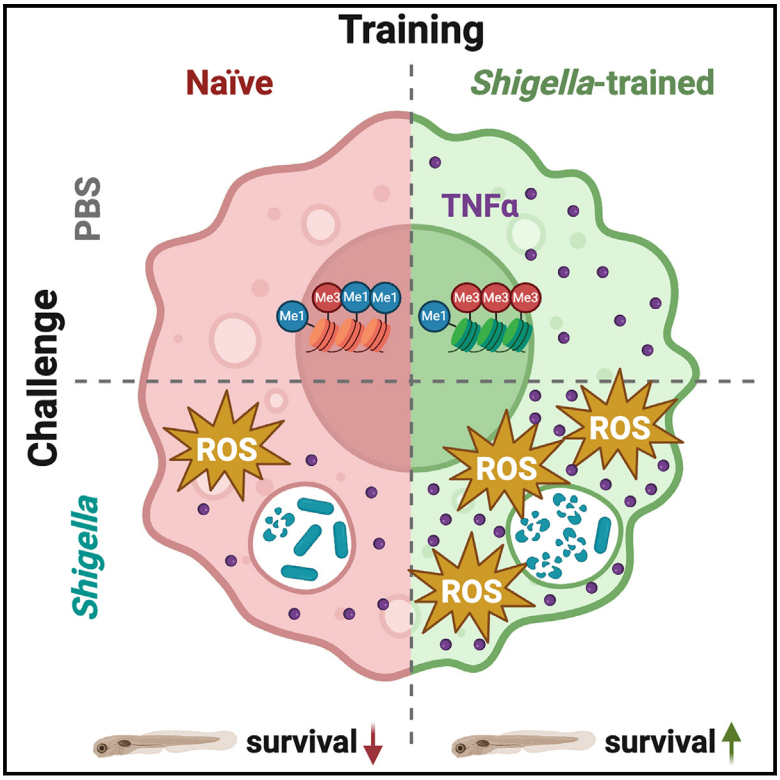
Cell Reports

Shigella-trained pro-inflammatory macrophages protect zebrafish from secondary infection

Graphical abstract



Highlights

- Zebrafish are valuable in studying *Shigella*-phagocyte interactions and immune training
- Trained macrophages are crucial for zebrafish survival during *Shigella* re-infection
- Bacteria clear during training, and protection is independent of re-infection site
- Trained macrophages show altered H3K4me1/me3 and shift to a pro-inflammatory state

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In brief

Trained immunity enhances host innate defenses against secondary infections. Gomes et al. innovate zebrafish infection models to investigate *Shigella*-induced training, demonstrating that *Shigella* epigenetically reprograms macrophages to be pro-inflammatory and promote antibacterial responses.

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Report

Shigella-trained pro-inflammatory macrophages protect zebrafish from secondary infection

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SUMMARY

Shigella is an important human pathogen that has no licensed vaccine. Despite decades of seminal work suggesting that its pathogenicity relies on inflammatory cell death of macrophages, the *in vivo* role of macrophages in controlling *Shigella* infection remains poorly understood. Here, we use a zebrafish model of innate immune training to investigate the antibacterial role of macrophages following a non-lethal *Shigella* infection. We found that macrophages are crucial for zebrafish larvae survival during secondary *Shigella* infection. Consistent with signatures of trained immunity, we demonstrate that bacteria are cleared during training and that protection is independent of the secondary infection site. We show that following *Shigella* training, macrophages have altered mono- and tri-methylation on lysine 4 in histone 3 (H3K4me1/me3) deposition and shift toward a pro-inflammatory state, characterized by increased tumor necrosis factor alpha (TNF-α) expression and antibacterial reactive oxygen species (ROS) production. We conclude that macrophages are epigenetically reprogrammed by *Shigella* infection to enhance pro-inflammatory and protective responses.

INTRODUCTION

Shigella is a human-adapted pathogen and leading cause of enteric infections worldwide.[1–3] Despite significant efforts to develop vaccines against shigellosis, an effective vaccine for its prevention has yet to be discovered, partly because the complete immunological response to *Shigella* is not yet known.[4] *Shigella* is an intensively studied pathogen that employs a type III secretion system (T3SS) during infection to manipulate the host cell and promote its survival, replication, and dissemination.[5] A crucial aspect of *Shigella* virulence is the induction of cell death in macrophages.[6] From decades of seminal work, mostly performed *in vitro* using tissue culture cells, *Shigella* has been shown to activate inflammasome assembly and trigger pyroptosis (pro-inflammatory cell death), rendering these cells unable to control infection.[7–9] In contrast, the antibacterial role of macrophages in *Shigella* infection control *in vivo* has been poorly understood.

There are no non-primate animal models that can fully replicate natural shigellosis. To overcome this limitation, the zebrafish infection model has emerged as valuable in understanding the host response to *Shigella*.[3,10–12] Over 10 years of work has shown that the main hallmarks of *Shigella* infection are reproduced during the infection of zebrafish larvae, including macrophage cell death and pro-inflammatory responses.[12] Moreover, the infection of zebrafish has been used to investigate the host response to epidemiologically successful *Shigella* isolates,[3,13–16] bacteria cell-cell interactions,[17,18] and killing of drug-resistant *Shigella* by *Bdellovibrio*, antibiotics, or bacteriophage.[17,19,20]

