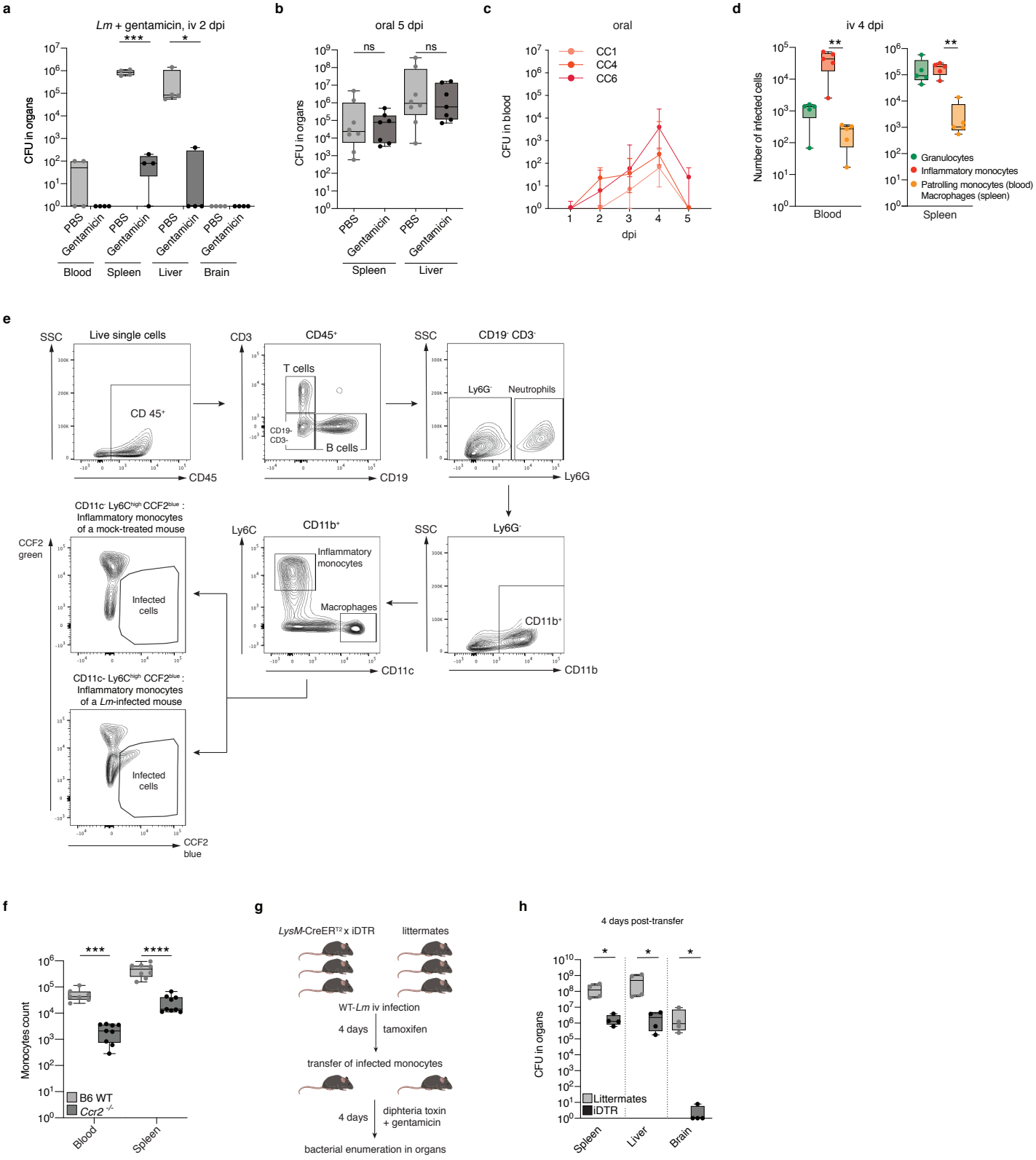
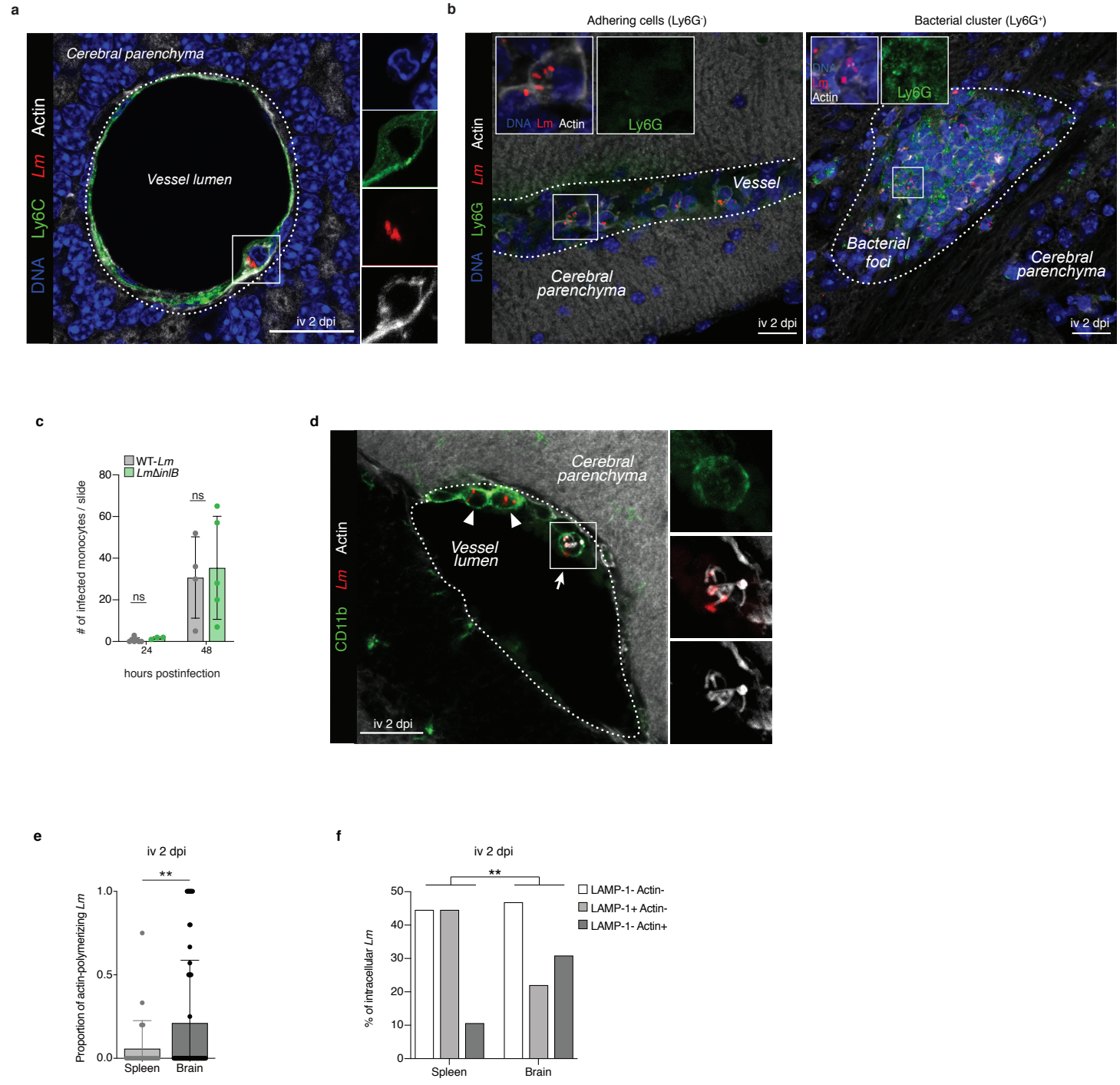
Extended Data Figure 1



