Supplemental Material

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

Flávia Viana[1,§,#,*], Laurent Boucontet[2,§], Daniel Schator[1,3], Valerio Laghi[2], Marine Ibranosyan[4], Sophie Jarraud[4,5], Emma Colucci-Guyon[2,€,*] and Carmen Buchrieser[1,€,*]

Supplementary Movies

Figure legends

Figure S1: Comparison of three methods to estimate the bacterial burden of infected zebrafish larvae at different points post nfection. A) For bacterial burden measure by fluorescent pixel counts, the pictures corresponding to the GFP channel were analysed to count pixels, using the ImageJ software. Here we plotted individual larvae injected with WT-GFP Low Dose (LD) (blue symbols) or High Dose (HD) (red symbols) or infected with Δ*dotA*-GFP HD (green symbols). **B**) For FACS analyses, infected larvae were lysed and then GFP bacteria were counted on a MACSQuant VYB FACS (Miltenyi Biotec) **C**) CFUs were enumerated by plating serial dilutions of lysed infected larvae in BCYE agar supplemented with Chloramphenicol and *Legionella* Selective Supplement GVPN (Sigma).

Figure S2: Replicative vacuoles in infected zebrafish larvae 3 days post infection. Confocal fluorescent microscopy analysis of macrophage- *L. pneumophila* interaction at 72hpi showing live *L. pneumophila* inside macrophages. A single 2m Z-Stack is shown.

Figure S3: Macrophage and neutrophil depletion by morpholino impacts larvae survival and cytokine gene expression upon *Legionella* infection Comparison of the impact of *spi1b* morpholino injection that blocks macrophage development or csf3r morpholino injection that blocks neutrophil development were administered. Macrophages (red symbols) and neutrophils (green symbols) were counted in CTRL (open symbols) or morphant (full symbols) conditions. **A)** effect of spe1b morpholino on macrophages and neutrophils, showing that spe1b morpholino injection leads to the specific depletion of macrophages and not neutrophils. B) effect of Csf3R morpholino on macrophages and neutrophils, showing that Csf3R morpholino injection leads to the specific depletion neutrophils and slightly impairs the number of macrophages **C-F)** Cytokine (*il1b*, *tnfa, ifng1, ifng2*) induction was measured from non-injected larvae as control (CTRL, dashed black curves) and individual zebrafish larvae injected with a LD (blue curves) or a HD (red curves) of WT-GFP, or a HD of Δ*dotA*-GFP (green curves), and). Data plotted are from 2 experiments (n=10 larvae for each condition) for *il1b* and *tnfa,* and from 1 experiment (n=5 larvae for each condition) for *ifng1* and *ifng2*; individual values are shown, and curves correspond to the medians. Statistical analysis is shown as a table under each graph.

Figure S4: *Legionella* invades the yolk only upon bloodstream inoculation and only blood borne *L. pneumophila* WT proliferate in the yolk region of zebrafish. A) Scheme of 3dpf larva indicating the sites of bacterial injection. B. Representative images of *L. pneumophila* dissemination, determined by live imaging using a fluorescence stereomicroscope, of zebrafish AB larvae infected with a HD WT-, in closed compartments (otic vesicle, the hind brain ventricle) or in the bloodstream. Infected

larvae were live imaged 4h, 24h, 48h, and 72h post *L. pneumophila* injection. Only GFP fluorescence is shown. Site of injection is indicated by dashed boxes. **B)** Survival curves of embryonated chicken eggs inoculated with WT strain (in red, n=9), Δ*dot*A strain (in blue, n=7) or PBS (in grey, n=7). Survival expressed in percentage and time in days. **C)** Quantification of *L. pneumophila*, expressed in $\log_{10}$ CFU/mL, in yolk sac of WT-infected embryos (n=9) and Δ*dotA*-infected embryos (n=6). Dead embryos are represented with black dots and those euthanized (alive at day 6) with white dots. The inoculum in the yolk sac after infection was estimated by taking into account the count of *L. pneumophila* in the inoculum (WT and Δ*dotA*) before injection and the volume of the yolk sac.

