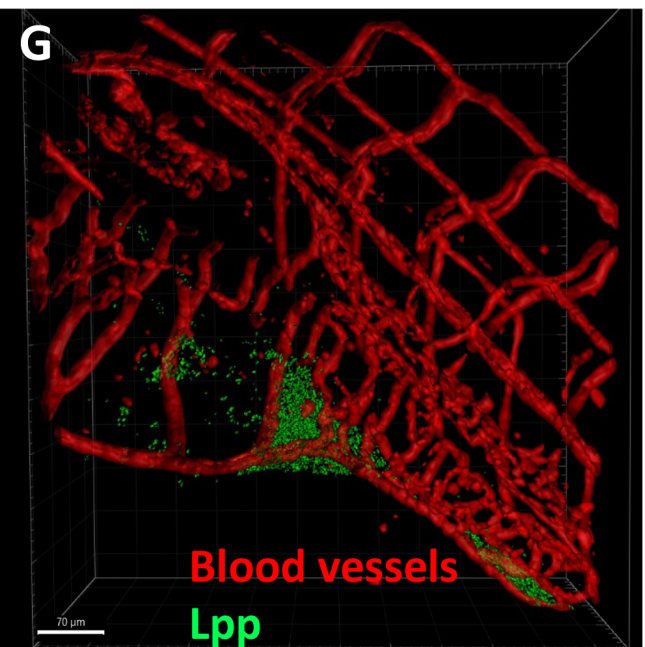
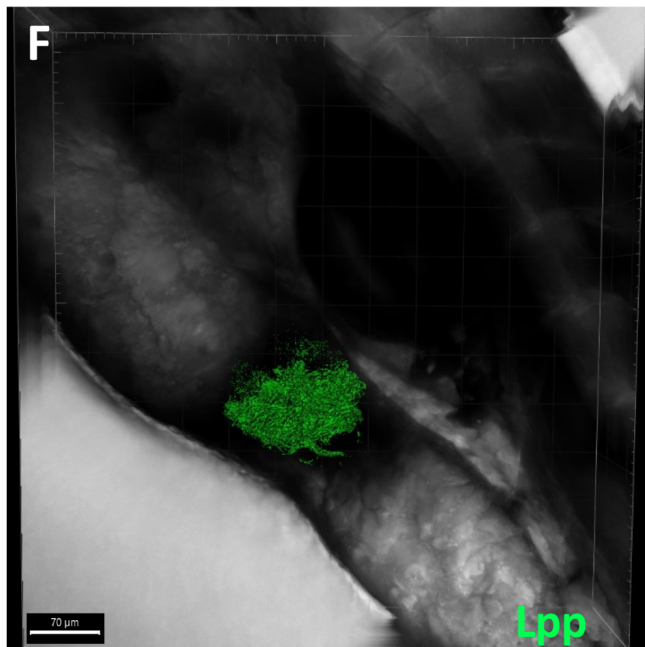
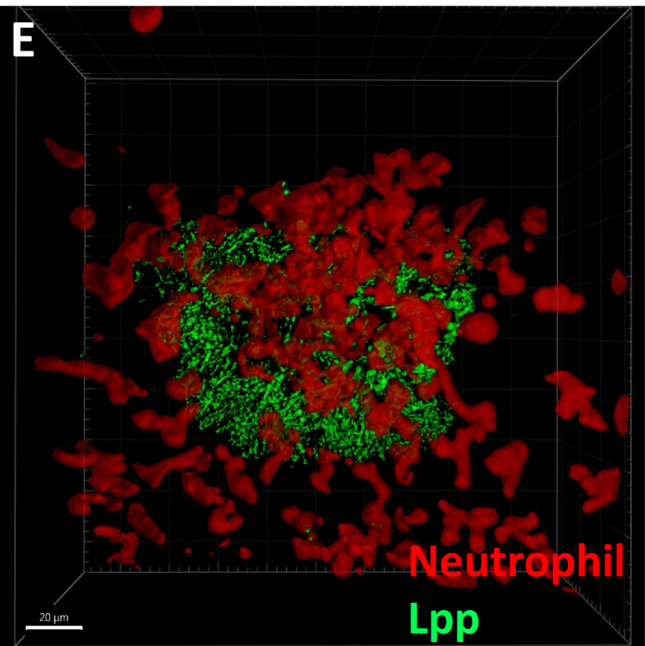
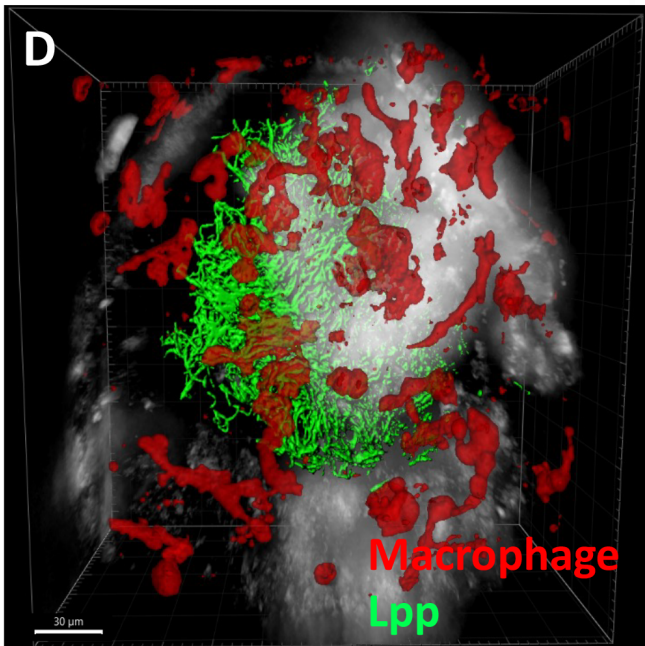
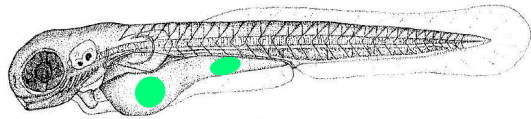
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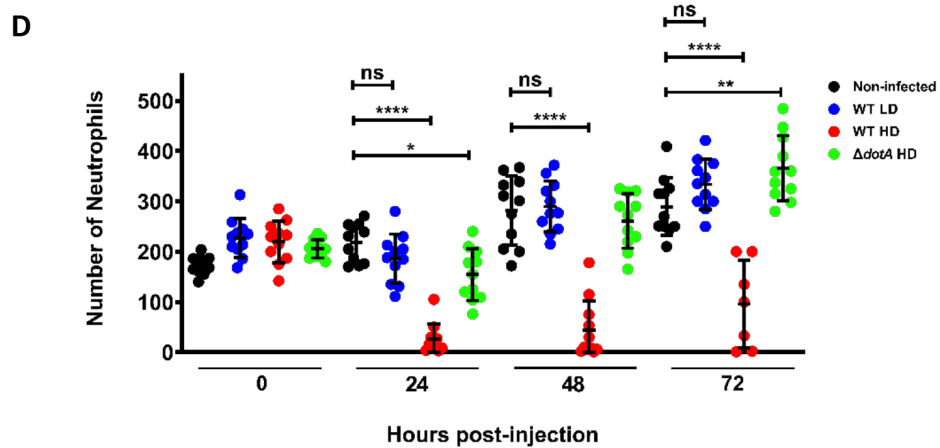
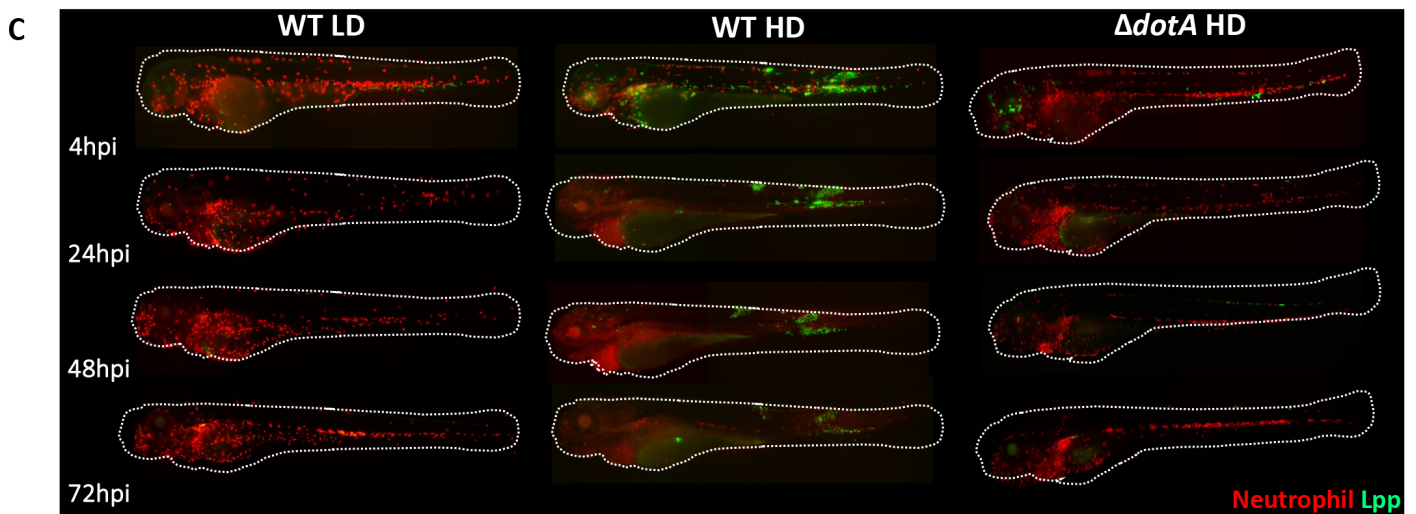
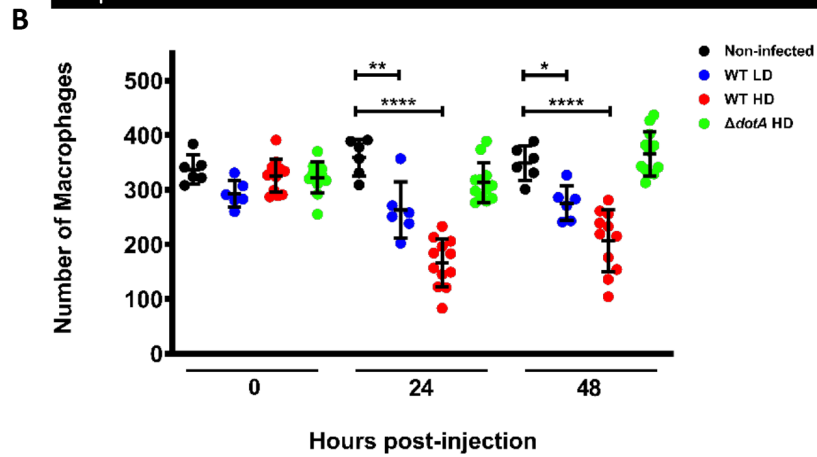
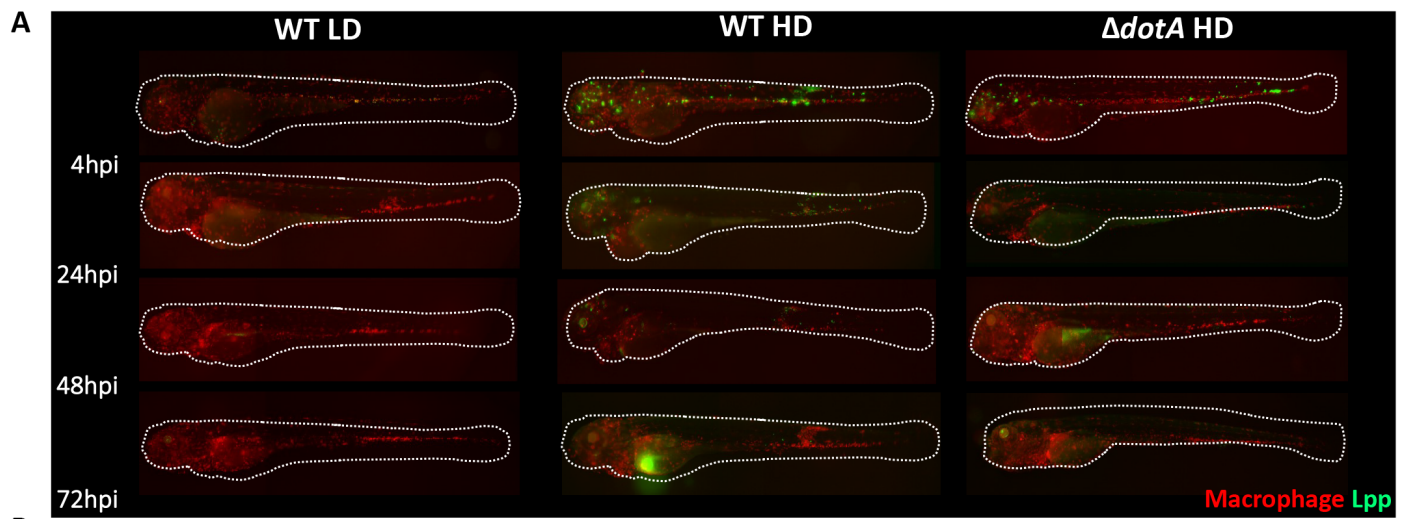
963 **Figure 7. *L. pneumophila* replication in the yolk of zebrafish larvae is T4SS dependent. A)** Survival  
964 curves of zebrafish larvae injected with WT-GFP Low Dose (LD) (blue curve) or High Dose (HD) (red  
