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Neutrophil plays critical role during *Edwardsiella piscicida* immersion infection in zebrafish larvae

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ABSTRACT

Edwardsiella piscicida is a facultative intracellular pathogen that causes hemorrhagic septicemia and haemolytic ascites disease in aquaculture fish. During bacterial infection, macrophages and neutrophils are the first line of host innate immune system. However, the role of neutrophils in response to *E. piscicida* infection *in vivo* remains poorly understood. Here, through developing an immersion infection model in the 5 day-post fertilization (dpf) zebrafish larvae, we found that *E. piscicida* was mainly colonized in intestine, and resulted into significant pathological changes in paraffin sections. Moreover, a dynamic up-regulation of inflammatory cytokines (TNF-α, IL-1β, GCSFb, CXCL8 and MMP9) was detected in zebrafish larvae during *E. piscicida* infection. Furthermore, a significant recruitment of neutrophils was observed during the *E. piscicida* infection in *Tg(mpx:eGFP)* zebrafish larvae. Thus, we utilized the CRISPR/Cas9 system to generate the neutrophil-knockdown (*gcsfr*$^{-/-}$ crispants) larvae, and found a comparative higher mortality and bacterial colonization in *gcsfr*$^{-/-}$ crispants, which reveals the critical role of fish neutrophils in bacterial clearance. Taken together, our results developed an effective *E. piscicida* immersion challenge model in zebrafish larvae to clarify the dynamic of bacterial infection *in vivo*, which would provide a better understanding of the action about innate immune cells during infection.

1. Introduction

Edwardsiella piscicida, previous named as *E. tarda* [1], is a kind of gram-negative intracellular pathogen, which can cause hemorrhagic septicemia and ascites in more than twenty species of fish, bringing great economic loss in aquaculture [2]. The genome of this pathogen has been sequenced and several virulence-associated genes have been identified previously [3–6]. There are several virulence secretion system effectors also have been characterized, such as EseG [7] and EseJ [8] secreted by type III secretion system (T3SS), and EvpP [9] secreted by type VI secretion system (T6SS), which are proved to be critical for the pathogenesis of *E. piscicida*. However, a better bacterial-fish interaction model still needs to be established to analysis the action of innate immune cells in response to *E. piscicida* infection.

Zebrafish (*Danio rerio*) are increasingly used as a star model to study the vertebrate immunology and diseases [10]. There are several advantages by using zebrafish larvae to study the host-pathogen interactions: (1) Zebrafish larvae are optical transparency in early development stage and make it possible to directly visualize the microbial infection process [11]. (2) Readily genetic manipulations such like generating fluorescent-labeled transgenic lines allow us to observe the host physiological changes response to infection, some gene tools (Morphlino [12] and CRISPR/Cas9 [13]) are applied to study the specific gene function. (3) Zebrafish are susceptible to many pathogens, including *Listeria monocytogenes* [14], *Salmonella typhimurium* [15], *mycobacterium tuberculosis* [16], *Shigella flexneri* [17] and *Edwardsiella tarda* [18], which is an ideal model for pathogen-host interaction studies. (4) The adaptive immune of zebrafish are not mature until 4–6 weeks during zebrafish development [19], so the innate immunity can be specifically analyzed, especially for the actions of macrophages and neutrophils.

Neutrophils are the first line of innate immune system, which play an essential role in bacterial clearance and host immune homeostasis [20]. Neutrophils can be recruited to infection or injured site through Cxcr2-cxcl8 signaling pathway [21], and phagocytize the pathogens, release cytokines or reactive oxygen species (ROS) to promote inflammation [22]. Moreover, the neutrophils can also release the extracellular traps (NETs) by a program cell death called NETosis [23,24].

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Furthermore, to reduce the tissue damages during injury, the neutrophils could reverse migration through an oxygen-sensing transcription factor hypoxia-inducible factor-1α (HIF-1α)-mediated signaling [25,26]. Thus, the action of neutrophils response to the danger signaling might through different mechanisms [27]. However, there is no study about the function of fish neutrophils in response to *E. piscicida* infection.