Movie legends:

Movie S1: *L. pneumophila* growing in the yolk region at 72hpi: interactions with macrophages

Mfap4: mCherry (red macrophages) 72hpf larva was injected in the bloodstream with HD of *L. pneumophila* WT-GFP, and was analyzed with confocal high microscopy at 72hpi, to study the behavior of the bacteria growing in the yolk region and their interactions with macrophages. The infected larva was mounted ventrally and acquired using a 40X water-immersion objective. Only the yolk region containing the bacterial aggregates was imaged. The acquired Z-stack was deconvolved using Leica Lightening Plug-in and processed for 3D visualization and volume rendering, using IMARIS 9.6 (Bitplane). The interactions of macrophages (red cells) with the growing bacterial aggregates (green), and the yolk region (bright field) are shown at various magnifications (scale bar indicated on the movie) and various rotation angles to highlight the complex filamentous bacterial structures and the recruited macrophages, that recognize the growing bacteria, but fail to penetrate the yolk content, and to engulf the bacterial aggregates. Due to the peculiar yolk composition and thickness, it was impossible to acquire the fluorescence of the bacteria growing inside the yolk distal to the objective, thus appearing as big dark spots.

Movie S2: *L. pneumophila* growing in the yolk region at 72hpi: interactions with neutrophils.

Lys:DsRed (red neutrophils) 72hpf larva was injected in the bloodstream with HD of *L. pneumophila* WT-GFP, and was analyzed with confocal high microscopy at 72hpi, to study the behavior of the bacteria growing in the yolk region and their interactions with neutrophils. The infected larva was mounted laterally and acquired using a 20X oil-immersion objective. The acquired Z-stack was deconvolved using Leica Lightening Plug-in and processed for 3D visualization and volume rendering, using IMARIS 9.6 (Bitplane). The interactions of neutrophils (red cells) with the growing bacterial aggregates (green), and the yolk region (bright field) are shown at various magnifications (scale bar indicated on the movie) and various rotations angles to highlight the complex filamentous bacterial structures and the recruited neutrophils, that recognize and sense the growing bacteria, migrate to them, but fail to penetrate the yolk content, and to engulf the big bacterial aggregates. Due

to the peculiar yolk composition and thickness, it was impossible to acquire the fluorescence of the bacteria growing inside the yolk distal to the objective, thus appearing as big dark spots.

Movie S3: *L. pneumophila* growing in the yolk region at 72hpi: localization in the yolk in AB wild type larva. AB wild type larva 72hpf was injected in the bloodstream with HD of *L. pneumophila* WT-GFP*,* and was analyzed with confocal high microscopy at 72hpi, to study the behavior of the highly growing bacteria in the yolk region. The infected larva was mounted laterally and acquired using a 20X oil-immersion objective. The acquired Z-stack was deconvolved using Leica Lightening Plug-in and processed for 3D visualization and volume rendering, using IMARIS 9.6 (Bitplane). Note the complex, filamentous, highly aggregate structures (green) formed by the growing *Legionella* in the yolk region (visualized by the bright field)**.**

Movie S4: *L. pneumophila* growing in the yolk region at 72hpi: interactions with blood vessels. *kdrl*:mCherry (red blood vessels) 72hpf larva was injected in the bloodstream with HD of *L. pneumophila* WT-GFP, and was analyzed with confocal high microscopy at 72hpi, to study the behavior of the highly growing bacteria in the yolk region and their interactions with the yolk vasculature. The infected larva was mounted laterally and acquired using a 20X oil-immersion objective. The acquired Z-stack was deconvolved using Leica Lightening Plug-in and processed for 3D visualization and volume rendering, using IMARIS 9.6 (Bitplane). The interactions of the blood vessels (red cells) with the growing bacterial aggregates (green), and the yolk region (bright field) are shown at various magnifications (scale bar indicated on the movie) and various rotations angles to highlight the complex filamentous bacterial structures and their interactions with the blood vessels. Due to the peculiar yolk composition and thickness, it was impossible to acquire the fluorescence of the bacteria growing inside the yolk distal to the objective, thus appearing as big dark spots.

Movie S5: Macrophage - *L. pneumophila* interactions (LD, HD). *Mfap4*: mCherry (red macrophages) 72hpf larvae were injected in the bloodstream with LD (left panel) or HD (middle panel) of *L. pneumophila* WT-GFP or with HD OF *L. pneumophila* Δ*dotA*-GFP (right panel), mounted laterally and acquired using high resolution confocal microscopy to analyze macrophages (red cells) bacteria (green) interactions immediately upon bacteria injection. The infected larvae were acquired over time from 20 min to approximately 16 hours post injection. Maximum projections of the acquired Z-stacks are shown. The 3D movies generated were combined using Image J software, to have them side by side, to compare the macrophage-bacteria interaction over time in the various conditions. Left panel: *mfap4*: mCherry (red macrophages) 72hpf larva injected in the bloodstream with LD wt GFP *Legionella* (green). Note that macrophages are recruited to the injected bacteria, engulf them, and the bacteria are cleared progressively from the bloodstream. Middle panel: *mfap4*: mCherry (red macrophages) 72hpf larva injected in the bloodstream with HD wt GFP *Legionella* (green).