965 curve), or with  $\Delta dotA$ -GFP LD (magenta curve) or HD (green curve). Non-injected larvae (black  
966 curves) were used as control. n= 48 larvae per conditions. All larvae were incubated at 28°C. Data  
967 plotted are from two pooled independent experiments. **B)** Bacterial burden quantification of  
968 zebrafish larvae injected with *L. pneumophila* in the yolk cell, by enumerating live bacteria in  
969 homogenates from individual larvae infected with WT-GFP LD (blue symbols) or HD (red symbols), or  
970 with  $\Delta dotA$ -GFP Low Dose (LD) (magenta symbols) or HD (green symbols). They were measured by  
971 plating onto BCYE agar plates supplemented with Chloramphenicol and the *Legionella* Selective  
972 Supplement GVPN immediately after *L. pneumophila* injection and 24h, 48h and 48h post *Legionella*  
973 injection. n=10 larvae for each condition. **C-D)** Representative images of *L. pneumophila*  
974 dissemination, determined by live imaging using a fluorescence stereomicroscope, of  
975 Tg(*LysC::DsRed*)<sup>nz50</sup> not infected zebrafish larvae, or infected with a Low Dose of WT-GFP or  $\Delta dotA$  -  
976 GFP (C), or infected with a High Dose of WT-GFP or  $\Delta dotA$  -GFP (D). The same infected larvae were  
977 live imaged 4h, 24h, 48h, and 72h post *L. pneumophila* injection. Overlay of GFP and mCherry  
978 fluorescence is shown.