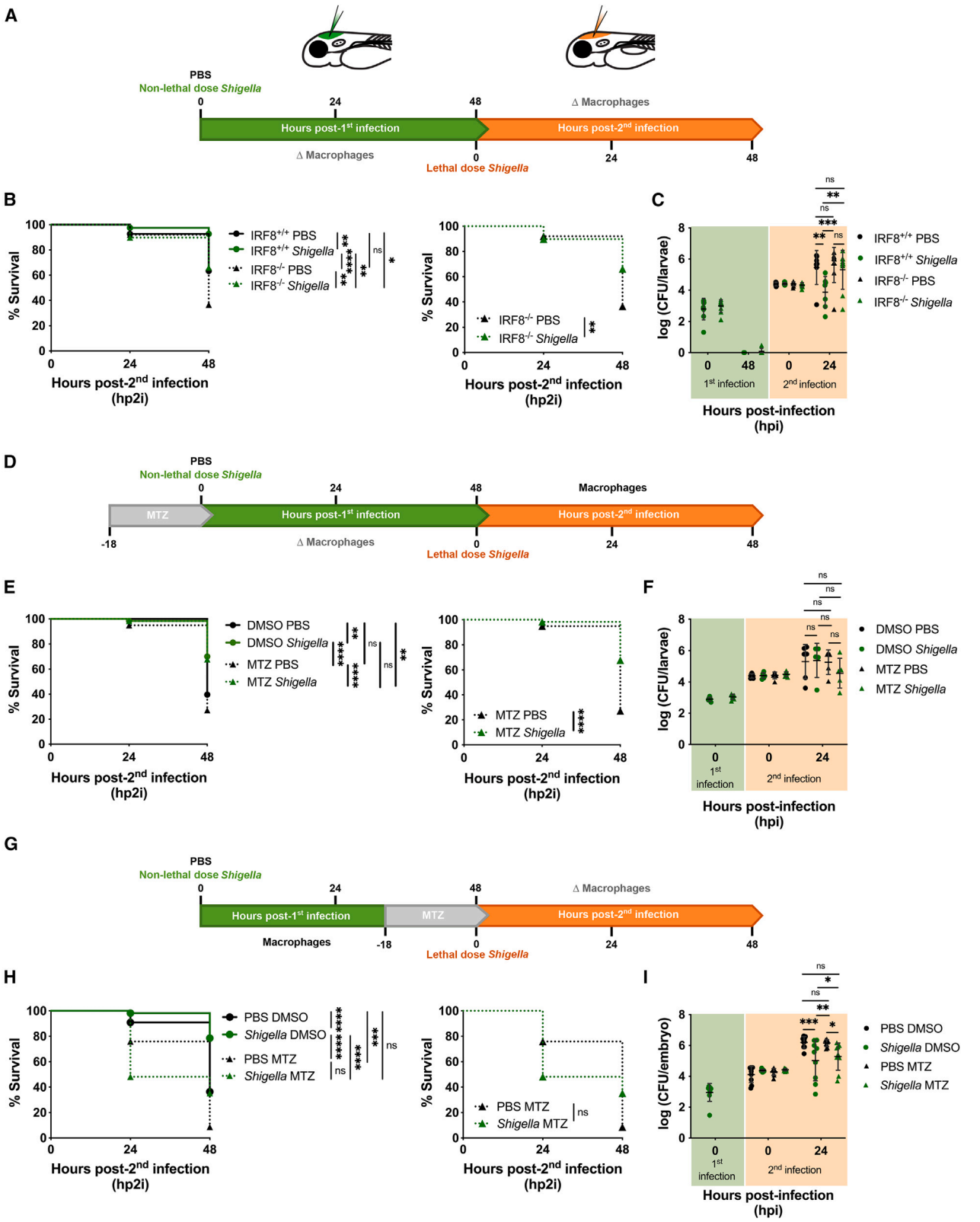
To progress toward a more complete understanding of the immune response to *Shigella*, we have been using zebrafish to study trained immunity.[21] Trained immunity is an epigenetic and metabolic rewiring of hematopoietic stem cells (HSCs) and myeloid progenitor cells toward a more effective response against secondary infection.[22] We and others have shown that *Shigella*, *Salmonella*, Bacillus Calmette–Guérin (BCG), and β-glucan can all train immunity in zebrafish larvae.[21,23] In the case of *Shigella*-trained zebrafish larvae, we have shown that neutrophils become more efficient at killing bacteria using mitochondrial reactive oxygen species (mtROS) and more resilient to cell death.[21]

Here, in this report, we show using zebrafish that macrophages trained by *Shigella* become more responsive to secondary infection, shifting toward a pro-inflammatory phenotype characterized by the increased expression of tumor necrosis factor alpha (TNF-α) and production of ROS. Together, the results of our zebrafish model of innate immune training reveal a previously unknown role for macrophages during infection control *in vivo*.

RESULTS

Shigella-trained macrophages reduce bacterial burden and promote zebrafish survival

Shigella induces trained immunity for better infection control by epigenetically reprogramming neutrophils,[21] but the role of macrophages was unknown. To assess the macrophage contribution to immune training and infection control, we used an interferon



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regulatory factor-8 (*irf8*) zebrafish mutant that is macrophage deficient (as hematopoiesis is skewed toward neutrophil production[24]). The immune system of larvae was trained by injecting a non-lethal dose of bacteria into the hindbrain ventricle (HBV) 2 days post-fertilization (dpf), when inflammatory responses have returned to basal levels.[21] The secondary infection challenge was done 48 h post-1st infection (hp1i) with a lethal dose of *Shigella* in the HBV (Figure 1A). Significantly increased survival was observed following *Shigella* training in both wild-type (*IRF8*$^{+/+}$) and mutant (*IRF8*$^{-/-}$) larvae, with 30% and 20% increased survival, respectively, compared to PBS-injected (naive) larvae (Figure 1B). However, in the absence of macrophages (*IRF8*$^{-/-}$ larvae), survival was significantly reduced in both naive and *Shigella*-trained larvae compared to *IRF8*$^{+/+}$ larvae (by 20% and 30%, respectively). The non-lethal infection was cleared in both wild-type and mutant larvae within 48 hp1i (Figure 1C). In agreement with larvae survival, bacterial burden at 24 h post-2nd infection (hp2i) is significantly reduced in *Shigella*-trained *IRF8*$^{+/+}$ larvae but not in *IRF8*$^{-/-}$ larvae (Figure 1C). These results suggest that trained macrophages contribute to reduce bacterial burden and promote host survival following *Shigella* secondary infection.

To test if the absence of macrophages during training is responsible for reduced survival upon infection challenge (as seen in Figure 1B), we performed macrophage ablation prior to inducing *Shigella* training. Using the nitroreductase (NTR)-metronidazole (MTZ) system specifically expressed in macrophages, we ablated macrophages 18 h prior to infection (i.e., −18 hp1i), and as larvae were not kept in MTZ, macrophages could be produced after 24 h[25] (Figures 1D and S1A). The survival rates of non-treated (DMSO) and MTZ-treated larvae were not significantly different, demonstrating that the absence of macrophages during training does not impact host survival increased by *Shigella* training (Figure 1E). In agreement, no differences in bacterial burden upon *Shigella* secondary infection were found between DMSO- and MTZ-treated larvae trained by *Shigella* (Figure 1F).

Considering this, we depleted larvae of macrophages before secondary infection, i.e., −18 hp2i (Figure 1G). In this case, the survival of MTZ-treated larvae recapitulates the results observed for *IRF8*$^{-/-}$ larvae (compare Figures 1B and 1H), where protection driven by *Shigella* training is observed, but MTZ-treated larvae have lower survival as compared to DMSO-treated larvae. This reduced survival may be driven by higher bacterial burden since bacterial levels for *Shigella*-trained MTZ-treated larvae are significantly increased at 24 hp2i compared to *Shigella*-trained DMSO-treated larvae but not in naive larvae (Figure 1I). Using RT-qPCR, we investigated the contribution of macrophages to cytokine expression upon secondary infection. We found that secondary infection induces pro- and anti-inflammatory responses, but these were not significantly different between DMSO- and MTZ-treated larvae at 3 or 18 hp2i (Figures S1B and S1C). These results suggest that trained macrophages are crucial during secondary infection, as their depletion before the secondary challenge reduces survival and increases bacterial burden.