Macrophages are recruited upon bacteria injection but failed to eliminate them over time; the phagocytosing macrophages round-up, suggesting cell death. Right panel: *mfap4*: mCherry (red macrophages) 72hpf larva injected in the bloodstream with HD GFP Δ*dotA Legionella* (green). Note that the recruited macrophages efficiently engulf and eliminate the injected bacteria, clearing them progressively from the blood and the mesenchyme near the point of injection**.**

Movie S6: Neutrophil - *L. pneumophila* (LD, HD) interactions. (*Lys*: DsRed (red neutrophils) 72hpf larvae were injected in the bloodstream with LD (left panel) or HD (middle panel) *L. pneumophila* WT-GFP, or with HD of Δ*dotA*-GFP (right panel), mounted laterally and acquired using high resolution confocal microscopy to analyze neutrophil (red cells) bacteria (green) interactions immediately upon bacteria injection. The infected larvae were acquired over time from 20 min to approximately 16 hours post injection. Maximum projections of the acquired Z-stacks are shown. The 3D movies generated were combined using ImageJ software, to have them side by side, to compare neutrophil-bacteria interactions over time in the various conditions. Left panel: *Lys*: DsRed (red neutrophils) 72hpf larva injected in the bloodstream with LD of WT-GFP (green). Note that neutrophils are recruited to the injected bacteria, engulfing the bacteria trapped in the mesenchyme near the site of injection, cooperating with macrophages (DsRed – cells, GFP+ having engulfed large amount of GFP bacteria), clearing progressively the infection. Middle panel: *Lys*:DsRed (red neutrophils) 72hpf larva injected in the bloodstream with HD of WT-GFP (green). Neutrophils are massively recruited upon bacterial injection but failed to eliminate them over time; the phagocytosing neutrophils round-up and loose DsRed fluorescence, suggesting cell death. Right panel: *lys:*DsRed (red neutrophils) 72hpf larva injected in the bloodstream with HD of Δ*dotA*-GFP *L. pneumophila* (green). Note that the recruited neutrophils engulf and eliminate the injected bacteria, clearing them progressively from mesenchyme near the point of injection, efficiently cooperating with macrophages in controlling the infection**.**

Figure S1

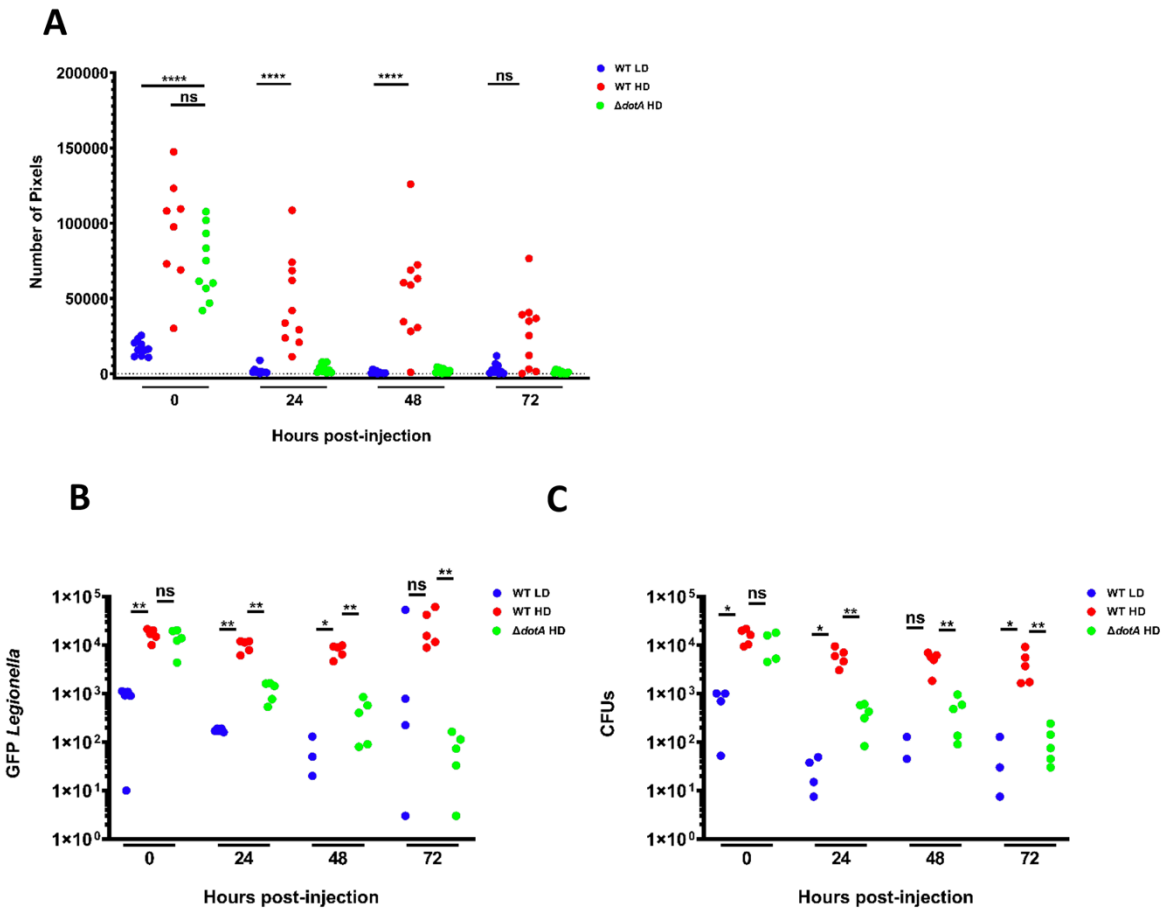
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Figure S2