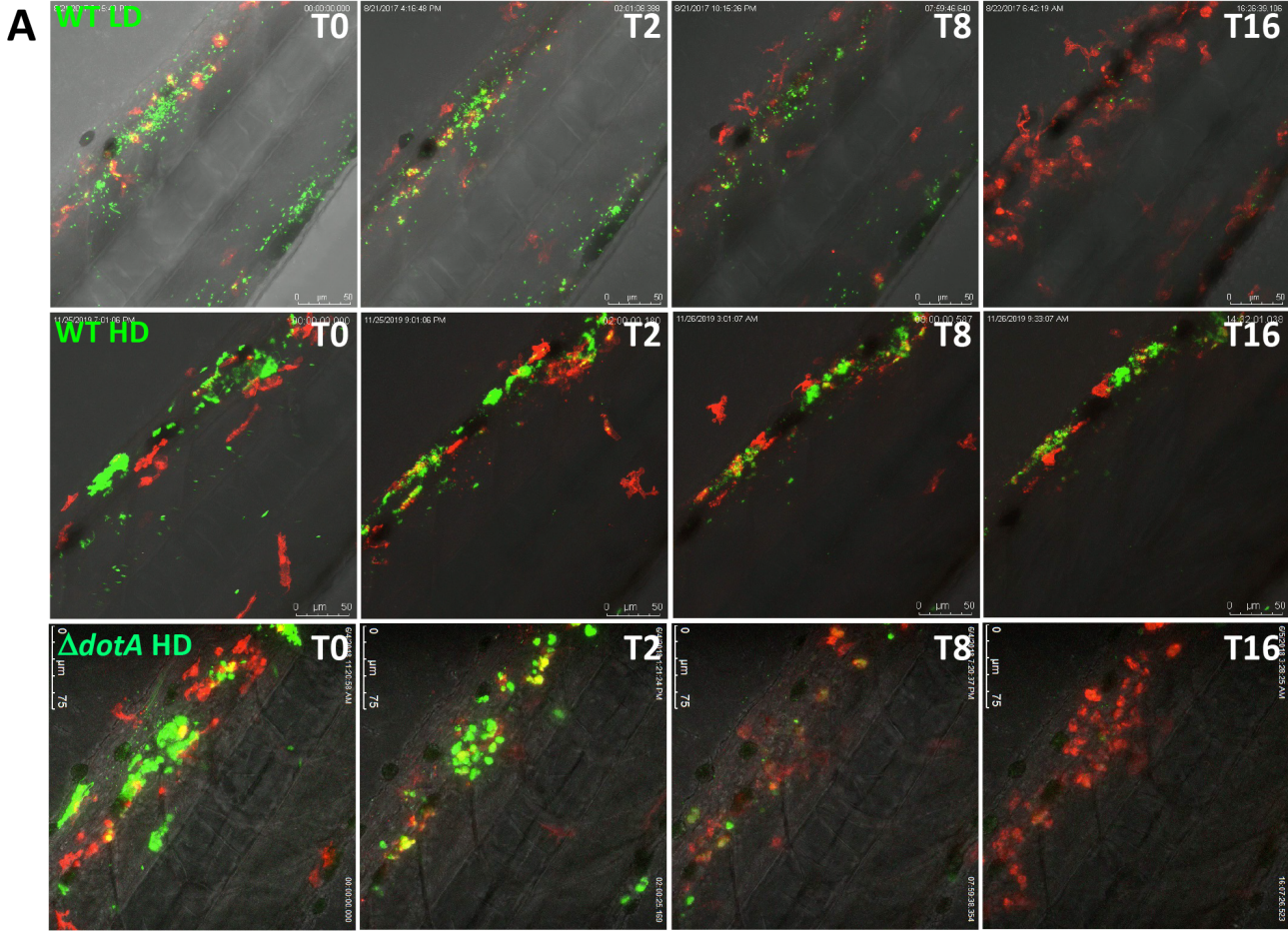


**C**

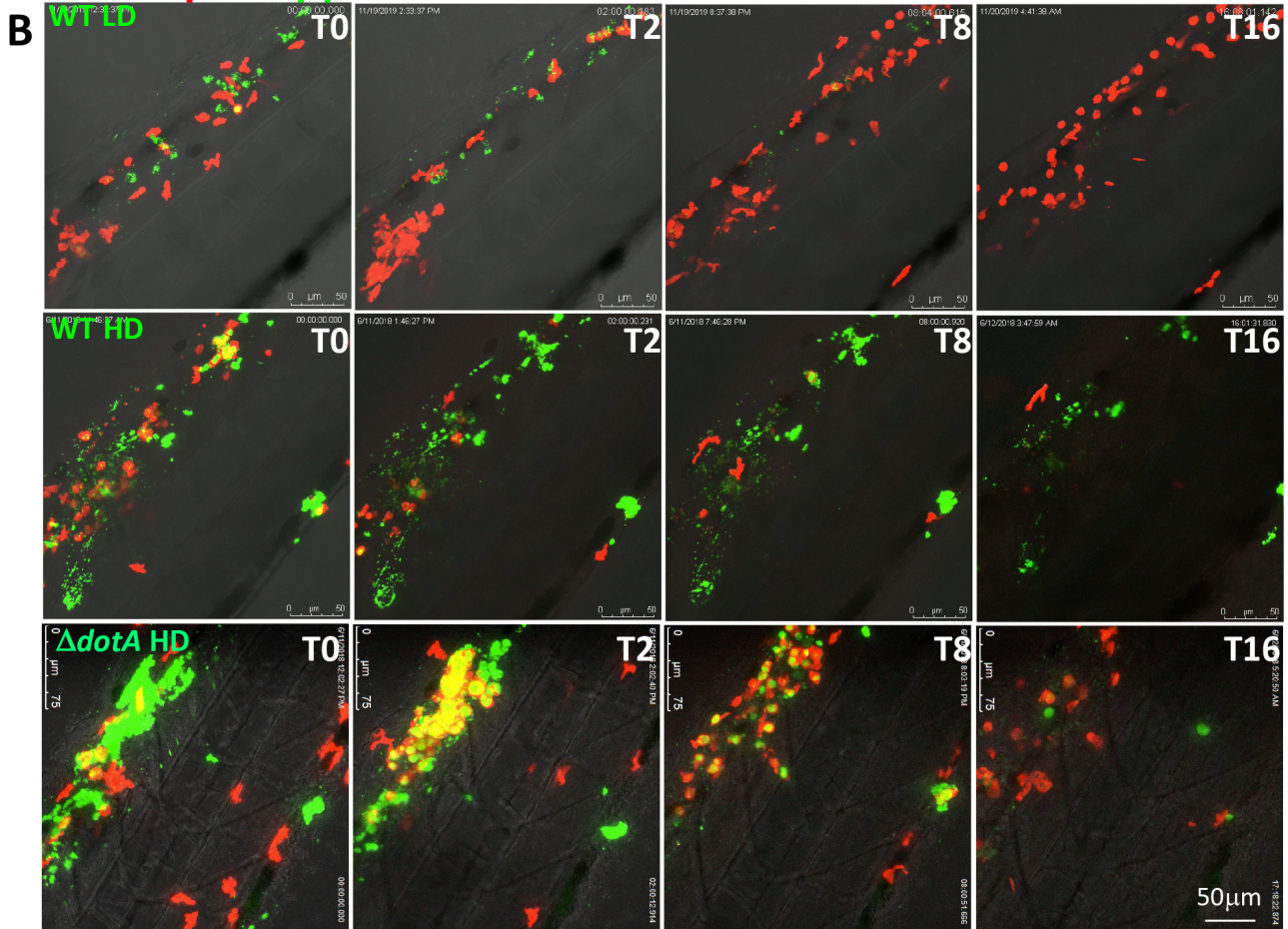




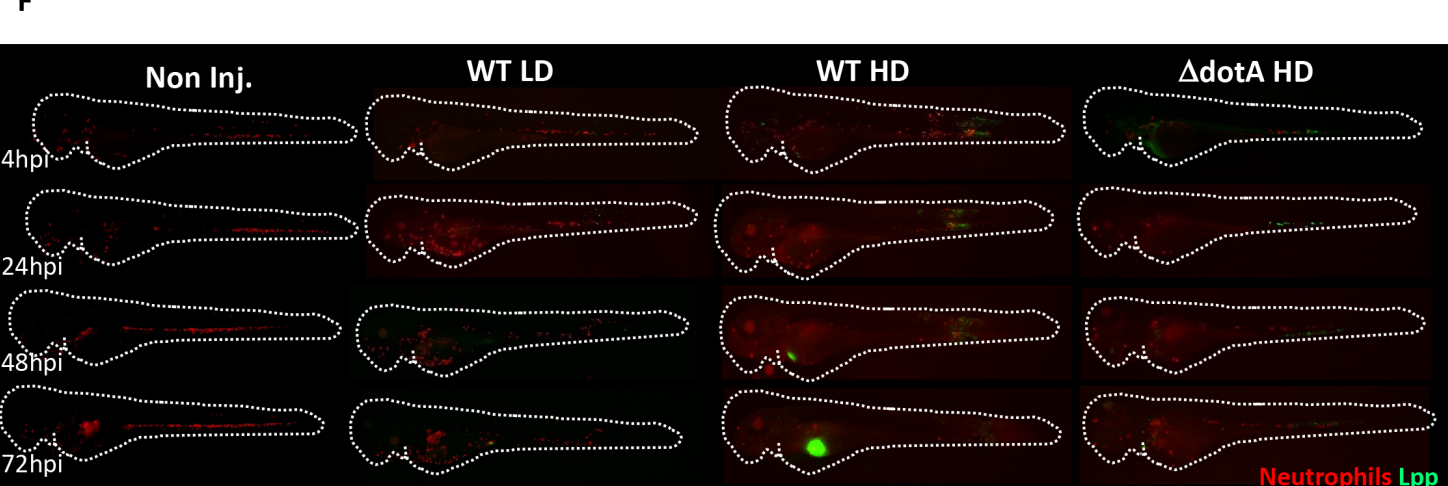