In this study, we established an immersion infection model in zebrafish larvae to analyze the dynamics of *E. piscicida in vivo,* and observed a significant neutrophil infiltration in paraffin sections at 72 h-post infection (hpi), within a significantly up-regulation of inflammatory cytokines (TNF-α, IL-1β and GCSFb) and chemokines (CXCL8 and MMP9). Furthermore, we found that neutrophils can be recruited to the bacterial colonization site in early phase of infection, and the number of neutrophils gradually decreased after 24 hpi especially in the caudal hematopoietic tissue (CHT) of zebrafish larvae. Thus, we developed the CRISPR/Cas9 mediated neutrophils knockdown (*gcsfr*$^{-/-}$ crispants) zebrafish larvae, and revealed that the neutrophils play critical role in response to *E. piscicida* infection.

2. Materials and methods

2.1. Edwardsiella piscicida strain and culture condition

Wild type *E. piscicida* strain (EIB202, CCTCC No. M208068) was isolated from pathogenetic *Scophthalmus maximus* from Yantai, China [28]. The red fluorescent protein (RFP)-expressing EIB202 (EIB202-mcherry) was constructed by electro-transforming (2000 V, 3 mS) a ready-made recombinant plasmid pUTt-mcherry [29] with a constitutive expression promoter *0456* gene by micropulser electroporator (Bio-rad, USA). The positive transformant was identified by fluorescence observation. The bacteria were cultured in Trypticase Yeast Broth (TYB) medium with 16.7 μg/ml Colistin (Col^{+}) at 30 °C and 200 rpm shaker, the EIB202-mcherry strain was supplied with 16.7 μg/ml Colistin (Col^{+}) and 100 μg/ml Ampicillin (Amp^{+}).

2.2. Zebrafish line and larvae maintenance

The zebrafish mainly used in this study is AB line wild type (WT) and *Tg(mpx:eGFP)*, which were purchased from China Zebrafish Resource Center (CZRC, Wuhan, China). The *Tg(mpx:eGFP)* is a transgenic zebrafish line derived from wild type AB zebrafish line by gene manipulation, and this transgenic line are labeled with enhanced green fluorescent protein (eGFP), so that neutrophils behavior could be observed clearly in zebrafish larvae. The *Tg(mfap4:GFP)* is a transgenic line that macrophages are labeled with green fluorescent protein (GFP), which was a gift from Dr. Li Li from Southwest University. Zebrafish larvae were maintained with E3 medium in 28 °C incubator accordingly [14].

2.3. Immersion infection

The bacteria were cultured overnight at 30 °C and 200 rpm shaker, then inoculated into fresh medium as the ratio of 1:50, and cultured at 30 °C incubator for another 4–5 h. Then, the bacteria were collected and washed with PBS for 3 times, and the starting concentration (1×10^{9} CFU/ml) of bacteria was calculated by NanoDrop (Thermo, USA), other concentration was obtained by gradient dilution. For immersion infection, five day-post fertilization (dpf) zebrafish larvae were transferred into the six-well plate (n = 30 per well) and immersed with 5 ml indicated concentrations of bacteria for 2 h, PBS immersion was used as the negative control. Then, the larvae were washed three times with E3 medium and maintained the larvae in another six well plate with E3 medium for subsequent study (Fig. S1).

2.4. Bacterial enumeration

At indicated time points (2, 24, 48, 72, 96, and 120 hpi), five larvae were randomly collected and sufficiently grinded in 200 μl PBS, pipetting 20 μl grinding fluid to 180 μl PBS for a 10-fold dilution and mixed together by series dilution. Then, 10 μl of serial diluent were plated on the deoxycholate hydrogen sulfide lactose (DHL) agar plate, and enumerated the black colonies after culturing in 37 °C incubator for 24 h.