Shigella-trained macrophages shift to a pro-inflammatory state

Macrophages are highly plastic cells that adopt different activated states ranging from pro-to anti-inflammatory, depending on microenvironment and stress/infection signals such as tissue-specific signals, cytokines, and pathogen-associated molecular patterns (PAMPs).[26,27] To test the state of macrophages before secondary infection, we used the transgenic reporter for the pro-inflammatory cytokine TNF-α in macrophages (Tg(*tnfa*:GFP)xTg(*mpeg1*:G/U*nsfB.*mCherry)) as an indicator of macrophage polarization to pro- (GFP-positive) or anti- (GFP-negative) inflammation.[28,29] Imaging of the caudal hematopoietic tissue (CHT) at 48 hp1i revealed that macrophages in *Shigella*-trained larvae become *tnfa*:GFP positive, whereas in naive larvae, they remain GFP-negative (Figure 2A). Quantification by flow cytometry shows that 47% of macrophages from naive larvae do not express *tnfa* (Figures 2B and S2A). In *Shigella*-trained larvae, we found a significant shift in the macrophage population toward the expression of *tnfa* (85% of the population). These data clearly show that *Shigella* training shifts macrophages toward a pro-inflammatory state.

Since heightened pro-inflammatory responses in macrophages are a signature of trained immunity by BCG and β-glucan,[22] we tested for changes in the epigenome of macrophages. We performed immunostaining against the epigenetic marks mono- and tri-methylation on lysine 4 in histone 3 (H3K4me1 and H3K4me3,

Figure 1. Macrophages are required for *Shigella* training and contribute to zebrafish survival

(A, D, and G) Schematic of the zebrafish immune training model in the HBV in the absence of macrophages using IRF8 zebrafish mutants (A) and MTZ treatment during the 1st infection (D) or 2nd infection (G).

(B) Survival rates of *IRF8*$^{+/+}$ and *IRF8*$^{-/-}$ zebrafish larvae (N = 3 with >8 larvae per experiment). Left graph represents collated data and right graph represents only *IRF8*$^{-/-}$ larvae, a subset of the data on the left.

(C) Bacterial burden of naive and *Shigella*-trained (*IRF8*$^{+/+}$ 1.1 × 10^3 ± 8.3 × 10^2 colony-forming units [CFUs] and *IRF8*$^{-/-}$ 1.3 × 10^3 ± 7.6 × 10^2 CFUs) larvae upon secondary infection (*IRF8*$^{+/+}$ PBS 2.6 × 10^4 ± 7 × 10^3 CFUs, *IRF8*$^{+/+}$ *Shigella* 2.7 × 10^4 ± 7.5 × 10^3 CFUs, *IRF8*$^{-/-}$ PBS 2.7 × 10^4 ± 7.5 × 10^3 CFUs, and *IRF8*$^{-/-}$ *Shigella* 2.3 × 10^4 ± 6.8 × 10^3 CFUs).

(E) Survival rates of DMSO- and MTZ-treated zebrafish larvae (N = 3 with >18 larvae per experiment). Left graph represents collated data and right graph represents only MTZ-treated larvae, a subset of the data on the left.

(F) Bacterial burden of naive and *Shigella*-trained (DMSO 8 × 10^2 ± 2.4 × 10^2 CFUs and MTZ 1.2 × 10^3 ± 4 × 10^2 CFUs) larvae upon secondary infection (DMSO PBS 2.6 × 10^4 ± 8 × 10^3 CFUs, DMSO *Shigella* 2.7 × 10^4 ± 1.1 × 10^4 CFUs, MTZ PBS 2.9 × 10^4 ± 1 × 10^4 CFUs, and MTZ *Shigella* 3.3 × 10^4 ± 1.2 × 10^4 CFUs).

(H) Survival rates of DMSO- and MTZ-treated zebrafish larvae (N = 3 with >14 larvae per experiment). Left graph represents collated data and right graph represents only MTZ-treated larvae, a subset of the data on the left.

(I) Bacterial burden of naive and *Shigella*-trained (1.3 × 10^3 ± 6.8 × 10^2 CFUs) larvae upon secondary infection (PBS DMSO 2.1 × 10^4 ± 1.5 × 10^4 CFUs, *Shigella* DMSO 2.5 × 10^4 ± 4.7 × 10^3 CFUs, PBS MTZ 2.2 × 10^4 ± 8.8 × 10^3 CFUs, and *Shigella* MTZ 2.7 × 10^4 ± 5.1 × 10^3 CFUs).

ns, non-significant; *p < 0.05, **p < 0.01, ***p < 0.01, and ****p < 0.0001. Error bars represent standard deviation (SD). (B, E, and H): log rank (Mantel-Cox) test. (C, F, and I) Two-way ANOVA with Tukey's multiple comparisons test.

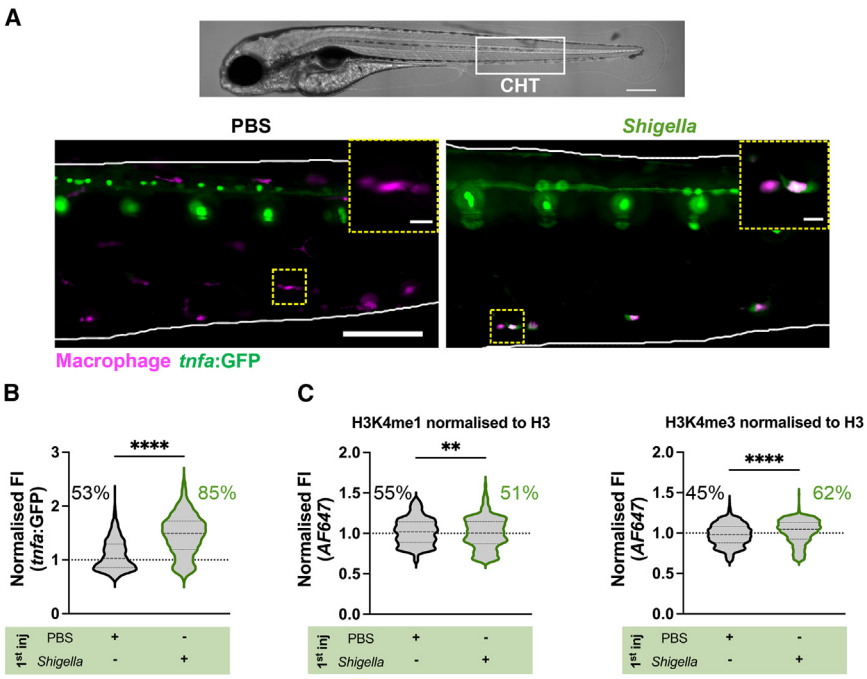

Figure 2. *Shigella*-trained macrophages shift to a pro-inflammatory state

(A) Representative images from the CHT region (top, white box indicates area imaged) of macrophages (magenta) expressing TNF-α (green) in naive (PBS, left) and *Shigella*-trained (right) larvae at 48 hp1i. Scale bar: 100 μm. Scale bars in insets: 10 μm.

(B) Quantification of *tnfa*:GFP expression.