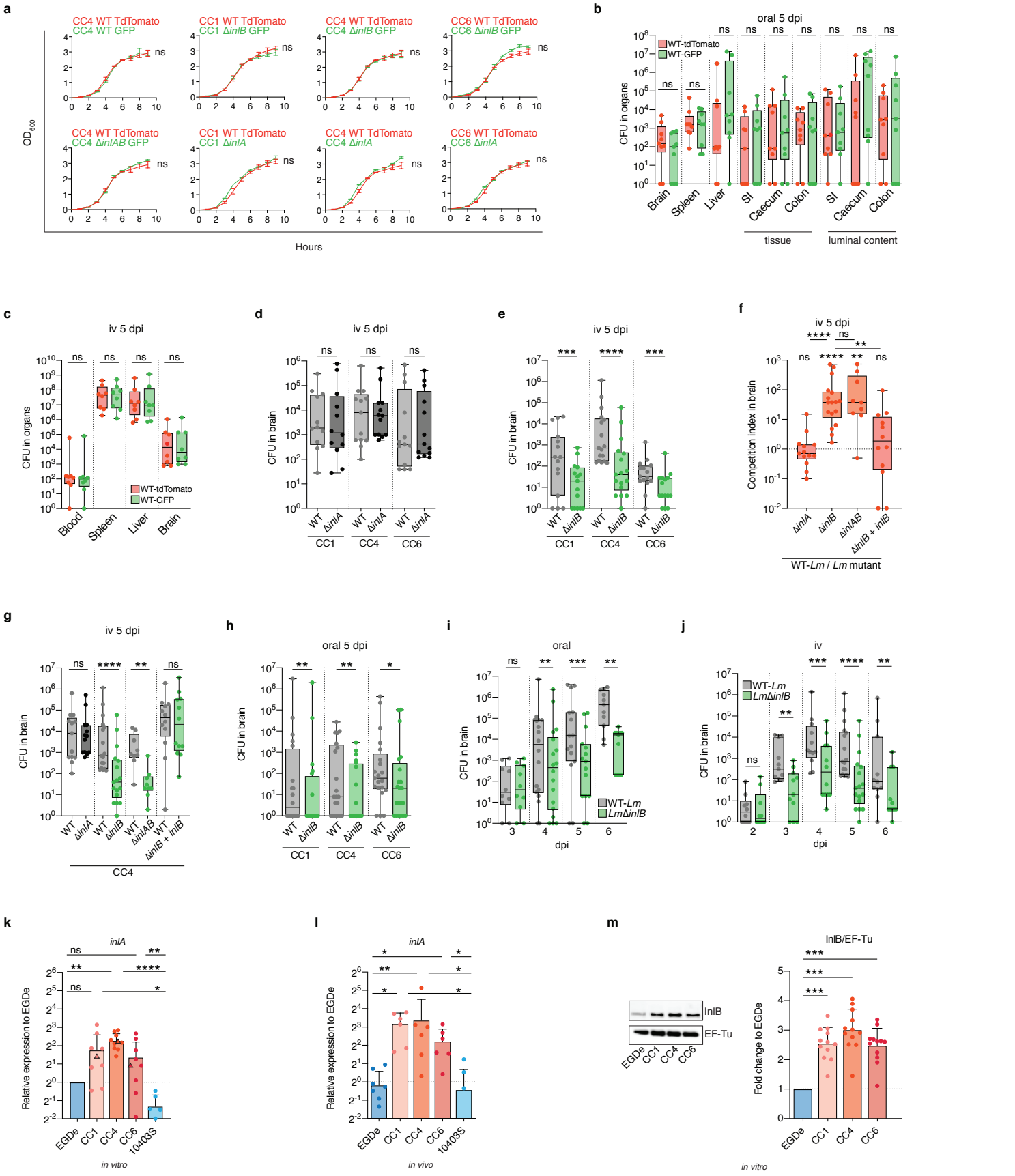
Extended Data Fig. 1. Infected inflammatory monocytes are necessary for *Lm* to invade the CNS. (**a**) Bacterial load in organs of KIE16P mice 2 days after iv inoculation with 10^4 CFU CC4-*Lm* immediately followed by injection of gentamicin, assessing the bactericidal effect of gentamicin on extracellular circulating *Lm*. (**b**) Bacterial load in the spleen and liver 5 days after oral inoculation with 2×10^8 CFU CC4-*Lm*, in KIE16P mice treated with gentamicin every day from day 1 post-inoculation, related to Fig. 1A. (**c**) Bacterial load in the blood of KIE16P mice after oral inoculation with 2×10^8 CFU of CC1/CC4/CC6-*Lm*, related to Fig. 1A. (**d**) Repartition of the 3 main infected cell subsets in the blood and spleen of KIE16P mice 4 days after iv inoculation with 10^4 CFU of CC4-*Lm*. (**e**) Representative dot plots of the gating strategy used for flow cytometry analysis. Infected cells are identified through the shift of fluorescence, upon excitation with the 405 nm laser, of the CCF2-AM substrate from green (518 nm) to blue (447 nm) in presence of β-lactamase expressing-*Lm*. (**f**) Number of inflammatory monocytes in the blood and spleen of B6-WT or *Ccr2*$^{-/-}$ mice. (**g**) Schematic pipeline of the transfer experiment in *LysM*-CreER^{T2} × iDTR mice. (**h**) Bacterial load in the spleen, liver and brain of gentamicin- and diphtheria toxin-treated recipient *LysM*-CreER$^{T2+/-}$ × *Rosa26*-iDTR$^{+/-}$ and littermates mice, 4 days after injection of infected monocytes harvested from n = 3 donor tamoxifen-treated *LysM*-CreER$^{T2+/-}$ × *Rosa26*-iDTR$^{+/-}$ or n = 3 littermates mice, 4 days after iv inoculation with 10^4 CC4-WT. Data were obtained from two (a) or three (b-d, f) and four (h) independent experiments and are presented as median ± interquartile (box) and extreme values (lines) (a-b, d, f and h) or as median ± interquartile (c). Samples are compared with an unpaired Mann-Whitney test (a-b, f and h) and number of infected cells with the Friedman test (d). ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$, ***: $p<0,001$, ****: $p<0.0001$.

Extended Data Figure 2



Extended Data Fig. 2. Infected inflammatory monocytes transfer *Lm* to the CNS. (**a-b**) Representative fluorescence microscopy images of infected inflammatory monocytes adhering to endothelial cells 2 days after iv inoculation with 5×10^5 CFU CC1-*Lm* in KIE16P mice. Adhering infected cells are $Ly6C^+$ (a) and $Ly6G^-$ (b left panel; to ensure the specificity of the $Ly6G^-$ staining we show in the right panel a positive control staining for Ly6G in a parenchymal bacterial cluster). (**c**) Quantification of infected monocytes in brain vessels of KIE16P mice 24 and 48 hours after iv inoculation with 5×10^5 CFU CC4-*Lm* and CC4Δ*inlB*. Each dot corresponds to the average number of monocytes counted on two slides (representative median sagittal sections, 40 μm thickness) for one mouse. (**d**) Representative fluorescence microscopy images of infected inflammatory monocytes adhering to endothelial cells 2 days after iv inoculation of 5×10^5 CFU CC1-*Lm* in KIE16P mice, in which intra-monocytic *Lm* are found polymerizing host actin. (**e, f**) Proportion of actin-polymerizing *Lm* (e) and *Lm* co-localizing with either LAMP-1 or Actin (f) in spleen and brain of KIE16P mice 3 days after iv inoculation with 10^4 CC4-*Lm*. Scale bars: 20 μm, **a** and **d** are maximum intensity projections over a *z*-stack. Data were obtained from three independent experiments and are presented as mean ± SD. Samples are compared with an unpaired Mann-Whitney test (c and e) and a one-way ANOVA (f). ns: $p>0.05$, **: $p<0.01$.