2.5. Microscopy and imaging

For analyzing the bacteria colonization and actions of neutrophils, zebrafish larvae were anesthetized in 200 μg/ml tricaine (sigma, USA) and visualized by Lei DMI3000B (Leica, Germany) in bright field and fluorescence field. Excitation light was supplied by mercury lamp with corresponding optical filter. The images were processed by photoshop CC (Adobe, USA). Fluorescence intensity was quantified by ImageJ (National Institutes of Health, USA).

2.6. Histopathological analysis

The zebrafish larvae were fixed with 4% Paraformaldehyde (PFA), dehydrated using ethanol and vitrificated by dimethylbenzene. The larvae were embedded in paraffin blocks and sliced with paraffin slicing machine (5 μm, Leica, Germany). After deparaffinage and rehydration, hemotoxylin and eosin (H&E) staining and stereomicroscopy were used for histopathological analysis.

2.7. RNA isolation and qRT-PCR

At indicated time-points, pooled 10 larvae in RNase-free centrifuge tube, added 200 μl RNAstore Reagent (Tiangen, Beijing, China) and stored at −80 °C. RNA of individual sample was isolated by Trizol (Invitrogen, USA) and chloroform. The cDNA was synthesized by FastQuant RT Kit (Tiangen, Beijing, China), then analyzed with real-time quantitative PCR (Bio-rad,USA) by using SuperReal PreMix Plus (SYBR Green) (Tiangen, Beijing, China) according to procedures. The results were analyzed by $2^{-\Delta\Delta Ct}$ method. Primers used in this study were listed in Table 1.

2.8. Neutrophil knockdown crispants generation

Two sgRNA sequences specifically targeted granulocyte colony-stimulating factor receptor (*gcsfr*) [30–32] were listed in Table 2. We microinjected 2 mixed sgRNAs into one-cell stage embryos together with Cas9 protein (Novoprotein, China) at a final concentration of 1000 ng/μl. At 48 h-post fertilization (hpf), cDNA template was obtained from 30 embryos microinjected with two-guide Cas9 RNP mix, or from untreated embryos, respectively. Genotyping PCR and sequencing were subsequently executed to verify the validity of gene editing. Genotyping primers listed in Table 2 were designed by PrimerQuest Tool (IDT,

Table 1
qRT-PCR primers used in this study.

Primers	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
tnf-α	GCGCTTTTCTGAATCCTACG	TGCCCAGTCTGTCTCCTTCT
il-1β	GGCTGTGTGTTTGGGAATCT	TGATAAACCAACCGGGACA
il-10	ATTTGTGGAGGGCTTTCCTT	AGAGCTGTTGGCAGAATGGT
cxcl8	GTCGCTGCATTGAAACAGAA	CTTAACCCATGGAGCAGAGG
gcsfa	TCTGACCCAACACCTGAAAAG	GGGTCTCTGTGATGGATATGTC
gcsfb	AGAGAACCTACTGAACGACCT	CTTGAACTGGCTGAGTGGAG
mmp9	CATCCGCAACTACAAGACATTC	GGTCCAGTATTCATCGTCATCA
gcsfr	CACAAAAGGTTTATGCGGTCC	TTCAGAGGATCAGTGGTTGTG
mpeg1	CCGTTAATGCTGATGTTTCCTTC	GGGTACGCCTTTACTGTGTAC
β-actin	ATGGATGAGGAAATCGCTGCC	CTCCCTGATGTCTGGGTCGTC

Table 2
gcsfr sgRNA targets and genotyping primers used in this study.

sgRNA	Sequence (5′-3′)
Target #1	TGCCCAGCCTCCGGTGTGAC
Target #2	CGTAGAAGTGCTGCCCGGGT
Primers	**Sequence (5′-3′)**
Genotyping F	CTTGTGTGTGTTCAGGAGGA
Genotyping R	GGGTGAGTTTCAGAGGATCAG

USA). Moreover, qRT-PCR was also performed to confirm the *gcsfr* knockout efficiency. The neutrophil knockdown efficiency was also confirmed by microscopic image analysis (Leica, Germany).