(C) Quantification of H3K4me1 (left) and H3K4me3 (right) deposition in macrophages from naive (black) and *Shigella*-trained (green) larvae at 48 hp1i.

Data were pooled from 3 independent experiments, with >20 larvae per experiment. $^{**}p < 0.01$ and $^{****}p < 0.0001$. (B and C) Student's unpaired t test. Percentages indicate population with normalized values ≥ 1.

respectively). These post-translational modifications indicate active gene expression, where H3K4me1 is commonly associated with enhancer regions and H3K4me3 with promoter regions.[30] Flow cytometry analysis of macrophages at 48 hp1i showed significant changes in detecting both methylation marks, with a decrease in H3K4me1 detection (from 55% to 51% of the population; Figures 2C and S2B) and a sharp enrichment for H3K4me3 (from 45% to 62% of the population; Figures 2C and S2C).

Together, these data suggest that macrophages polarize toward a pro-inflammatory state due to epigenetic reprogramming by *Shigella* training.

Trained macrophages are more responsive upon secondary infection

To better understand the role of trained macrophages in infection control, we changed the secondary infection site from the HBV to the duct of Cuvier (Figure 3A), inducing a systemic infection to promote macrophage-*Shigella* interactions.[7,8,13,31] In this case, *Shigella* training also promotes significantly higher larvae survival, suggesting that protective mechanisms observed are independent of where the 1st infection occurred (Figure 3B). Bacterial clearance in systemic infection is not as effective compared to HBV infection (compare Figures 3C and 1C, 1F, and 1I, respectively), supporting the prevailing view that neutrophils are the main drivers in *Shigella* infection control *in vivo*. However, upon *Shigella* training, macrophages significantly contribute to bacterial clearance by 24 hp2i. Flow cytometry analysis shows that by 3 hp2i, naive and *Shigella*-trained macrophages have similar rates of phagocytosis (Figures S3A and S3B), and differences in bacterial killing are not yet evident at this time point (Figure S3C).

Quantification of macrophages by flow cytometry indicates that at 3 hp2i, TNF-α expression in naive larvae is significantly increased upon infection (83% compared to 50% non-infected, Figures 3D and S3D), as expected. In *Shigella*-trained larvae, we observed that infection does not increase the *tnfa*-expressing population (84% and 83% non-infected, Figures 3D and S3D), possibly because these cells have reached maximum expression levels prior to secondary infection. However, infected macrophages from *Shigella*-trained larvae have much stronger TNF-α expression compared to naive larvae (see single data points in Figure 3D). Together, these data show that *Shigella*-trained macrophages contribute to infection control and significantly increase TNF-α expression in response to secondary infection.

Shigella-trained macrophages produce more ROS

We observed that the population of infected macrophages was smaller in naive larvae compared to *Shigella*-trained larvae (Figures S3D and S3F). Since macrophage death is a hallmark of *Shigella* infection,[6–8,13] we analyzed the internalization of live/dead staining by flow cytometry. Although we observed a smaller population of macrophages having internalized live/dead stain (43% compared to 47% naive; Figures 3E and S3E), the survival of *Shigella*-trained macrophages upon infection was not significantly different under these experimental conditions.

Considering that pro-inflammatory macrophages respond better to infection, we assessed whether the production of ROSs was increased as an antibacterial mechanism. Flow cytometry analysis showed that *Shigella*-trained macrophages produce more ROS in the steady state than naive macrophages (Figures 3F and S3F). Upon secondary infection, the ROS levels in macrophages from both naive and *Shigella*-trained larvae are reduced, possibly due to potent ROS scavenger enzymes produced by *Shigella*.[32] However, when macrophages are infected, ROS production is better sustained in *Shigella*-trained macrophages compared to naive macrophages (see single data points in Figure 3F). Considering results previously showing that total macrophage numbers are reduced in *Shigella*-trained larvae,[21] these results highlight that the altered functional state of trained

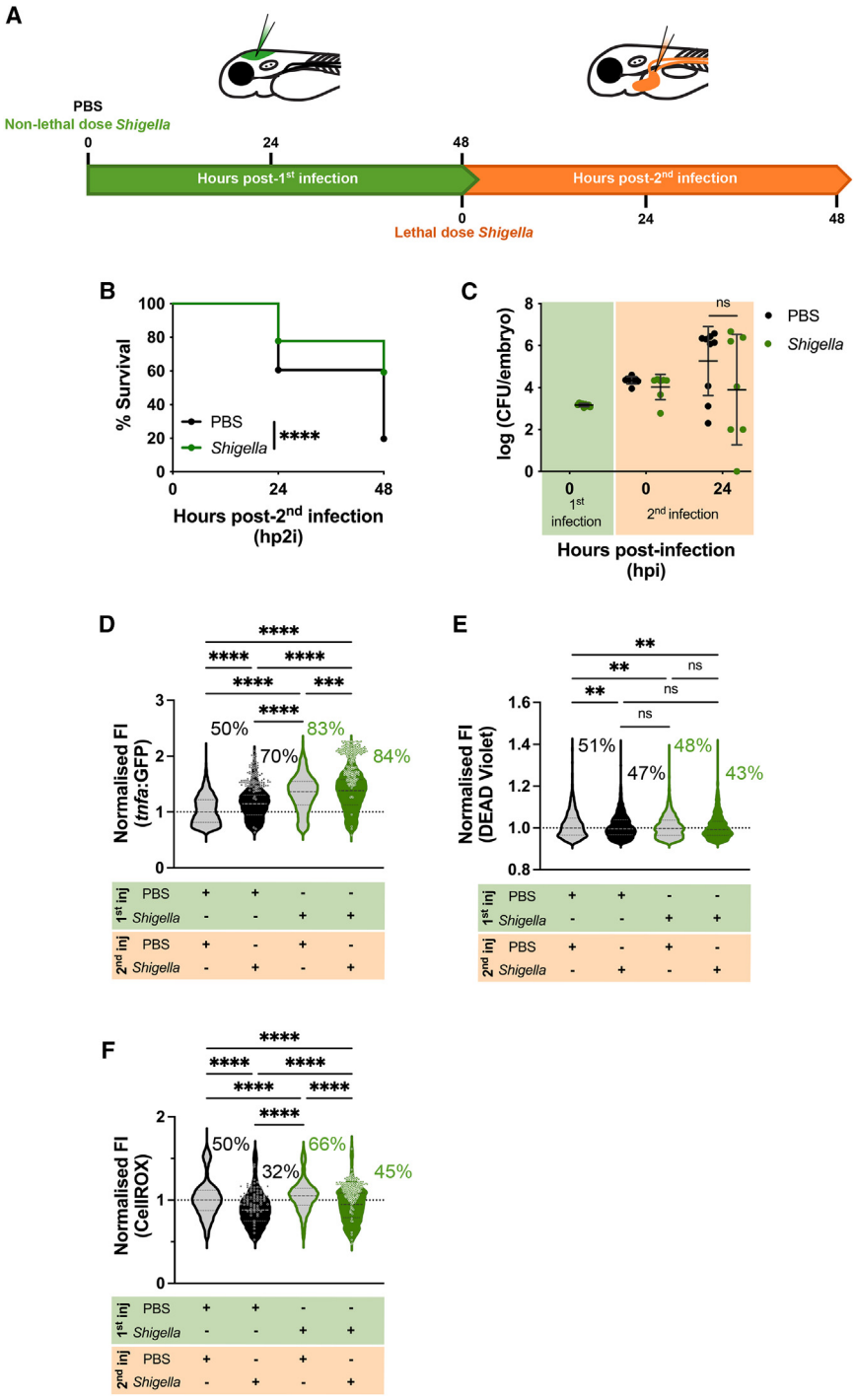

Figure 3. *Shigella*-trained macrophages produce more ROS

(A) Schematic of the zebrafish immune training model in the HBV and re-infection in the duct of Cuvier.