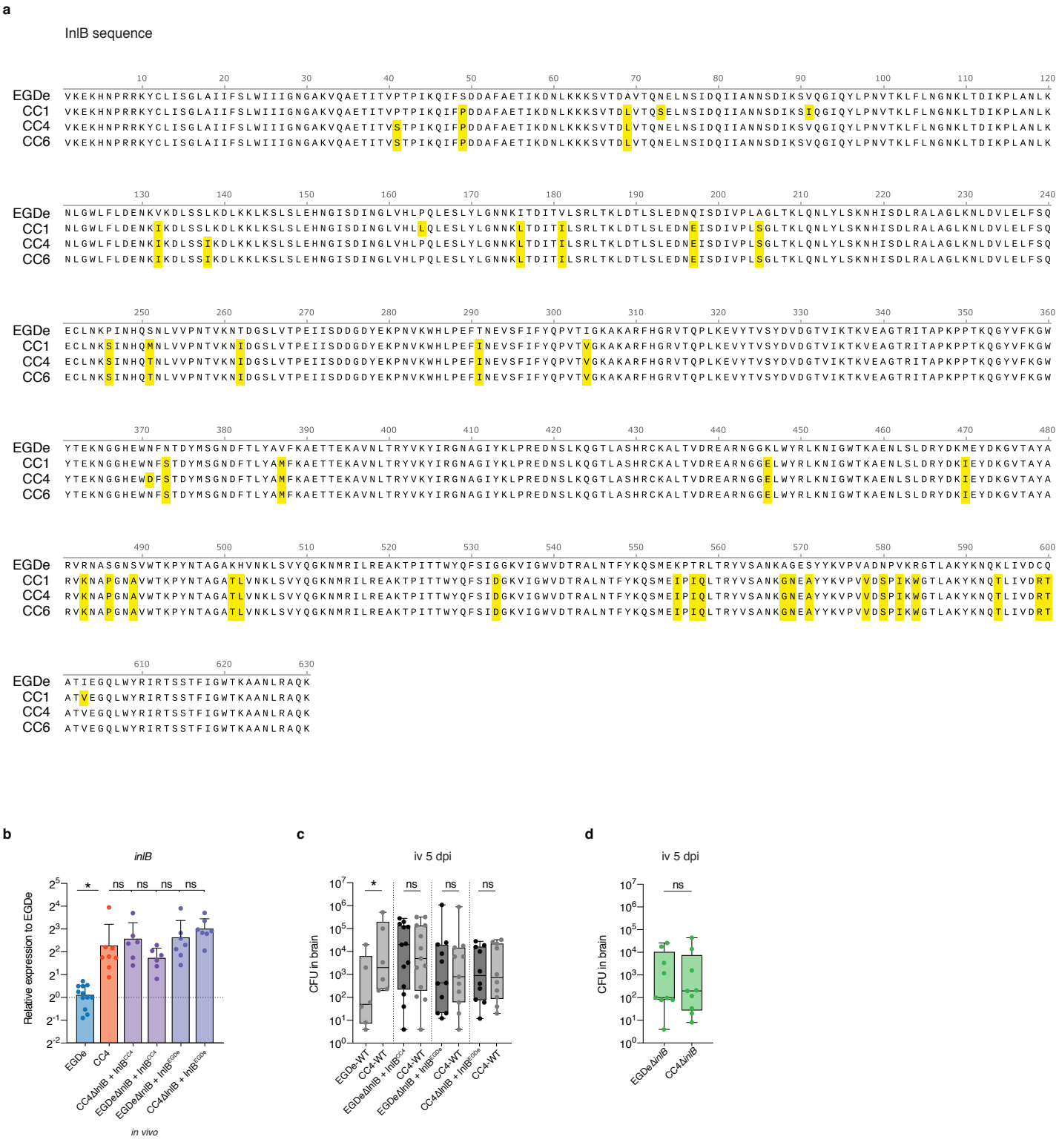
Extended Data Figure 3



Extended Data Fig. 3. InlB is a major determinant of *Lm* neuroinvasiveness whereas InlA is not. (a) Optical density of indicated bacterial strains measured every hour for 9 hours after 1:100 dilution in BHI of an overnight culture. (**b** and **c**) Bacterial load in organs or luminal contents of KIE16P mice 5 days after oral inoculation with 2×10^8 CFU (b) or after iv inoculation with 10^4 CFU (c) of 1:1 CC4-WT expressing TdTomato or GFP. (**d**) Bacterial load in brain of KIE16P mice 5 days after iv inoculation with 10^4 CFU of 1:1 mix of WT and Δ*inlA* isogenic strains, related to Fig. 2a. (**e**) Bacterial load in brain of KIE16P mice 5 days after iv inoculation with 10^4 CFU of 1:1 mix of WT and Δ*inlB* isogenic strains, related to Fig. 2b. (**f, g**) Competition index (f) and bacterial load (g) in brain of KIE16P mice 5 days after iv inoculation with 10^4 CFU of 1:1 mix of CC4-WT and either CC4Δ*inlA,* CC4Δ*inlB,* CC4Δ*inlAB* or CC4Δ*inlB* complemented with *inlB*, related to Fig. 2a-b and panels d,e. (**h**) Bacterial load in brain of KIE16P mice 5 days after oral inoculation with 2×10^8 CFU of 1:1 mix of WT strain and Δ*inlB* isogenic strains, related to Fig. 2c. **(i, j)** Bacterial load in brain of KIE16P mice at indicated times after oral inoculation with $2x10^8$ CFU (i) and after iv inoculation with 10^4 CFU (j) of 1:1 CC4-WT and CC4Δ*inlB*, related to Fig. 2f, g. (**k**) Transcription levels of *inlA* relative to EGDe in mid-log phase in BHI. For CC1/4/6, each dot corresponds to a different clinical isolate and triangles represent the strains used throughout the rest of the study and referred to as CC1, CC4 and CC6, related to Fig. 2h. (**l**) Transcription levels of *inlA* relative to EGDe in infected splenocytes 2 days after iv inoculation with $2x10^5$ CFU in KIE16P mice, related to Fig. 2i. (**m**) Representative Western blot (left) and quantification (right) of InlB expression, normalized to that of EF-Tu, relative to EGDe in mid-log phase in BHI. Data were obtained from three (a-l) and four (m) independent experiments and are presented as mean ± SD (a, k-m) or as median ± interquartile (box) and extreme values (lines) (b-j). Curves were fitted with a Gompertz model and the lag phases (k) for each pair of *Lm* strains were compared with the