2.9. Statistical analysis

Statistical analysis was performed by Prism 7.0 (Graphpad, USA). Each experiment was performed at least three times with 3 parallel repeats. The statistical significance of neutrophils fluorescence and survival differences between $gcsfr^{-/-}$ crispants and wild type larvae were analyzed by Students' *t*-test (Fig. 4D and F). The statistical significance of cytokines expression, neutrophils counts, $gcsfr^{-/-}$ crispants phenotype analysis and bacterial burden after infection were determined by two-way ANOVA (Fig. 2, Fig. 3C, Fig. 4E and G). The results were presented as mean ± SD, $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$; $^{****}p < 0.0001$.

3. Results

3.1. Evidence of E. piscicida systemic infection in zebrafish larvae

To establish an infection model in zebrafish, we developed an immersion challenge model by utilizing 5 dpf zebrafish larvae with 10^7, 10^8, or 10^9 CFU/ml *E. piscicida*, respectively (Fig. S1). After 2 h immerse infection, we found a dose-dependent mortality, compared with the PBS-treated controls (Fig. 1A). According to the Reed-Muench method, we calculated the LD_{50} is approximately 1.13×10^8 CFU/ml. Furthermore, we found the bacterial loads increased significantly in infected larvae (starting from 1000 CFU and increased to 50000 CFU) from 2 to 48 h-post infection (hpi), and gradually decreased to a basic level till 120 hpi (Fig. 1B).

To better visualize the colonization of *E. piscicida in vivo*, we constructed the EIB202-mcherry strain, and confirmed that this bacterium could constitute express RFP but didn't influence the bacteria growth and pathogenesis, compared with the wild type *E. piscicida* (Fig. S2). To track the colonization of the *E. piscicida in vivo*, we found that the bacteria were mainly colonized in the intestine of zebrafish larvae, within the fluorescence signal gradually migrated along the bowel lumen and basically disappeared after 72 hpi (Fig. 1C). Moreover, we analyzed the histopathological changes in intestinal paraffin sections at 72 hpi zebrafish larvae, the inflammatory cells were significantly accumulated in the lumen, along with the tissue infiltration and intestinal epithelial cells detachment, compared with the PBS-treated controls (Fig. 1D). Taken together, these results suggest that the intestinal mucosal immune system might play an important role during *E. piscicida* infection *in vivo*.