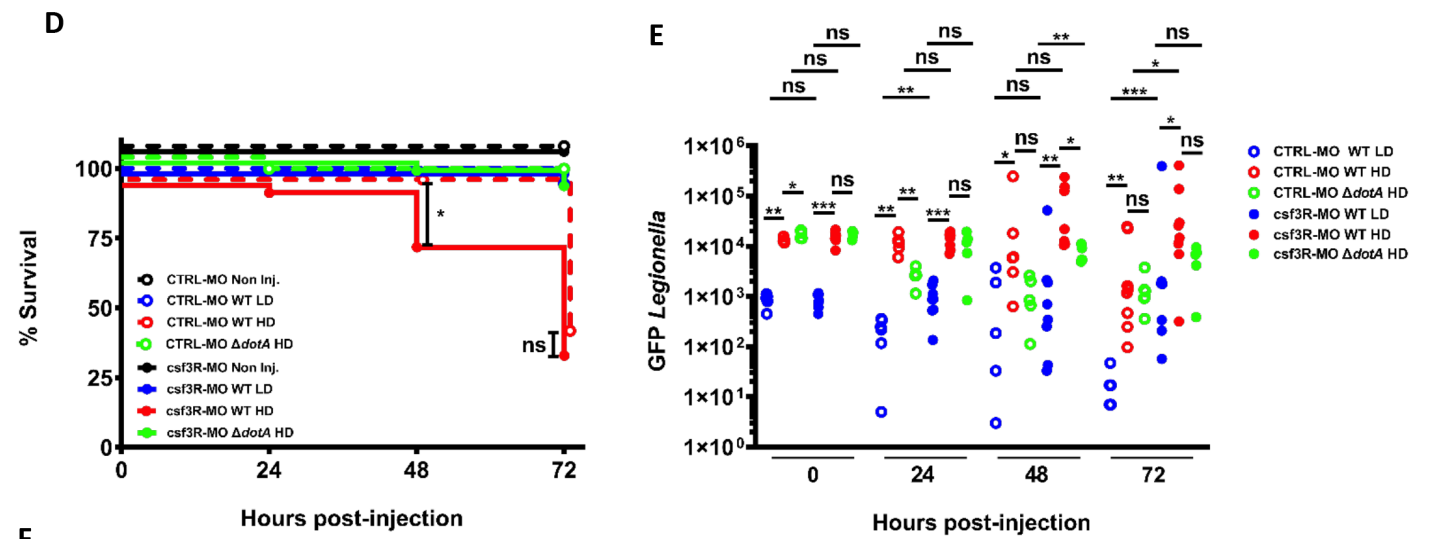
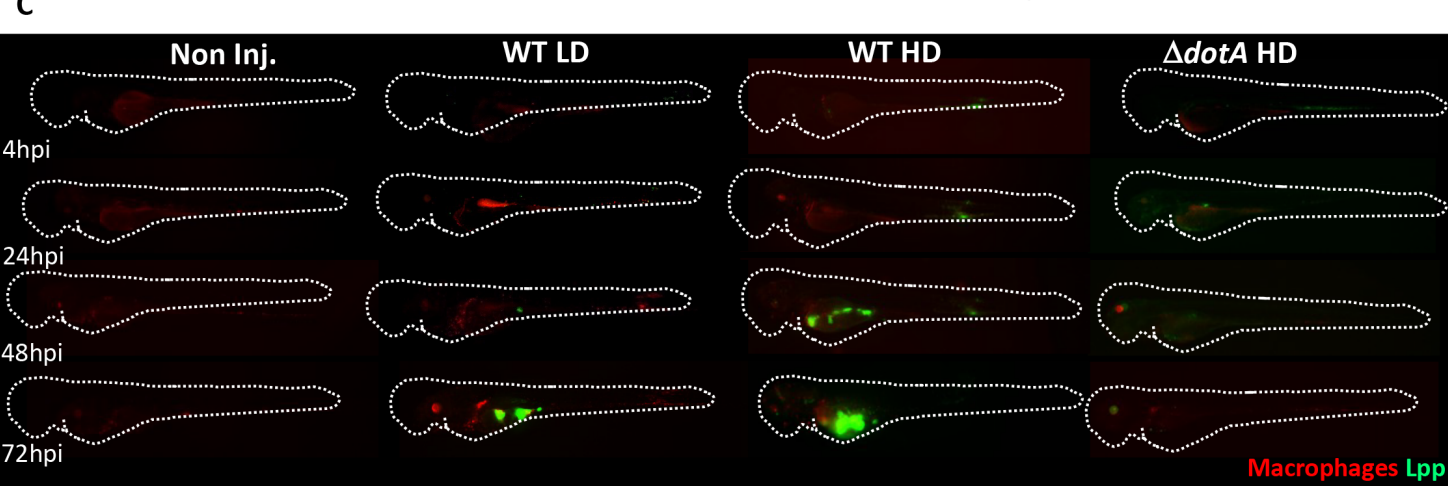
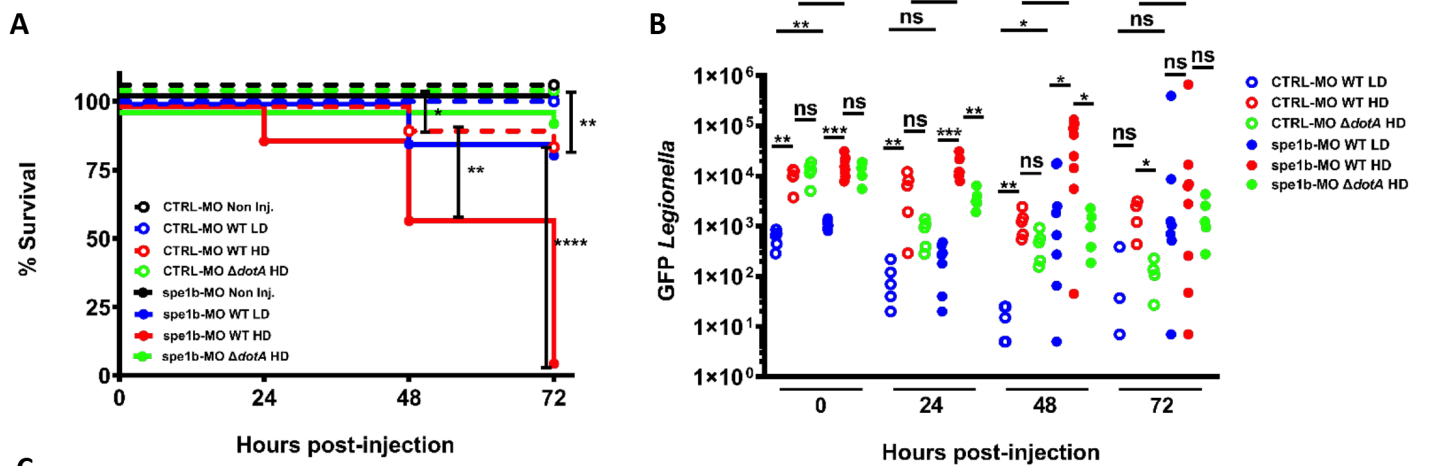
# Macrophage-*Lpp* interactions over time