(B and C) Survival rates (B; $N = 3$ with >14 larvae per experiment) and bacterial burden (C) of naive and *Shigella*-trained ($1.5 \times 10^3 \pm 2.5 \times 10^2$ CFUs) larvae upon systemic secondary infection (PBS $2.4 \times 10^4 \pm 8.2 \times 10^3$ CFUs and *Shigella* $1.7 \times 10^4 \pm 9.9 \times 10^3$ CFUs).

(D) Quantification of *tnfa*:GFP expression in naive and *Shigella*-trained macrophages at 3 hp2i ($N = 3$ with >20 larvae per experiment). Single dots indicate *Shigella*-infected macrophages.

(E) Quantification of LIVE/DEAD staining internalized by macrophages in naive and *Shigella*-trained larvae at 3 hp2i ($N = 4$ with >20 larvae per experiment).

(F) Quantification of CellROX in naive and *Shigella*-trained macrophages at 3 hp2i ($N = 3$ with >20 larvae per experiment). Single dots indicate *Shigella*-infected macrophages.

ns, non-significant; ****$p < 0.0001$. Error bars represent standard deviation (SD). (B) Log rank (Mantel-Cox) test. (C) Two-way ANOVA with Sidak's multiple comparisons test. (D–F) One-way ANOVA with Tukey's multiple comparisons test). Percentages indicate population with normalized values ≥ 1.

macrophages has a crucial role in response to secondary infections, irrespective of their numbers.

DISCUSSION

Macrophages are widely recognized as ineffective in controlling *Shigella* infection due to their susceptibility to bacterial evasion strategies and induced cell death.[8,9] Despite this, the broader implications of macrophage susceptibility to *Shigella in vivo* have been poorly understood. Our study demonstrates that macrophages play a crucial role in zebrafish survival upon *Shigella* secondary infection. We found that immune training with *Shigella* promotes macrophages to shift toward a pro-inflammatory state, characterized by increased production of antimicrobial TNF-α and ROS, that contributes to infection control.

Shigella has evolved sophisticated mechanisms to evade killing by macrophages. It expresses T3SS effectors, including IpaD,[33] MxiI,[34] IpaB,[35] and IpaH7.8,[36] which activate inflammasomes and induce macrophage cell death via pyroptosis.[37–39] Although this is an effective strategy to evade immune cell killing, our recent findings show that macrophage pyroptosis is critical for inflammation and *Shigella* infection control *in vivo*.[7] These findings are consistent with our results showing that macrophages are essential for zebrafish survival during *Shigella* secondary infection (Figure 1). As previously identified in the case of *Shigella*-induced neutrophil training,[21] it is likely that the T3SS also plays an important role in training the macrophage protective response.

Previous studies on trained immunity have focused on individual cell types, delivering detailed mechanistic insights.[40] The impact of trained immunity induced by BCG was first described in macrophages and subsequently in neutrophils.[41,42] In our case, we first investigated the impact of trained immunity in neutrophils, examining how *Shigella* infection reprograms these cells at the epigenetic level. These neutrophils demonstrated significant changes in their epigenome and higher antimicrobial activity.[21] Importantly, using the zebrafish model, Darroch et al. have shown, using adoptive transfer of HSCs, that these cells carry the epigenetic remodeling to generate trained neutrophils.[23] Here, we show that *Shigella* training induces epigenetic changes in macrophages, rendering them more effective in controlling secondary infections. It is next of great interest to investigate the collaboration between macrophages and neutrophils in the context of trained immunity. In the longer term, we envision that this understanding can be used to guide *Shigella* vaccine strategies.

Limitations of the study

The precise role of the *Shigella* T3SS (a key regulator of host immune responses[9]) on macrophage training remains unclear and warrants further investigation. Using our zebrafish model of immune training, we previously demonstrated that a functional T3SS is required for full protection against secondary infection and plays a crucial role in defining the training signature in neutrophils.[21,43] Due to the pleiotropic nature of T3SS effectors and their diverse impacts on host responses,[9] delineating their specific contributions to training remains challenging. Furthermore, *Shigella* training is multifactorial, relying on both an active T3SS and innate immune responses.[21]

In this study, we used different macrophage depletion methods. Although results from both approaches were concordant, macrophage ablation based on the NTR system with the *nsfB* variant and MTZ treatment achieved only partial depletion (i.e., some macrophages could still be detected; Figure S1A). To address this limitation, future research could adopt the recently developed NTR2.0 system, which significantly enhances cell depletion efficiency in zebrafish models and reduces the exposure of larvae to MTZ by 100-fold.[44] This advancement would allow more precise evaluation of immune training and infection control in the total absence of macrophage biology.

Finally, long-term studies are essential to elucidate the role of trained immunity over extended periods of time, an issue central to the field. Using our zebrafish model of immune training, we previously showed that protective effects can be observed after 5 dp1i.[21] However, future work designed to specifically address this issue will require significantly longer intervals between training and challenge. We propose that adult zebrafish models hold great potential to provide valuable insights. However, their use introduces the complexity of adaptive immunity and ethical considerations (given their status as protected animals), which may complicate the targeted investigation of trained immunity *in vivo*.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Serge Mostowy (serge.mostowy@lshtm.ac.uk).