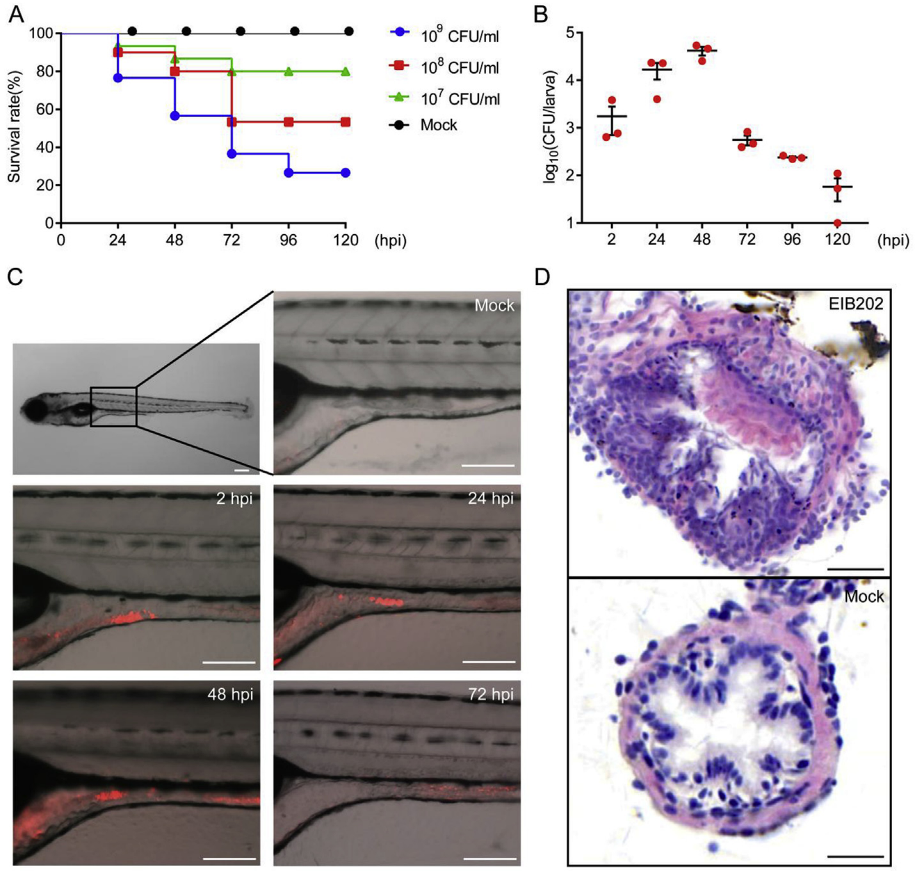

Fig. 1. Evidence of *E. piscicida* systemic infection in zebrafish larvae. (A) Percent survival of 5 dpf zebrafish larvae (n = 30) infected with indicated dose of EIB202. **(B)** Bacterial burden of zebrafish larvae immersed with 1×10^8 CFU/ml EIB202, data are presented as mean ± SD of three independent experiments. **(C)** Lateral images of infected zebrafish larvae at indicated time-points, red represents EIB202-mCherry colonization site. Scale bar, 100 μm. **(D)** Histopathological analysis of intestinal paraffin section of 72 hpi (or Mock) zebrafish larvae by H&E staining. Scale bar, 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