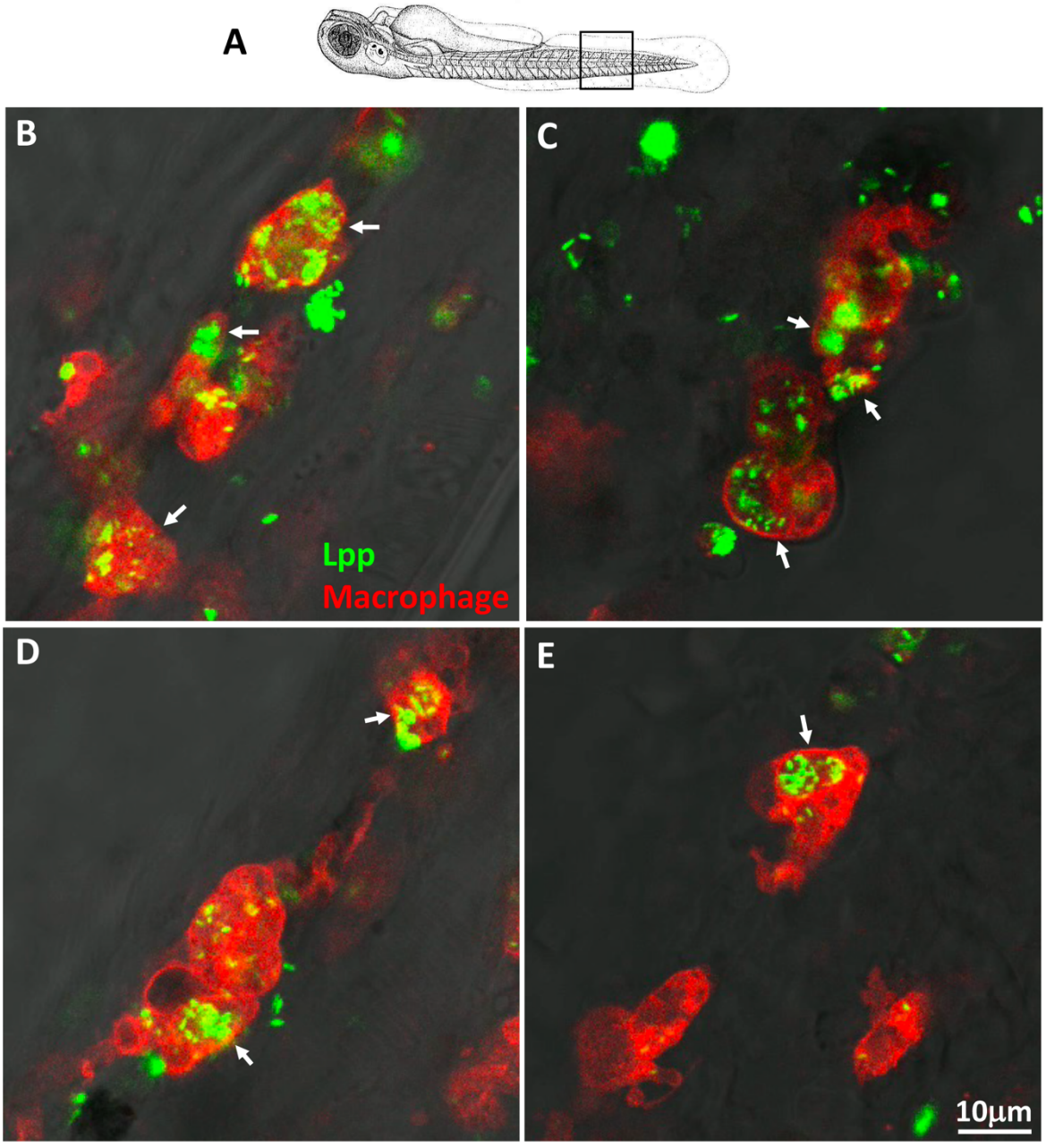

Figure S3

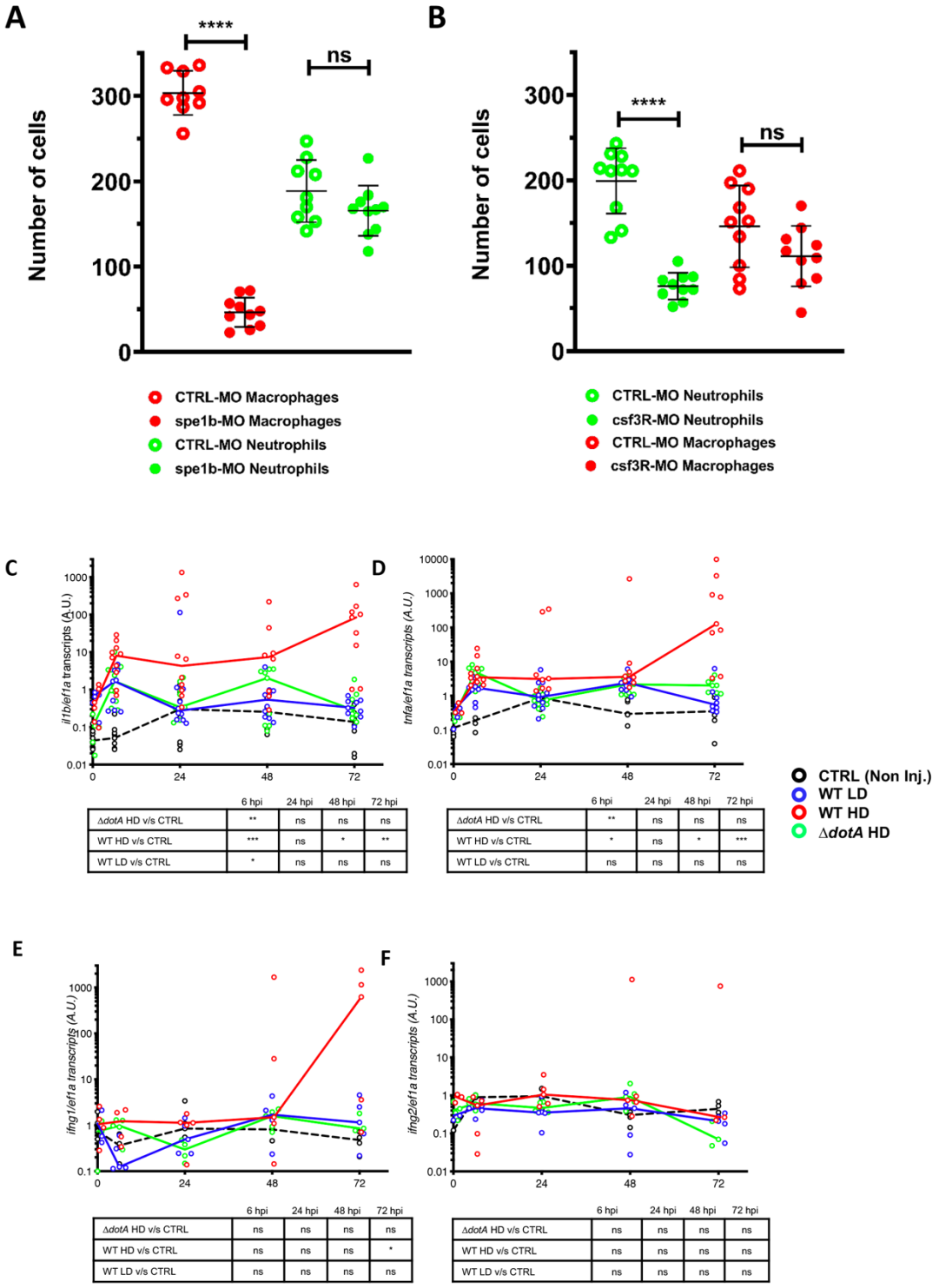
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