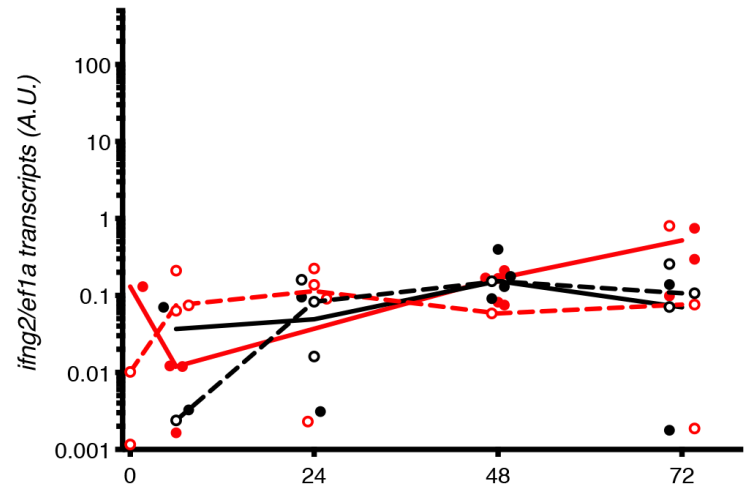
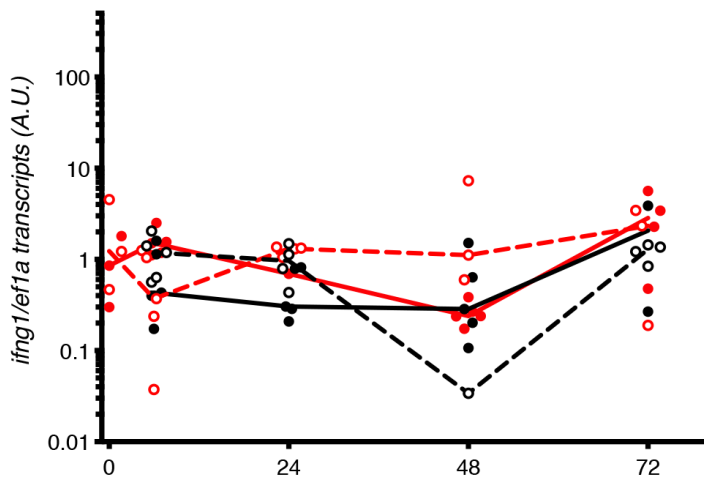
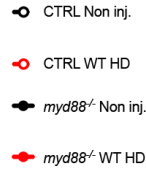
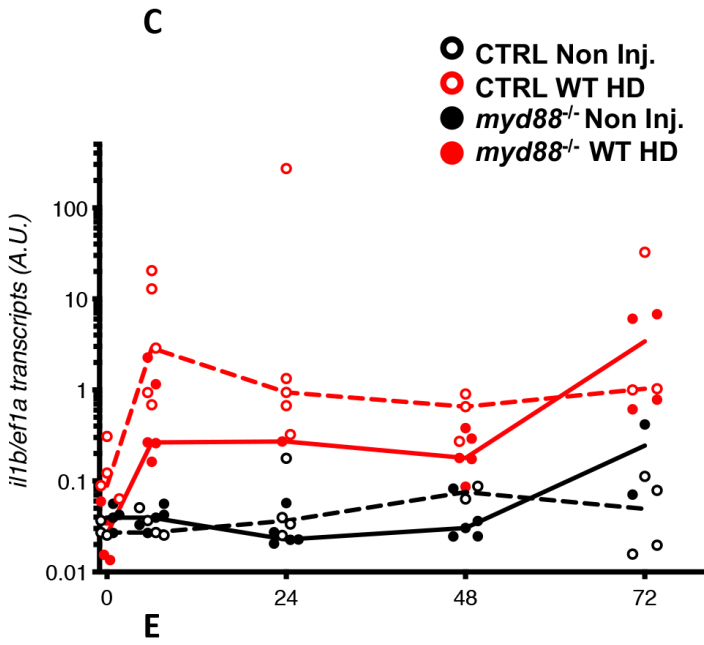