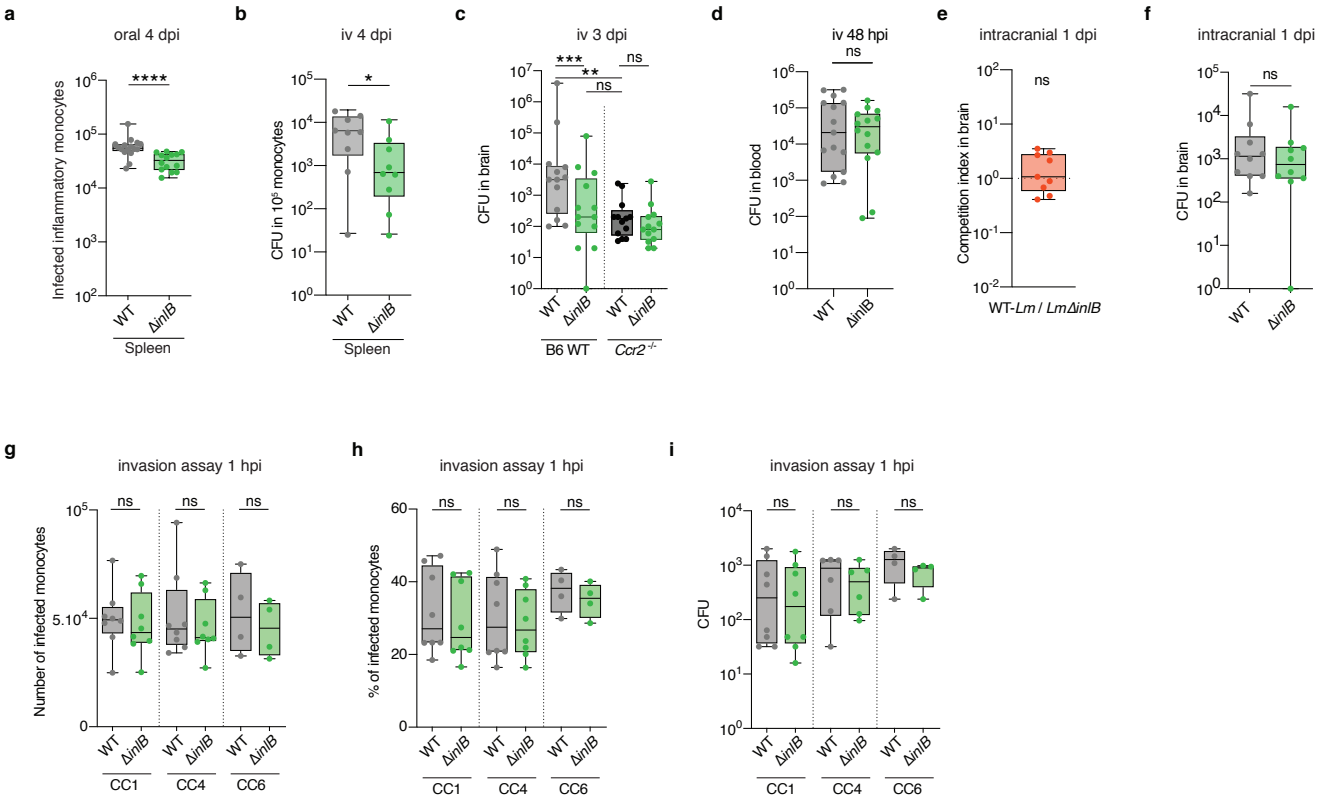
extra sum-of-squares F test (a). CFU in competition assays are compared with the Wilcoxon matched-pairs signed rank test (b-j) and samples compared with the Kruskal-Wallis test (f, k-m). ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.

Extended Data Figure 4



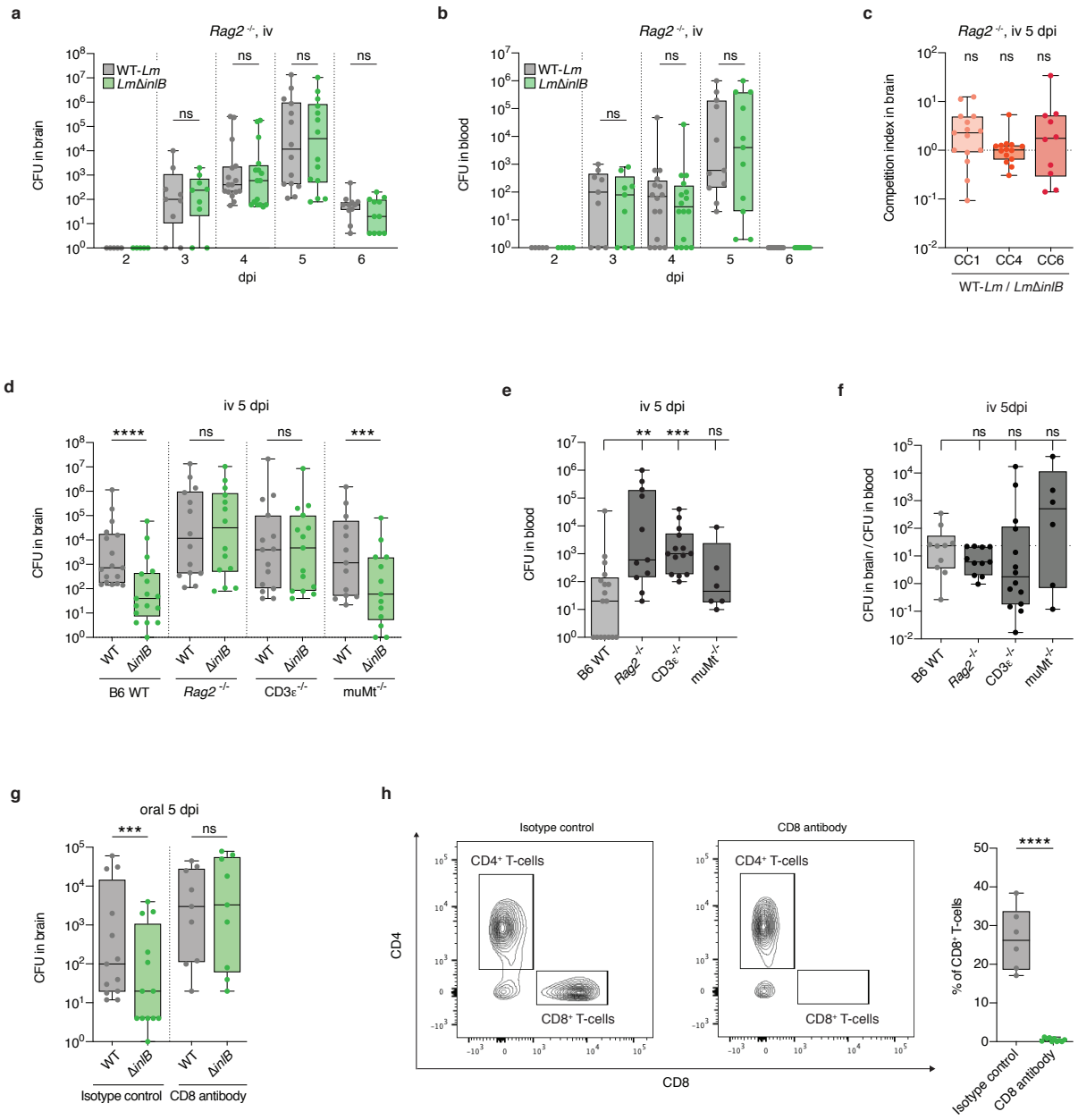
Extended Data Fig. 4. Levels of expression of InlB, and not allelic differences with EGDe, explain enhanced neuroinvasiveness of hypervirulent CC1, CC4 and CC6 strains. (a) Protein sequence alignment of InlB alleles from EGDe and from CC1, CC4 and CC6 strains. Mismatches are indicated in yellow. (**b**) Transcription levels of *inlB,* relative to EGDe, in infected splenocytes 2 days after iv inoculation with $2x10^5$ CFU in KIE16P mice of EGDe-WT, CC4-WT and strains complemented with either InlB from EGDe or from CC4. (**c**) Bacterial load in brain of KIE16P mice 5 days after iv inoculation with 10^4 CFU of a 1:1 mix of the indicated bacterial strains, related to Fig. 2j. (**d**) Bacterial load in brain of KIE16P mice 5 days after iv inoculation with $2x10^4$ CFU of a 1:1 mix of EGDeΔ*inlB* and CC4Δ*inlB*, related to Fig. 2j. Data were obtained from three independent experiments and are presented as mean ± SD (b) or as median ± interquartile (box) and extreme values (lines) (c-d). CFU in competition assays are compared with the Wilcoxon matched-pairs signed rank test (c-d) and samples compared with the Kruskal-Wallis test (b). ns: $p>0.05$, *: $p<0.05$.