Materials availability

Materials generated in this study will be shared upon request.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to re-analyze the data reported in this paper is available upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.C.G. and S.M.; methodology, M.C.G. and D.B.; software, M.C.G. and D.B.; validation, M.C.G.; formal analyses, M.C.G. and D.B.; investigation, M.C.G. and D.B.; data curation, M.C.G.; writing, M.C.G. and S.M.; supervision, S.M.; project administration, S.M.; funding acquisition, S.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Histone H3	Abcam	RRID: AB_302613
Anti-Histone H3K4me1	Abcam	RRID: AB_306847
Anti-Histone H3K4me3	Abcam	RRID: AB_306649
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647	Thermo Fisher Scientific	RRID: AB_2535813
Bacterial and virus strains		
Shigella flexneri: M90T	Mostowy et al. (2010)[45]	N/A
Shigella flexneri: M90T GFP	Mostowy et al. (2010)[45]	N/A
Shigella flexneri: M90T mCerulean	This paper	N/A
Shigella flexneri: M90T mCherry	Mostowy et al. (2013)[8]	N/A
Chemicals, peptides, and recombinant proteins		
0.5% Trypsin EDTA 10x no phenol	Thermo Fisher Scientific	CAT#15400054
Carbenicillin	Sigma-Aldrich	CAT#C1389-5G
CellROX Deep Red	Thermo Fisher Scientific	CAT#C10422
Congo-Red	Sigma-Aldrich	CAT#C6767
Ethyl 3-aminobenzoate methanesulfonate (Tricaine)	Sigma-Aldrich	CAT#E10521
Foetal calf serum	Thermo Fisher Scientific	CAT#11550356
LIVE/DEAD fixable violet dead cell stain	Thermo Fisher Scientific	CAT#L34964
Metronidazole	Sigma-Aldrich	CAT#611683
Phenol-red solution	Sigma-Aldrich	CAT#P0290
Phosphate buffered saline (PBS)	Sigma-Aldrich	CAT#P4417
Polyvinylpyrrolidone (PVP)	Sigma-Aldrich	CAT#PVP40
Triton X-100	Sigma-Aldrich	CAT#X100
Tryptic Soy Agar (TSA)	Sigma-Aldrich	CAT#22091
Tryptic Soy Broth (TSB)	Sigma-Aldrich	CAT#T8907
Critical commercial assays		
HiFi DNA Assembly Master Mix	New England Biolabs	CAT#E2621L
QuantiTect Reverse Transcription kit	Qiagen	CAT#205311
RNeasy Mini kit	Qiagen	CAT#74104
SYBR green master mix	Applied Biosystems	CAT#10187094
Experimental models: Organisms/strains		
Zebrafish: *irf8*^{st95}	Shiau et al.[24]	N/A
Zebrafish: Tg(*mpeg1*::Gal4-FF)^{gl25}/Tg(UAS- E1b::*nfsB*.mCherry)^{c264}	Ellett et al.[46]	N/A
Zebrafish: Tg(*tnfa*::GFP)	Marjoram et al.[47]	N/A
Oligonucleotides		
Cerulean_fwd: aaacagcgccGATTTTTTCGCATATTTTTCTTGCAAAG	This paper	N/A
Cerulean_rev: gcttgcatgcAGTCAAAAGCCTCCGGTC	This paper	N/A
BACKBONE_fwd: gcttttgactGCATGCAAGCTTTAATGC	This paper	N/A
BACKBONE_rev: cgaaaaaatcGGCGCTGTTTATGCTTCG	This paper	N/A
See Table S1 for qRT-PCR primers		
Recombinant DNA		
Plasmid: pTSAR1Ud2.1	Campbell-Valois et al.[48]	N/A
Plasmid: pFPV-mCherry	Addgene	CAT#20956
Plasmid: pFPV-mCerulean	This paper	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
GraphPad Prism 10	GraphPad Prism	RRID:SCR_002798
Life Technologies QuantStudio 5 Real Time PCR System	Thermo Fisher Scientific	RRID:SCR_020240
FIJI/ImageJ	ImageJ	RRID:SCR_002285
ZEISS ZEN Microscopy Software	Zeiss	RRID:SCR_013672
FlowJo 10.8.1	FlowJo LLC	RRID:SCR_008520

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Zebrafish lines and husbandry

Transgenic zebrafish lines used here were *irf8*^{st95} (referred to as IRF8),[24] Tg(*mpeg1*::Gal4-FF)^{gl25}/Tg(UAS-E1b::*nfsB*.mCherry)^{c264} (referred to as Tg(*mpeg1*::G/U::*nsfB.*mCherry))[46] and Tg(*tnfa*::GFP).[47] The lines Tg(*mpeg1*::G/U::*nsfB.*mCherry) and Tg(*tnfa*::GFP) were crossed to generate a double transgenic line in which macrophages express both mCherry and GFP. Embryos were obtained from naturally spawning adult zebrafish. For experiments, larvae were maintained at 28.5°C in 0.5x E2 medium. For injections and live microscopy, larvae were anaesthetised with 200 μg/ml tricaine in 0.5x E2 medium. In survival experiments, from 5 days post fertilisation larvae were monitored 2-3 times a day for appearance, righting reflex, reactive reflex and opercular movement. Larvae were not fed during the course of the experiment. Animal experiments were performed according to the Animals (Scientific Procedures) Act 1986 and approved by the Home Office (Project licenses: PPL P4E664E3C and PP5900632).

METHOD DETAILS

Zebrafish injections

Bacterial strains used in this study were *Shigella flexneri* M90T expressing a GFP,[45] mCherry[8] or mCerulean (in this study) plasmid. *Shigella* strains were grown on trypticase soy agar (TSA, Sigma-Aldrich #22091) plates supplemented with 0.01% Congo red (Sigma-Aldrich #C6767) and carbenicillin 100 μg/mL (Sigma-Aldrich #C1389-5G) when required. Individual colonies were grown overnight at 37°C and 200 rpm in 5 ml trypticase soy broth (TSB, Sigma-Aldrich #T8907) supplemented with the appropriate antibiotics as above. Prior to injections, bacteria were grown to log phase (optical density (OD) of 0.55–0.65 at 600 nm) by diluting the overnight culture 50x in fresh TSB supplemented with the appropriate antibiotics. Then, bacteria were spun down and washed in phosphate buffer saline (PBS, Sigma-Aldrich #P4417). The desired concentration was achieved by resuspension of bacteria in injection buffer (2% polyvinylpyrrolidone (PVP [Sigma-Aldrich #PVP40] in PBS and 0.5% phenol red [Sigma-Aldrich #P0290]). Control groups were injected with injection buffer (referred to as PBS/naïve group). For bacterial quantification, larvae were dissociated in 0.4% Triton X-100 (Sigma-Aldrich #93443) with the aid of mechanical pestles, and serial dilutions were plated in the appropriate medium at the indicated time points. Plates were incubated at 37°C and colony forming units (CFU) were enumerated when colonies were visible.

Design of pFPV-mCerulean plasmid

The plasmid pFPV-mCerulean was created using Gibson assembly. The mCerulean gene and its *rpsM* promoter were PCR-amplified from plasmid pTSAR1Ud2.1,[48] using the primers *Cerulean_fwd* (AAACAGCGCCGATTTTTTCGCATATTTTTCTTGCAAAG) and *Cerulean_rev* (GCTTGCATGCAGTCAAAAGCCTCCGGTC). The plasmid pFPV-mCherry (Addgene #20956) was used as the backbone and PCR-amplified using the primers *Backbone_fwd* (GCTTTTGACTGCATGCAAGCTTTAATGC) and *Backbone_rev (*CGAAAAAATC GGCGCTGTTTATGCTTCG).