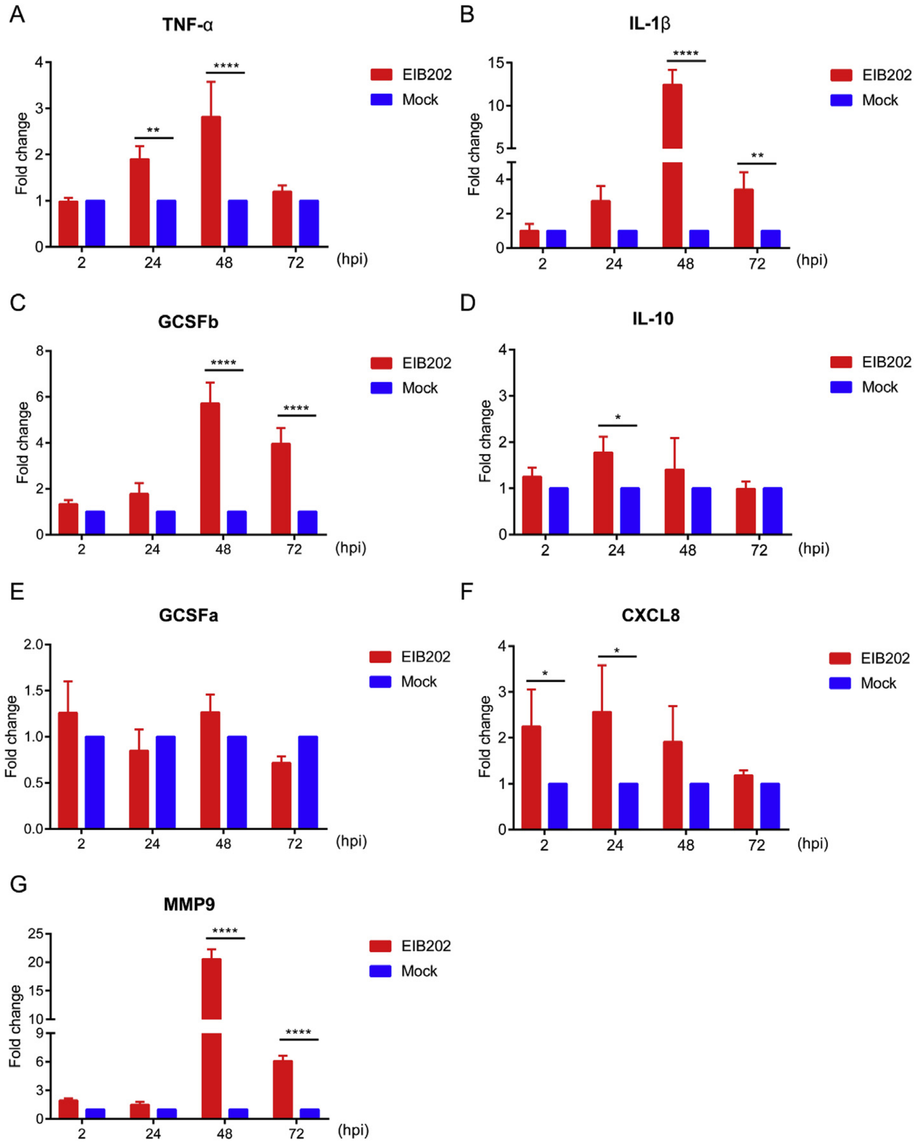

Fig. 2. Inflammatory cytokines expression during *E. piscicida* infection. (A-G) qRT-PCR analysis of TNF-α **(A)**, IL-1β **(B)**, GCSFb **(C)**, IL-10 **(D)**, GCSFa **(E)**, CXCL8 **(F)** and MMP9 **(G)** transcripts in infected zebrafish larvae. At indicated time-points post infection, 10 zebrafish larvae were pooled as samples for total RNA extraction and qRT-PCR analysis. Data are presented as mean ± SD of three independent experiments; $*p < 0.05$, $**p < 0.01$ and $****p < 0.0001$.

3.2. *Inflammatory cytokines expression during E. piscicida infection*

Inflammatory cytokines and chemokines secretion is a common manner in vertebrate hosts during bacteria invasion [33]. Here, we analyzed the expression profiles of inflammatory cytokines, the transcripts of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and the granulocyte colony-stimulating factor (GCSFb) in *E. piscicida* infection larvae were significantly increased compared with control groups (Fig. 2A, B and C), while the transcripts of IL-10 and GCSFa were not affected (Fig. 2D and E). Interestingly, we observed a significantly up-regulation of C-X-C motif chemokine ligand 8 (CXCL8) during the early phase of *E. piscicida* infection (Fig. 2F), while the matrix metalloprotein 9 (MMP9) was significantly up-regulated at the late phase of *E. piscicida* infection (Fig. 2G), which suggest a dynamic role of chemokines in regulating the function of neutrophils, and this might play critical role during this bacterial infection.

3.3. *Neutrophils recruit to the infection sites during early phase of infection*

To better analyze the function of neutrophils during *E. piscicida* infection *in vivo*, we utilized a transgenic zebrafish line *Tg(mpx:eGFP)*, the promoter of tissue-specific myeloperoxidase, *mpx*, could drive the eGFP expression in neutrophils [34], to visualize the action of neutrophils during *E. piscicida* infection. A robust neutrophils recruitment was found in zebrafish larvae infected with *E. piscicida* at 4 hpi (Fig. 3A), which suggest that the neutrophils might response to *E. piscicida* infection at the early phase. For better monitoring the changes of neutrophils, we visualized the zebrafish larvae at two parts, the caudal hematopoietic tissue (CHT) of zebrafish larvae located in the posterior of body, and the bacterial colonized in the anterior of body in the early phase of infection (Fig. 3B), and interestingly, in PBS-mock group, neutrophils were mainly existed in the CHT, while the numbers of neutrophils in the anterior of body is seldom in 5 dpf larvae. However, during *E. piscicida* infection, the number of neutrophils was increased in the anterior of body at 2 hpi, while decreased after 24 hpi (Fig. 3B and C). Moreover, the neutrophils in caudal hematopoietic tissue (CHT) of infected larvae were almost vanished at 72 hpi compared to the PBS-treated controls (Fig. 3B and C). These results provided us a clue that neutrophils were migrated from the CHT of zebrafish larvae to the *E. piscicida* infection sites, and might play a role called bacterial killing