Extended Data Figure 5



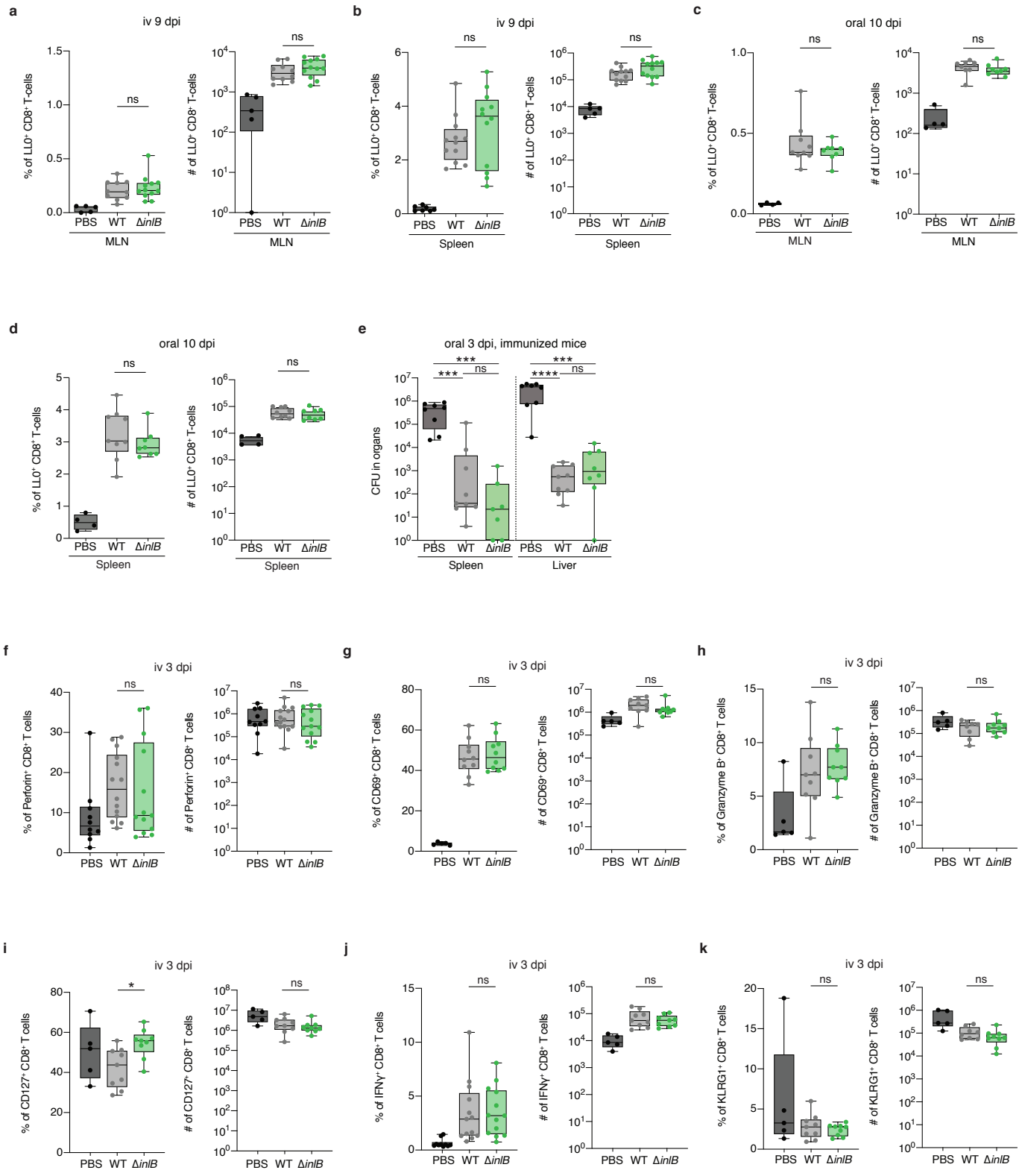
Extended Data Fig. 5. InlB is not involved in *Lm* invasion in monocytes. (**a**) Number of infected monocytes in the spleen of KIE16P mice 4 days after oral inoculation with 2×10^8 CFU of CC4-WT or CC4Δ*inlB*. (**b**) Bacterial enumeration from sorted monocytes retrieved from KIE16P mice iv infected for 4 days with 10^4 CFU of CC4-WT or CC4Δ*inlB*. (**c**) Bacterial load in brain of control or *Ccr2*$^{-/-}$ mice 3 days after iv inoculation with 10^4 CFU of 1:1 CC4-WT and CC4Δ*inlB*. (**d**) Bacterial load in the brain 48 hours after iv inoculation of KIE16P mice with 5×10^5 CFU of either CC4-WT strain or CC4Δ*inlB*. (**e, f**) Competition index (e) and bacterial load (f) in the brain of KIE16P mice 1 day after intracranial inoculation with 10^2 CFU of 1:1 mix of CC4-WT and CC4Δ*inlB*. (**g-i**) Number of infected monocytes (g), percentage of infected monocytes (h) and bacterial load (i) in monocytes 1 hour after *in vitro* infection of primary bone marrow mouse monocytes with WT-*Lm* or Δ*inlB* isogenic mutant, at a MOI of 5. Data were obtained from three (d-f) and four (a-c, g-i) independent experiments and are presented as median ± interquartile (box) and extreme values (lines). Samples are compared with the unpaired Mann-Whitney test (a-b, d, g-i) and the Kruskal-Wallis test (c), and CFU in competition assays are compared with the Wilcoxon matched-pairs signed rank test (c, e-f). ns: $p>0.05$.