Gibson assembly was performed at 50°C for 30 min using the HiFi DNA Assembly Master Mix (New England Biolabs #E2621L). The resulting plasmid (pFPV-mCerulean) was transformed into *S. flexneri*.

Zebrafish chemical treatment

For macrophage chemical depletion, Tg(*mpeg1*::G/U::*nsfB.*mCherry) larvae were treated with 10 mM metronidazole (Sigma-Aldrich #611683), which was added to embryo medium (in 1% dimethyl sulfoxide (DMSO)), for 18 h in the dark at 28.5°C.

RNA extraction, cDNA synthesis and RT-qPCR

For gene expression analysis using RT-qPCR, RNA was extracted from 5-10 snap-frozen larvae, purified using a RNeasy Mini kit (QIAGEN #74104) and reverse-transcribed using QuantiTect Reverse Transcription kit (QIAGEN #205311) according to the manufacturer's instructions. The QuantStudio 5 PCR system and SYBR green master mix (Applied Biosystems #10187094) were used for quantitative PCR (qPCR). Samples (technical duplicates) were run from three biological replicates. Primers used for PCR are described in Table S1 and have been validated previously (references in Table S1). The comparative Ct method was used for

gene expression quantification, and *ef1a1l1* was used as the housekeeping gene, and its expression was verified to not change across the different conditions.

Flow cytometry

Tg(*mpeg1*::G/U::mCherry) and Tg(*mpeg1*::G/U::mCherry)/Tg(*tnfa*::GFP) transgenic larvae were dissociated for flow cytometry as previously described.[21] For detection of dead cells, LIVE/DEAD™ fixable violet dead cell stain (Thermo Fisher Scientific #L34964) was used (1:2000). For ROS staining, 5μM CellROX™ Deep Red (Thermo Fisher Scientific #C10422) was used as described in.[21] To quantify histone marks, antibodies against H3 (1:200, Abcam #ab1791), H3K4me1 (1:200, Abcam #ab8895) and H3K4me3 (1:200, Abcam #ab8580) were used with Alexa Fluor 647 secondary antibody (Thermo Fisher Scientific #A-21245) for detection. In brief, larvae were dissociated and after fixation with 4% PFA, cells were permeabilised, slowly adding ice-cold 100% methanol. Cells were incubated for 30 min at -20°C. Cells were then washed with incubation buffer (0.5% w/v BSA in PBS) and divided into 3 aliquots of 100 μL of incubation buffer with each antibody separately. Upon 1 h incubation, cells were washed and incubated for 30 min at room temperature with 100 μL of incubation buffer with Alexa Fluor 647 secondary antibody. Single cells were measured on an Aurora (Cytek Biosciences). Data was analysed with FlowJo software v10.8.1.

Microscopy and image analysis

To assess the efficiency of macrophage ablation, anaesthetised larvae were placed on 1% agarose-E2 plates and imaged using a stereo fluorescent microscope Leica M205FA. Quantification was performed as described in.[21] Imaging of Tg(*mpeg1*::G/U::mCherry) xTg(*tnfa*::GFP) transgenic larvae was done using widefield microscopy in a Zeiss Celldiscoverer 7 (CD7), with a 20x/0.95 plan-apochromat objective with a 0.5x tube lens to acquire a 3 z-plane image. Anaesthetised larvae were placed on 96-well plates (Perkin Elmer) and embedded in 1% low melting agarose. Image files were processed using ImageJ/Fiji software.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical significance was determined using GraphPad Prism v10. The Log-rank (Mantel-Cox) test for differences in survival curves. Bacterial burden and gene expression levels were Log10-transformed, respectively. Pairwise comparisons were determined using a Student's unpaired t-test. For multiple comparisons, one-way or two-way ANOVA tests with Tukey's or Sidak's multiple comparison tests were used, as indicated in the figure legend.

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Supplemental information

Shigella-trained pro-inflammatory macrophages protect zebrafish from secondary infection