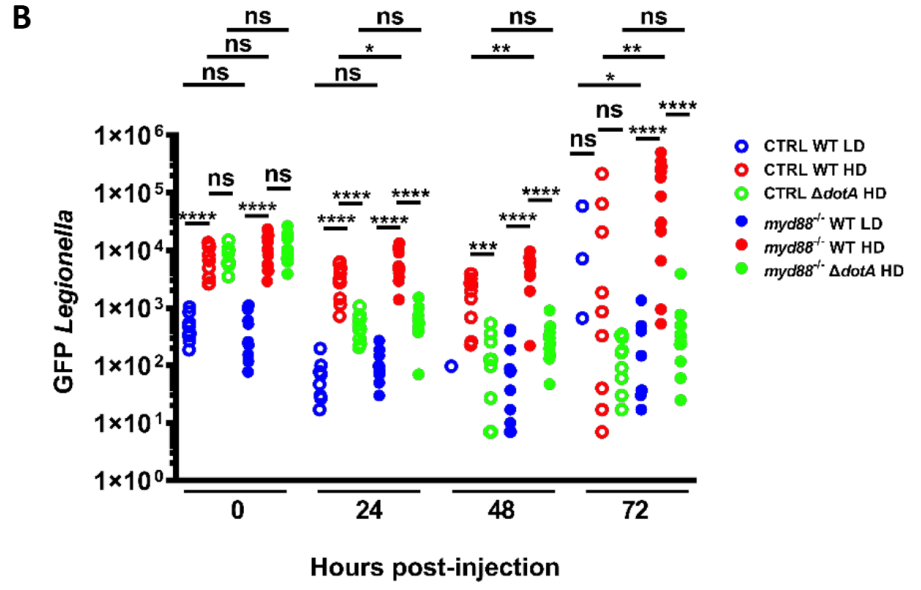
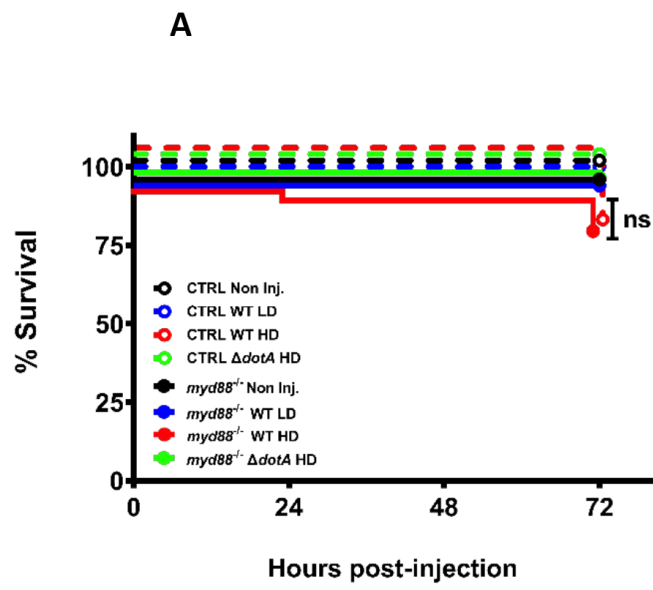


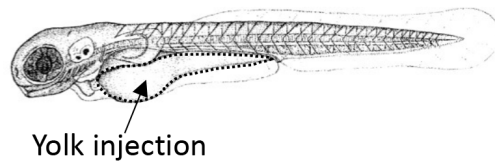
# Neutrophil-*Lpp* interactions over