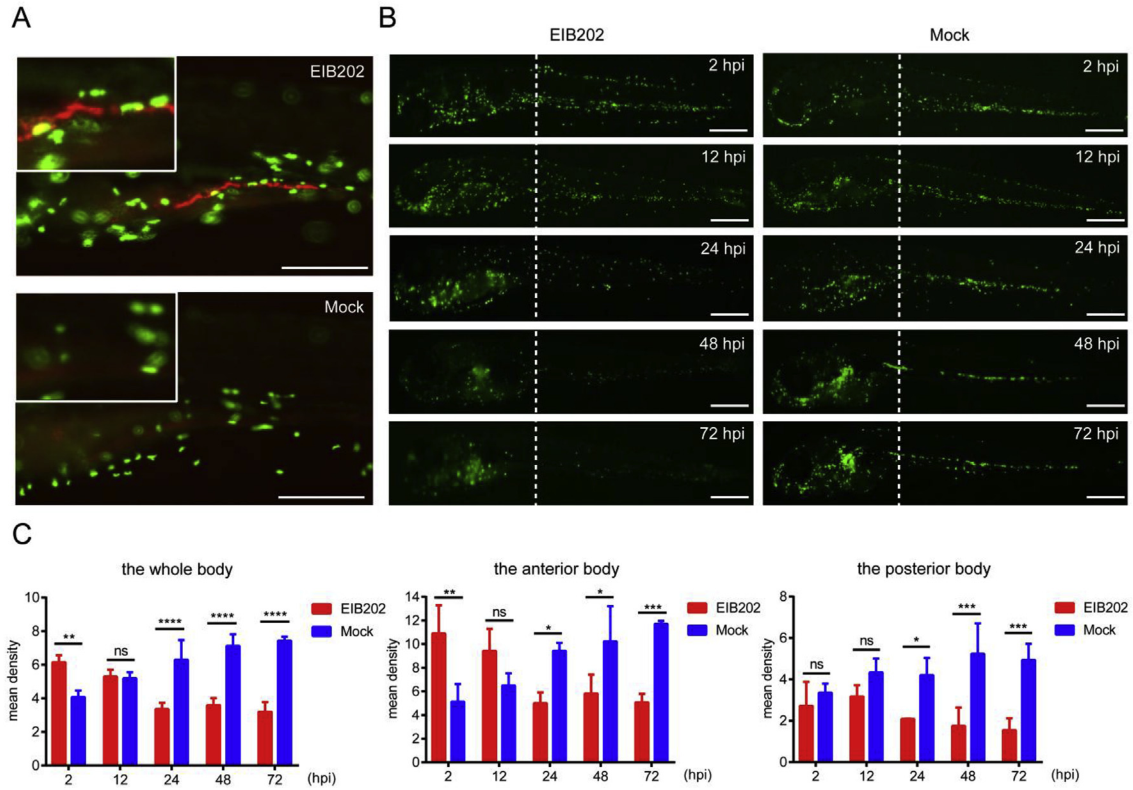

Fig. 3. Neutrophils recruit to the infectious sites during early phase of infection. **(A)** Lateral images of 5 dpf *Tg(mpx:eGFP)* zebrafish immerse infected with 1×10^8 CFU/ml EIB202-mCherry. Green, neutrophils; Red, EIB202-mCherry. Scale bar, 100 μm. **(B)** Lateral images of whole fish larvae from EIB202-infected or PBS-mocked *Tg(mpx:eGFP)* zebrafish larvae were analyzed at indicated time-points post infection. Green, neutrophils. Scale bar, 100 μm. **(C)** Fluorescence intensity quantification of the whole zebrafish larvae, the anterior body (left of white dotted line), and the posterior body (right of white dotted line) as in **(B)**. Mean density was calculated by the measured parameters (ratio of RawIntDen and Area) from ImageJ. Data are expressed as mean ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$ and $****p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

activity through its cell death program.