Extended Data Figure 6



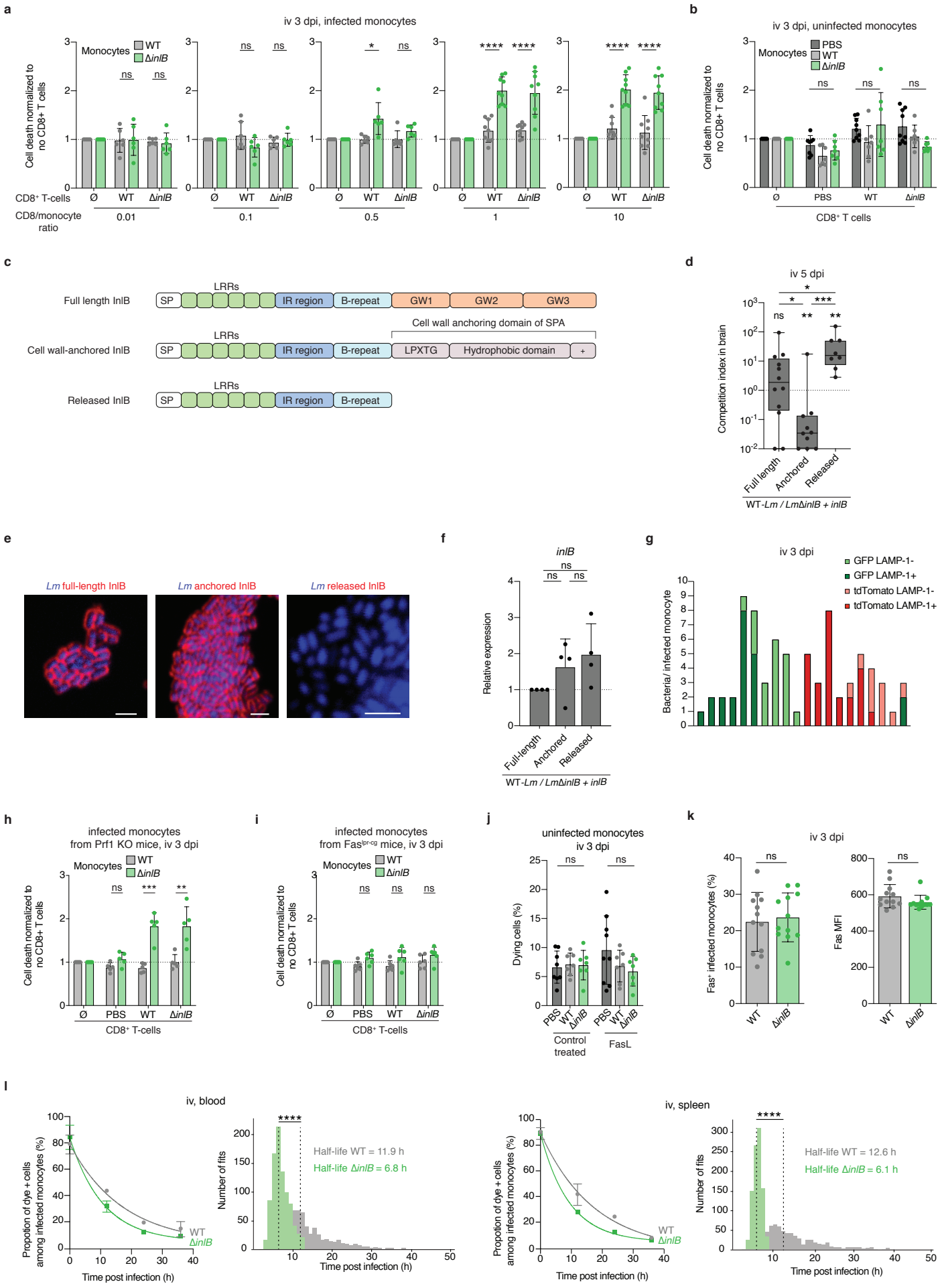
Extended Data Figure 6. InlB-mediated *Lm* neuroinvasiveness is abrogated in CD8$^+$ T-cells deficient mice. (**a, b**) Bacterial load in brain (a) and in blood (b) of *Rag2*$^{-/-}$ mice after iv inoculation with 10^4 CFU of a 1:1 mix of CC4-WT and CC4Δ*inlB*, related to Fig.3f (**c**) Competition index in brain of *Rag2*$^{-/-}$ mice 5 days after iv inoculation with 10^4 CFU of a 1:1 mix WT strain and Δ*inlB* isogenic strains. (**d, e**) Bacterial load in brain (d) and in blood (e) 5 days after iv inoculation with 10^4 CFU of 1:1 CC4-WT strain and CC4Δ*inlB* isogenic mutant in control mice and in mice lacking functional T (CD3ε$^{-/-}$), B lymphocytes (muMt$^{-/-}$) or both (*Rag2*$^{-/-}$), related to Fig. 3g. (**f**) Ratio of brain/blood bacterial load in control, *Rag2*$^{-/-}$, CD3ε$^{-/-}$ and muMt$^{-/-}$ mice, related to Fig. 3g. (**g**) Bacterial load in brain of KIE16P mice 5 days after oral inoculation with 2×10^8 CFU of 1:1 CC4-WT and CC4Δ*inlB* after CD8$^+$ T-cells depletion, related to Fig. 3h. (**h**) Representative dot plots (left) and proportion of CD8$^+$ T-cells (right) among CD45$^+$ CD3$^+$ cells in the spleen, after CD8$^+$ T-cells depletion, related to Fig. 3h. Data were obtained from three independent experiments and are presented as median ± interquartile (box) and extreme values (lines). CFU in competition assays are compared with the Wilcoxon matched-pairs signed rank test (a-d and g) and samples are compared with the Kruskal-Wallis test (e, f) and with the Mann-Whitney test (h). ns: $p>0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.

Extended Data Figure 7



Extended Data Figure 7. InlB does not alter the induction and differentiation of specific anti-*Lm* CD8$^+$ T-cells. (**a, b**) Percentage (left) and number (right) of LLO-specific CD8$^+$ T-cells in mesenteric lymph nodes (MLN) (a) and spleen (b) of BALB/c mice 9 days after iv inoculation with 1x10^3 of CC4-WT strain or CC4Δ*inlB*. (**c, d**) Percentage (left) and number (right) of LLO-specific CD8$^+$ T-cells in mesenteric lymph nodes (MLN) (c) and spleen (d) of iFABP-hEcad mice 10 days after oral inoculation with 2x10^7 of CC4-WT strain or CC4Δ*inlB*. (**e**) Bacterial load in spleen and liver 3 days after oral inoculation with 1x10^9 CFU of CC4-WT in KIE16P mice challenged 30 days before with 5x10^7 CFU of CC4-WT strain or CC4Δ*inlB*. (**f-k**) Percentage (left) and number (right) of Perforin$^+$ (f), CD69$^+$ (g), Granzyme-B$^+$ (h), CD127$^+$ (i), IFNγ$^+$ (j) and KLRG1$^+$ (k) CD8$^+$ T-cells 3 days after iv inoculation of KIE16P mice with 10^4 CFU of CC4-WT or CC4Δ*inlB*. Data were obtained from three independent experiments and are presented as median ± interquartile (box) and extreme values (lines). Samples are compared with the Mann-Whitney test. ns: $p>0.05$, ***: $p<0.001$, ****: $p<0.0001$.