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Figure S1

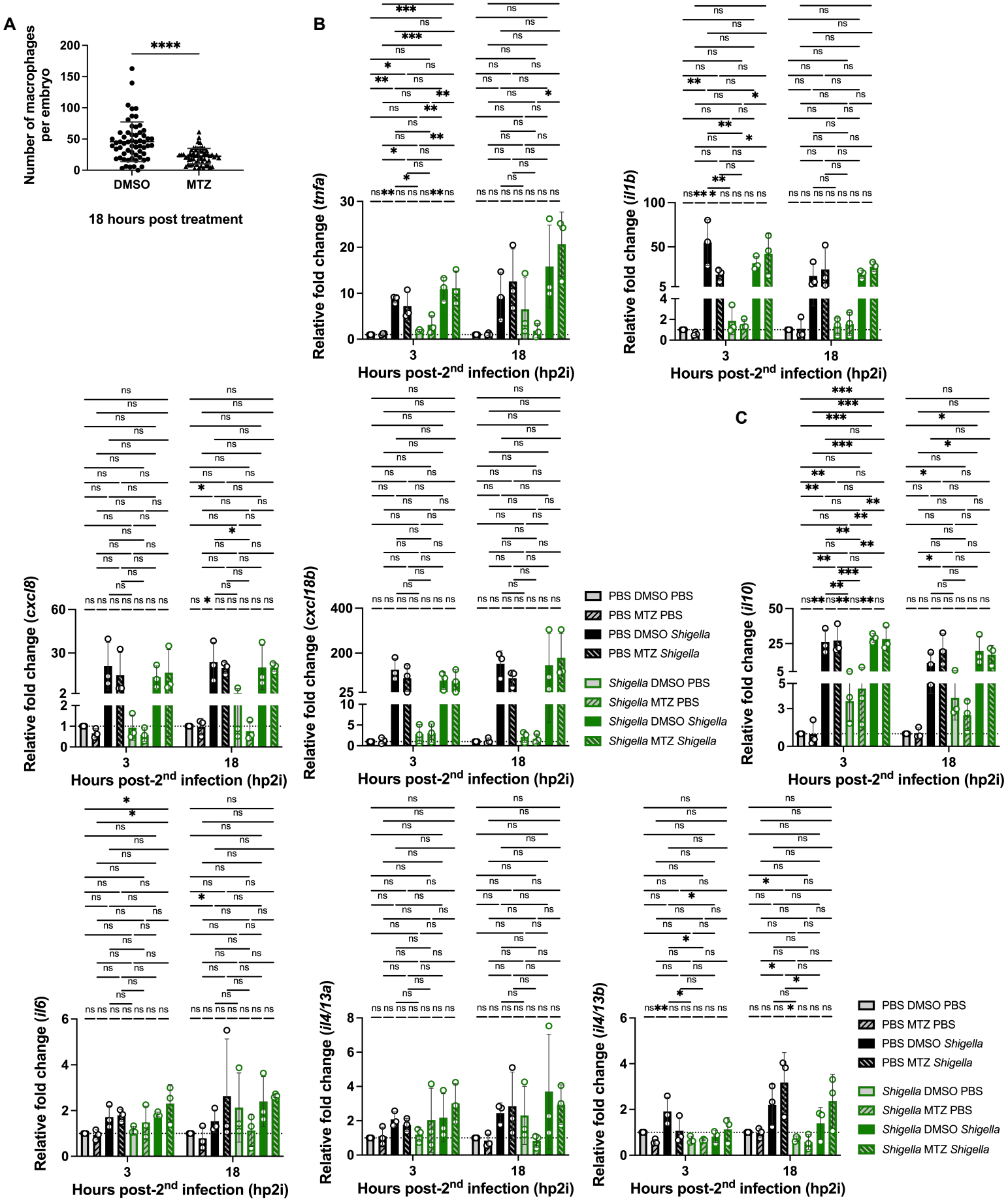


Figure S1 – Efficiency of macrophage ablation and quantification of the expression of inflammatory responses by qRT-PCR, related to Figure 1. (A) Quantification of macrophages upon 18 h of treatment with DMSO or metronidazole (MTZ). (B-C) Expression of (B) pro-inflammatory (*tnfa*, *il1b*, *cxcl8* and *cxcl18b*) and (C) anti-inflammatory (*il10*, *il6*, *il4/13a* and *il4/13b*) cytokines in naïve and *Shigella*-trained larvae in the presence or absence of macrophages at 3 and 18 hp2i by qRT-PCR. Data were pooled from 3 independent experiments (*p<0.05, **p<0.01, ***p<0.01, ****p<0.0001; A: Student's unpaired *t*-test; B, C: 1-way ANOVA with Tukey's multiple comparisons test).

Figure S2

A

TNF-α	Total cells analysed	Cells with normalised values => 1	%	Cells with normalised values => 1.5	%
PBS	3180	1698	53.40	321	10.09
Shigella	3180	2713	85.31	1557	48.96

B

H3K4me1	Total cells analysed	Cells with normalised values => 1	%
PBS	1144	624	54.55
Shigella	970	493	50.82

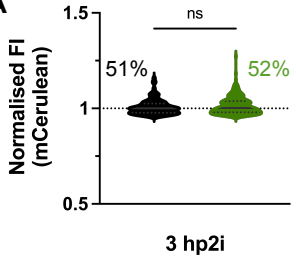
C

H3K4me3	Total cells analysed	Cells with normalised values => 1	%
PBS	2530	1141	45.10
Shigella	3806	2356	61.90

Figure S2 – In-depth analysis of flow cytometry data from *tnfa* expression and histone marks immunostaining, related to Figure 2. (A-C) Percentages of macrophages with normalised fluorescence values above 1 for *tnfa* expression (A) and immunostaining of H3K4me1 (B) and H3K4me3 (C).

Figure S3

A



B

mCerulean	Total cells analysed	Cells with normalised values => 1	%
PBS + *Shigella*	95	48	50.53
Shigella + *Shigella*	233	121	51.93

D

TNF-α	Total cells analysed	Cells with normalised values => 1	%	Total infected cells
PBS + PBS	7045	3537	50.21	--
PBS + *Shigella*	6307	4419	70.07	101
Shigella + PBS	6878	5724	83.22	--
Shigella + *Shigella*	6658	5584	83.87	232

E

DEAD stain	Total cells analysed	Cells with normalised values => 1	%
PBS + PBS	1162	588	50.60
PBS + *Shigella*	1441	673	46.70
Shigella + PBS	1109	536	48.33
Shigella + *Shigella*	1234	534	43.27

F

ROS	Total cells analysed	Cells with normalised values => 1	%	Total infected cells
PBS + PBS	7045	3492	49.57	--
PBS + *Shigella*	5089	1607	31.58	76
Shigella + PBS	6105	4002	65.55	--
Shigella + *Shigella*	5531	2513	45.43	207

Figure S3 – Macrophage phagocytosis and in-depth analysis of flow cytometry data for *tnfa* expression, cell death and ROS production, related to Figure 3. (A-B) Quantification of phagocytosis by naïve and *Shigella*-trained macrophages at 3 hp2i (A) with percentages of macrophages with normalised fluorescence values above 1 for mCerulean (B). (C) Quantification of bacterial clearance at 3 hp2i. Data were pooled from 3 independent experiments (ns: non-significant; A, C: Student's unpaired *t*-test). (D-F) Percentages of macrophages with normalised fluorescence values above 1 for *tnfa* expression (D), internalisation of LIVE/DEAD staining (E) and ROS production (F) at 3 hp2i. Total of infected cells detected is found in the last column.

Table S1 – qRT-PCR primers used in this study, related to STAR Methods.

Name	GeneID	Sequence (5'-3')	Reference
*eef1a1a*FW	ENSDARG00000039502	AAGCTTGAAGACAACCCCAAGAGC	[S1]
*eef1a1a*RV	ENSDARG00000039502	ACTCCTTTAATCACTCCCACCGCA	[S1]
*cxcl8a*FW	ENSDARG00000104795	TGTGTTATTGTTTTCCTGGCATTTC	[S2]
*cxcl8a*RV	ENSDARG00000104795	GCGACAGCGTGGATCTACAG	[S2]
*cxcl18b*FW	ENSDARG00000075045	TCTTCTGCTGCTGCTTGCGGT	[S3]
*cxcl18b*RV	ENSDARG00000075045	GGTGTCCCTGCGAGCACGAT	[S3]
*il1b*FW	ENSDARG00000098700	GAACAGAATGAAGCACATCAAACC	[S3]
*il1b*RV	ENSDARG00000098700	ACGGCACTGAATCCACCAC	[S3]
*il6*FW	ENSDARG00000102318	TCAACTTCTCCAGCGTGATG	[S4]
*il6*RV	ENSDARG00000102318	TCTTTCCCTCTTTTCCTCCTG	[S4]
*il10*FW	ENSDARG00000078147	CATAACATAAACAGTCCCTATG	[S5]
*il10*RV	ENSDARG00000078147	GTACCTCTTG CATTTCACCA	[S5]
*tnfa*FW	ENSDARG00000009511	AGACCTTAGACTGGAGAGATGAC	[S2]
*tnfa*RV	ENSDARG00000009511	CAAAGACACCTGGCTGTAGAC	[S2]
*il4/13a*FW	ENSDARG00000077809	GCACTGTATTCGTCTCGGGTTTTA	[S6]
*il4/13a*RV	ENSDARG00000077809	TTTTCCCCAGATCTACAAGGAAGA	[S6]
*il4/13b*FW	ENSDARG00000087909	GCAGGAATGGCTTTGAAGGGTAAA	[S6]
*il4/13b*RV	ENSDARG00000087909	AAACTCCTTCATTGTGCATTCCCC	[S6]

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