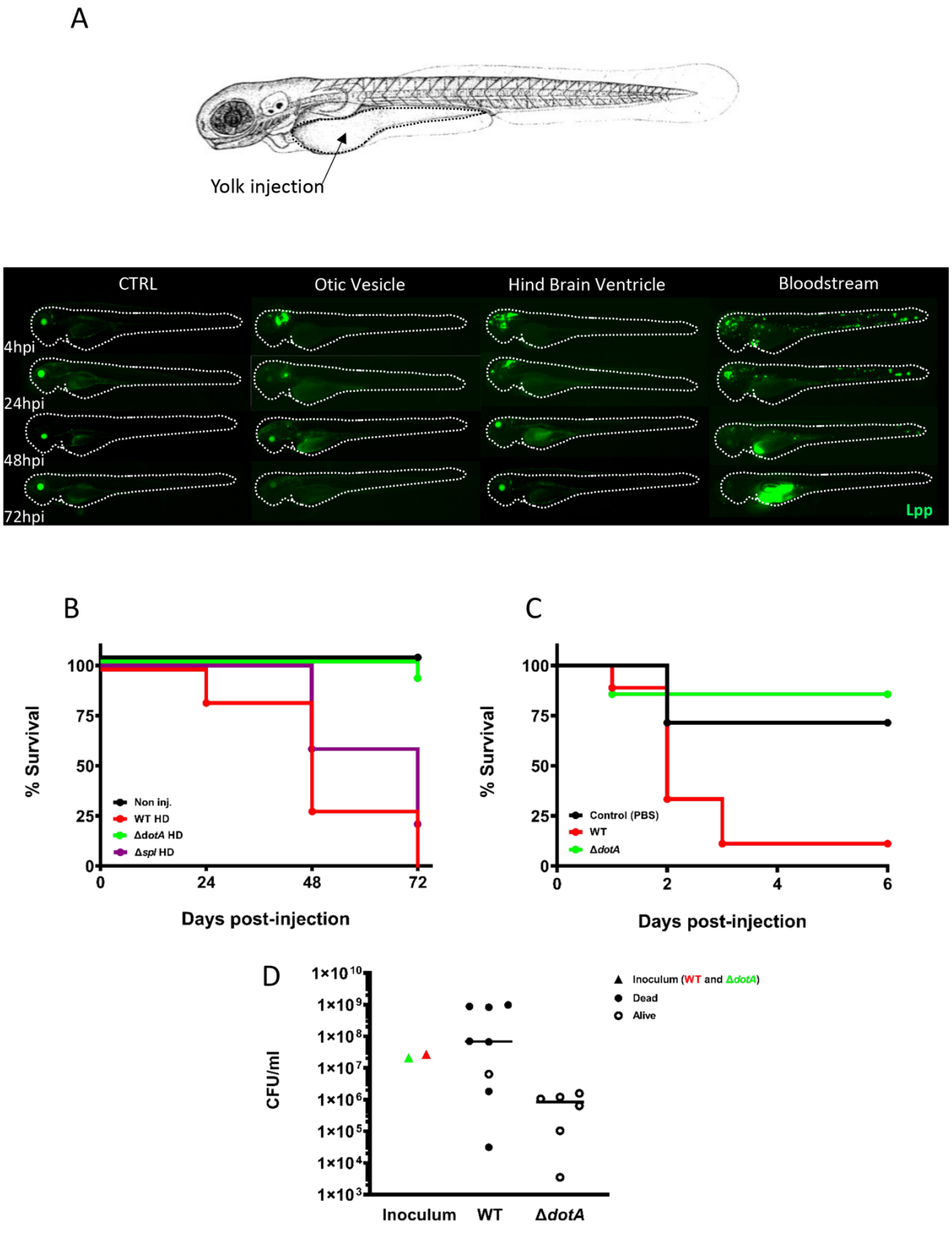
Figure S4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ΔdotA HD
Δspl HD
Days post-injection
C
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WT
ΔdotA
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Dead
Alive
Inoculum
WT
ΔdotA