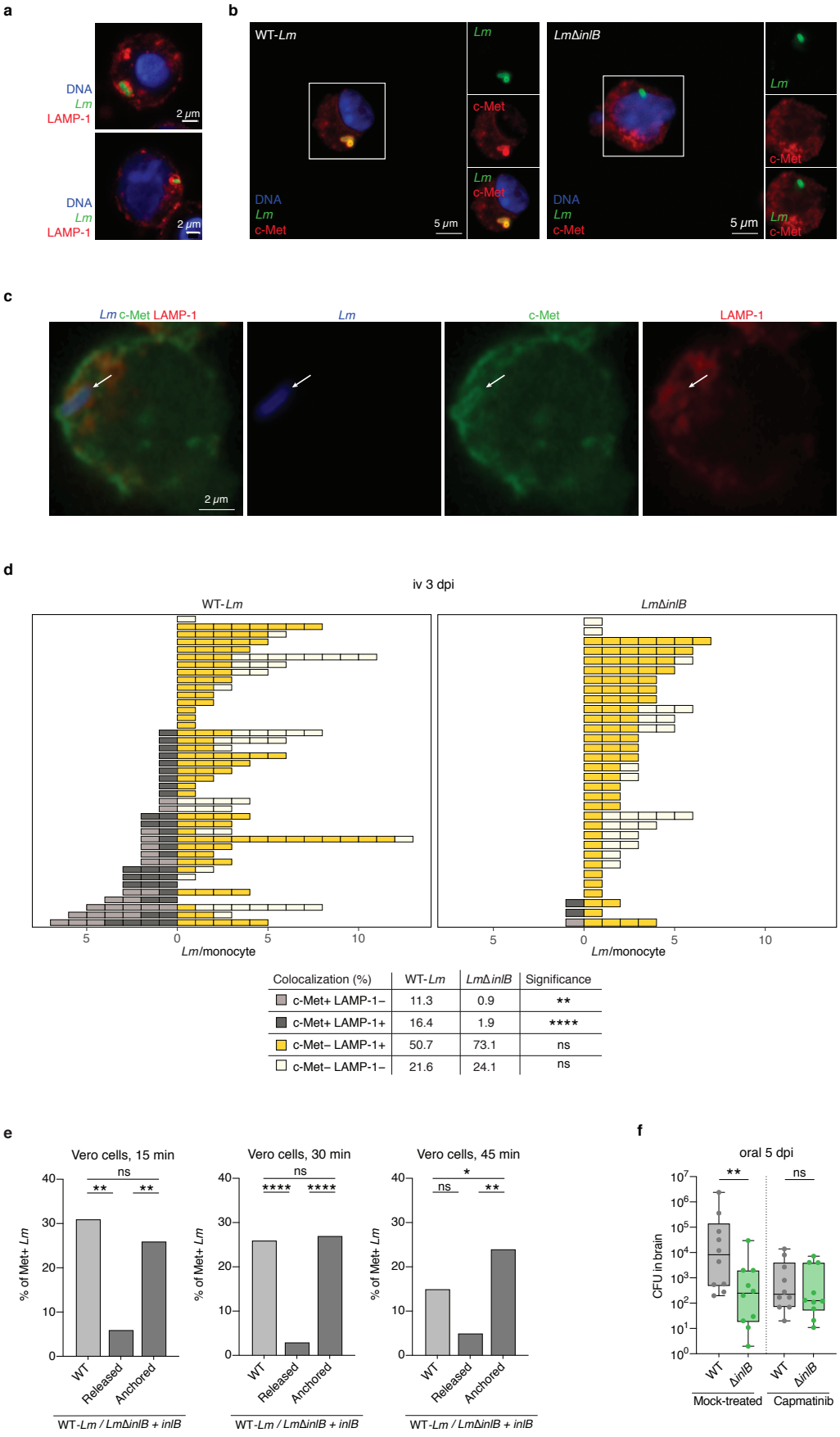
Extended Data Figure 8



Extended Data Figure 8. Membrane-associated InlB protects infected monocytes from CD8$^+$ T cells-mediated cell death. (a) Level of caspase-3 cleavage of infected spleen monocytes, harvested 3 days after iv infection with 10^4 CFU of CC4-WT or CC4Δ*inlB* of KIE16P mice, and incubated with CD8$^+$ T-cells from similarly infected (WT and Δ*inlB*) or control (PBS) mice at the indicated effector to target ratio, related to Fig. 3j. Results are normalized to the level of caspase-3 cleavage in absence of CD8$^+$ T cells. (**b**) Level of caspase-3 cleavage of uninfected spleen monocytes, harvested 3 days after iv infection with 10^4 CFU of CC4-WT or CC4Δ*inlB* of KIE16P mice, and incubated with CD8$^+$ T-cells from similarly infected (WT and Δ*inlB*) or control (PBS) mice at an effector to target ratio of 5, related to Fig. 3j. Results are normalized to the level of caspase-3 cleavage in absence of CD8$^+$ T cells. (**c**) Schematic representation of WT InlB and its anchored and released variants. (**d**) Competition index in the brain of KIE16P mice 5 days after iv inoculation with 10^4 CFU of 1:1 CC4-WT and CC4Δ*inlB* transformed with a plasmid expressing either full-length WT InlB, cell wall-anchored InlB or released InlB. (**e**) Representative fluorescence microscopy images of centrifugated CC4Δ*inlB* transformed with a plasmid expressing either full length InlB (left panel), anchored InlB (central panel) or released InlB (right panel). Scale bars: 5 μm. (**f**) Transcription level of *inlB* in CC4Δ*inlB* transformed with a plasmid expressing InlB variants in mid-log phase in BHI, relative to CC4Δ*inlB* expressing full length InlB. (**g**) Proportion of GFP- or tdTomato-expressing bacteria, co-localizing or not with LAMP-1, in 20 infected monocytes harvested 3 days after iv inoculation of KIE16P mice with 10^4 CFU of 1:1 mix of CC4-WT expressing GFP or tdTomato. (**h, i**) Level of caspase-3 cleavage of infected spleen monocytes, harvested from *Prf1 KO* (h) or *Fas*$^{lpr\text{-}cg}$ (i) mice, 3 days after iv inoculation with 10^4 CFU of CC4-WT or CC4Δ*inlB*, incubated with CD8$^+$ T-cells from similarly infected (WT and Δ*inlB*) or control (PBS) mice, at an effector to target ratio of 5. (**j**) Level of caspase-3 cleavage of