time



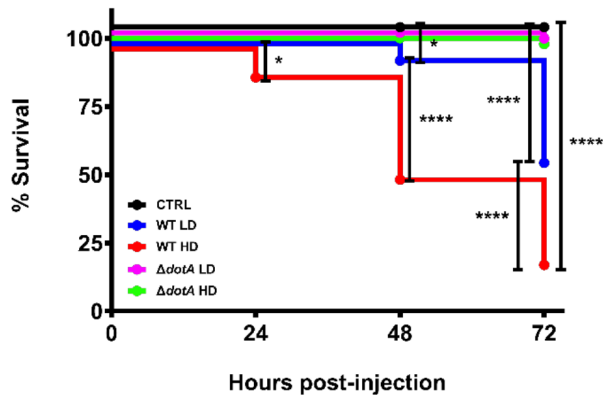




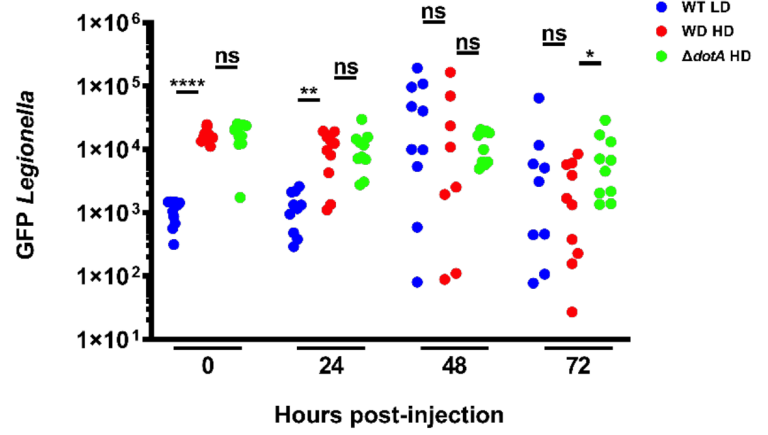




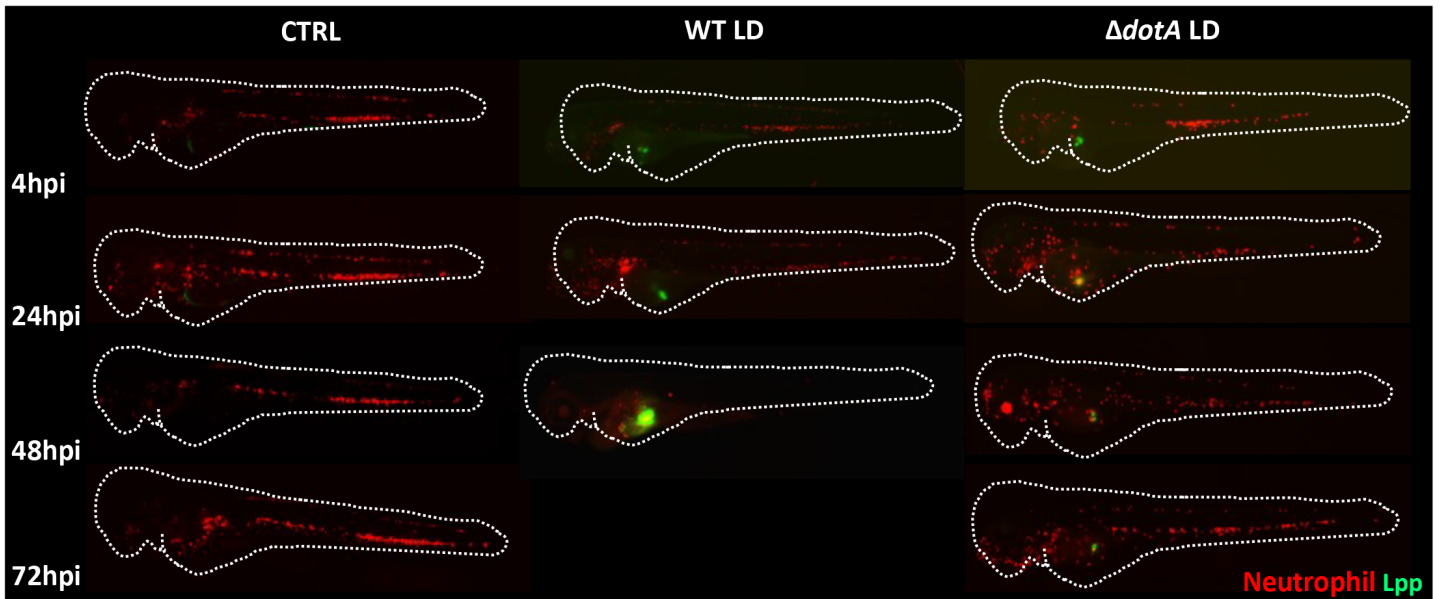
A



B



C



D