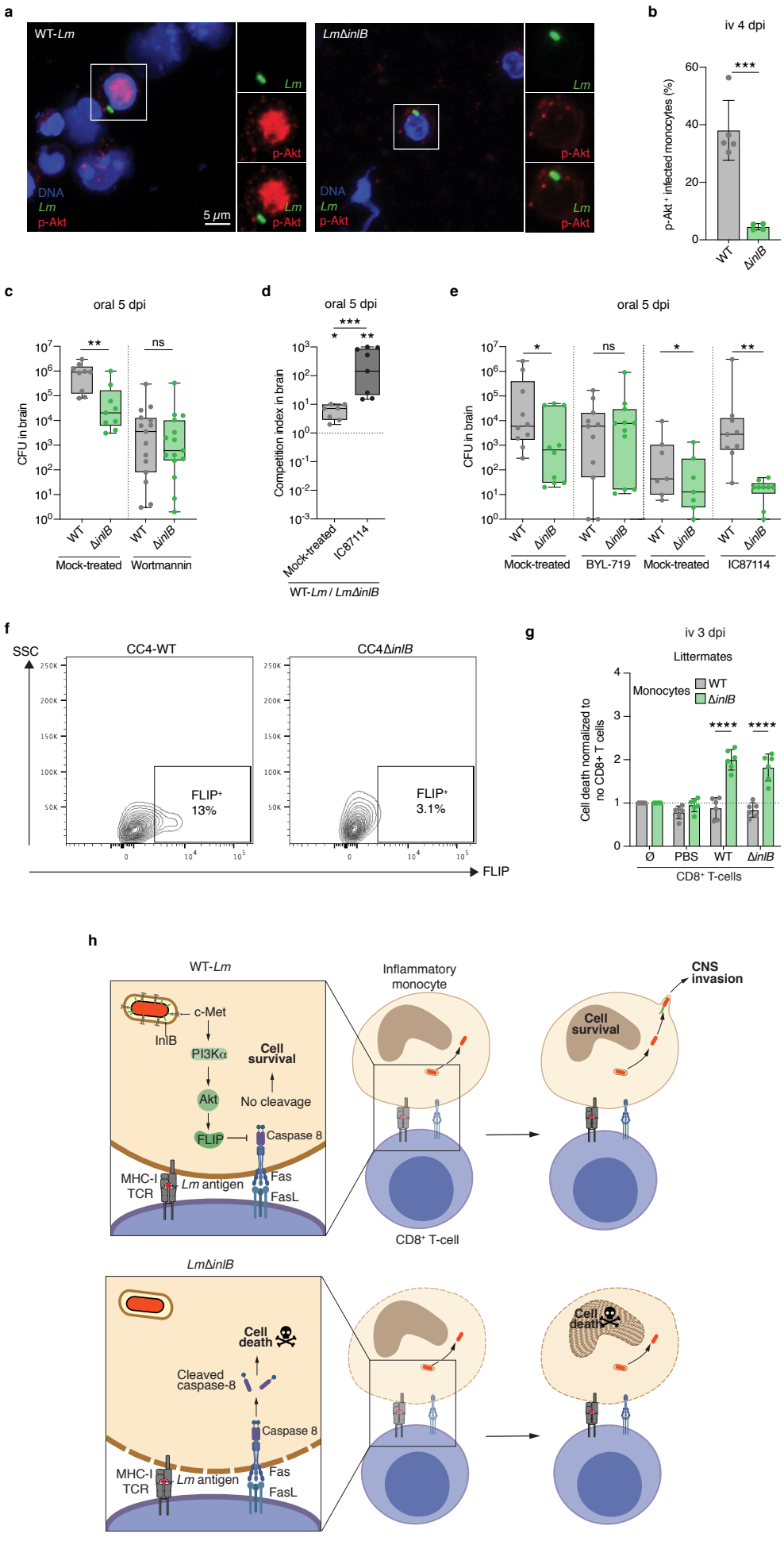
non-infected spleen monocytes, harvested from KIE16P mice iv infected for 3 days with 10^4 CFU of CC4-WT or CC4Δ*inlB*, incubated *ex vivo* with Fas ligand, related to Fig. 3k. (**k**) Percentage of infected spleen monocytes expressing Fas at their surface (left), and the mean fluorescence intensity (MFI) of Fas signal (right), 3 days after iv inoculation of KIE16P mice with 10^4 CFU of CC4-WT or CC4Δ*inlB*. (**l**) Proportion of dye-positive infected monocytes in the blood and the spleen after iv infection of KIE16P mice with 10^4 CFU of CC4-WT or CC4-Δ*inlB*. The repartition of the estimated half-lives and the median are shown besides each graph. Data were obtained from three independent experiments and are presented as mean ± SD (a, b, f, h-l) or median ± interquartile (box) and extreme values (lines) (d). Samples are compared with an unpaired student *t*-test (a, b, f, h-k), CFU in competition assays are compared with the Wilcoxon matched-pairs signed rank test (d), samples are compared with the Kruskal-Wallis test (d) and distribution of estimated half-lives (i) are compared with a Mood test. ns: $p>0.05$, *: $p<0.05$, ***: $p<0.001$, ****: $p<0.0001$.

Extended Data Figure 9



Extended Data Figure 9. InlB recruits c-Met in infected monocytes. (**a-c**) Representative fluorescence microscopy images of spleen monocytes harvested from KIE16P mice iv infected for 4 days with 10^4 CFU of CC4-WT or CC4Δ*inlB*, showing intra-vacuolar *Lm* surrounded with LAMP-1 (a), co-localizing with c-Met (b) and co-localizing with both c-Met and LAMP-1 (c). (a and b) are maximum intensity projection over a *z*-stack. (c) is a confocal single plane image. (**d**) Quantification of intracellular *Lm* co-localizing or not with c-Met and LAMP-1 in infected spleen monocytes harvested from KIE16P mice iv infected for 4 days with 10^4 CFU of CC4-WT or CC4Δ*inlB*. Individual cells are plotted in top panel and samples are compared in bottom panel. (**e**) Percentage of *Lm* co-localizing with c-Met *in vitro* in Vero cells 15 min (left), 30 min (middle) and 45 min (right) after infection at MOI 50 with CC4Δ*inlB* expressing either WT InlB, released InlB or cell wall-anchored InlB. (**f**) Bacterial load in the brain 5 days after oral inoculation with 2×10^8 CFU of 1:1 of CC4-WT and CC4Δ*inlB*, in KIE16P mice treated with capmatinib, related to Fig. 4a. Data were obtained from three independent experiments (e-f) or from three microscopic field of views (d). Median number of bacteria in each intracellular compartment were compared with the Mann-Whitney test (d), proportions of c-Met associated bacteria (e) with Fischer's exact test, and CFU in competition assays (f) compared with the Wilcoxon matched-pairs signed rank test. ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$, ****: $p<0.0001$.

Extended Data Figure 10



Extended Data Figure 10. InlB-mediated neuroinvasion involves the c-Met/PI3Kα/FLIP pathway in infected monocytes. (**a**) Representative fluorescence microscopy images of spleen monocytes harvested from KIE16P mice iv infected for 4 days with 10^4 CFU of CC4-WT or CC4Δ*inlB*, showing phosphorylation of Akt. Images are maximum intensity projection over a *z*-stack. (**b**) Proportion of infected spleen monocytes positive for p-Akt signal 4 days after iv inoculation of KIE16P mice with 10^4 CFU of CC4-WT or CC4Δ*inlB*. (**c**) Bacterial load in brain 5 days after oral inoculation with 2×10^8 CFU of 1:1 of CC4-WT and CC4Δ*inlB*, in KIE16P mice treated with wortmannin, related to Fig. 4d. (**d**) Competition index in brain 5 days after oral inoculation with 2×10^8 CFU of 1:1 of CC4-WT and CC4Δ*inlB*, in KIE16P mice treated with PI3Kδ inhibitor (IC87114). (**e**) Bacterial load in the brain 5 days after oral inoculation with 2×10^8 CFU of 1:1 of CC4-WT and CC4Δ*inlB*, in mice treated with BYL-719 or IC87114, related to Fig. 4e and Extended Data Fig. 10d. (**f**) Representative dot plot of FLIP expression in infected inflammatory spleen monocytes, 3 days after iv inoculation with 10^4 CFU of CC4-WT or CC4Δ*inlB*, related to Fig. 4f, g. (**g**) Level of caspase-3 cleavage of infected spleen monocytes, harvested 3 days after iv infection with 10^4 CFU of CC4-WT or CC4Δ*inlB* of tamoxifen-treated *Rosa26*-CreER^{T2} x *Cflar*$^{+/+}$ (FLIP$^{+/+}$) littermate mice and incubated with CD8$^+$ T-cells from similarly infected mice at an effector to target ratio of 5, related to Fig. 4h. (**h**) Representation of InlB-activated pathway of infected monocytes survival to Fas-mediated cell death. Data were obtained from three independent experiments and are presented as median ± interquartile (box) and extreme values (lines) (c-e) or mean ± SD (b, g). CFU in competition assays are compared with the Wilcoxon matched-pairs signed rank test (c-e) and samples with a Mann-Whitney test (c-e) or an unpaired student *t*-test (b, g). ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.

Movie S1. Polymerization of actin comet tail by *Lm* within a monocyte adhering to the blood-brain barrier.
CX3CR1$^{GFP/+}$ E16P KI humanized mice were infected with 5x10^5 CFUs of CC6 (isolate 2009-01092) via iv route. Mice were sacrificed 48 hours post infection. CX3CR1$^+$ are labeled in green, *L. monocytogenes* in red, actin (phalloidin) in white and nuclei (Hoechst) in blue. Forty-six optical sections of a 20 μm thick brain sample were imaged with a Zeiss LSM700 confocal microscope. 3D reconstruction was performed using the Arivis Vison 4D software.