3.4. Neutrophils play critical role in E. piscicida clearance

To test whether the neutrophils exert certain bactericidal activity during *E. piscicida* infection, we generated the neutrophil-knockdown zebrafish larvae through CRISPR/Cas9 system [32,35]. By microinjecting high concentration of *gcsfr* sgRNAs mix and Cas9 protein into yolk, we acquired the F_0 mosaic mutant embryos (crispants). Through PCR genotyping (Fig. 4A) and sequencing analysis (Fig. 4B), as well as qRT-PCR analysis for *gcsfr* transcriptional expression (Fig. S3C), we confirmed the efficiency of this gene editing. Moreover, through fluorescence observation, we found the neutrophils were significantly knocked down in *gcsfr*$^{-/-}$ crispants larvae (showed a phenotype of weak fluorescent), compared with the wild type *Tg(mpx:eGFP)* larvae (Fig. 4C and D). More than 80% of larvae showed this positive phenotype in all observed embryos (Fig. 4E). In addition, we also generated the *gcsfr*$^{-/-}$ crispants in *Tg(mfap4:GFP)* zebrafish larvae, a macrophages specific labeled zebrafish line [36], and confirmed that the *gcsfr* knockout has no effects on macrophages differentiation (Fig. S3). Furthermore, the expression of related inflammatory genes was not significantly changed in *gcsfr*$^{-/-}$ crispants compared to wild type larvae (Fig. S4). Based on the basic results above, to further analyze the role of neutrophils in response to infection, we immersion infected the wild type or *gcsfr*$^{-/-}$ crispants larvae with *E. piscicida*, respectively, and found a significantly higher mortality in *gcsfr*$^{-/-}$ crispants larvae (Fig. 4F), and consistently, the bacterial loads were also comparatively enhanced in *gcsfr*$^{-/-}$ crispants larvae (Fig. 4G), compared with the wild type larvae (Fig. 4F and G). Taken together, our results suggest that the fish neutrophils play essential role to mediate the immune homeostasis in response to *E. piscicida* infection *in vivo*.

4. Discussion

Intestinal mucosal barrier is the first line in host innate immune system and plays important roles in maintaining the gut homeostasis [37,38]. Several immune mechanisms of mucosal immunity about sensing pathogen, amplifying pro-inflammatory response, secreting anti-microbial peptides and recruiting neutrophils have been studied in mammals [39–42]. In the meanwhile, due to the conservation of homology genes between zebrafish and mammalians, zebrafish is increasingly used to study the intestinal inflammation and injury [43]. Chemically induced intestinal inflammatory in zebrafish model has been used to study the function of microbiota [44,45], as well as the inflammatory bowel diseases (IBD) [46]. Moreover, zebrafish is becoming an ideal model to analyze the infection-mediated intestinal mucosal immunity *in vivo* [47,48]. Here, we established an immersion model by using 5 dpf zebrafish larvae to analyze the intestinal mucosal immunity, and found that *E. piscicida* colonized in the gut lumen rather than other tissues, which suggest that the intestinal mucosal might restrict the spread of infection and prevent the host from systemic infection.

Inflammatory cytokines and chemokines play critical roles in mediating host inflammation responses during bacterial infection [49]. In response to infection, the TNF-α is a primary pro-inflammatory mediator involved in immune response [24], which could work as a regulator for neutrophils ROS generation [50]. Moreover, the inflammasome activation induced a robust IL-1β production, which could

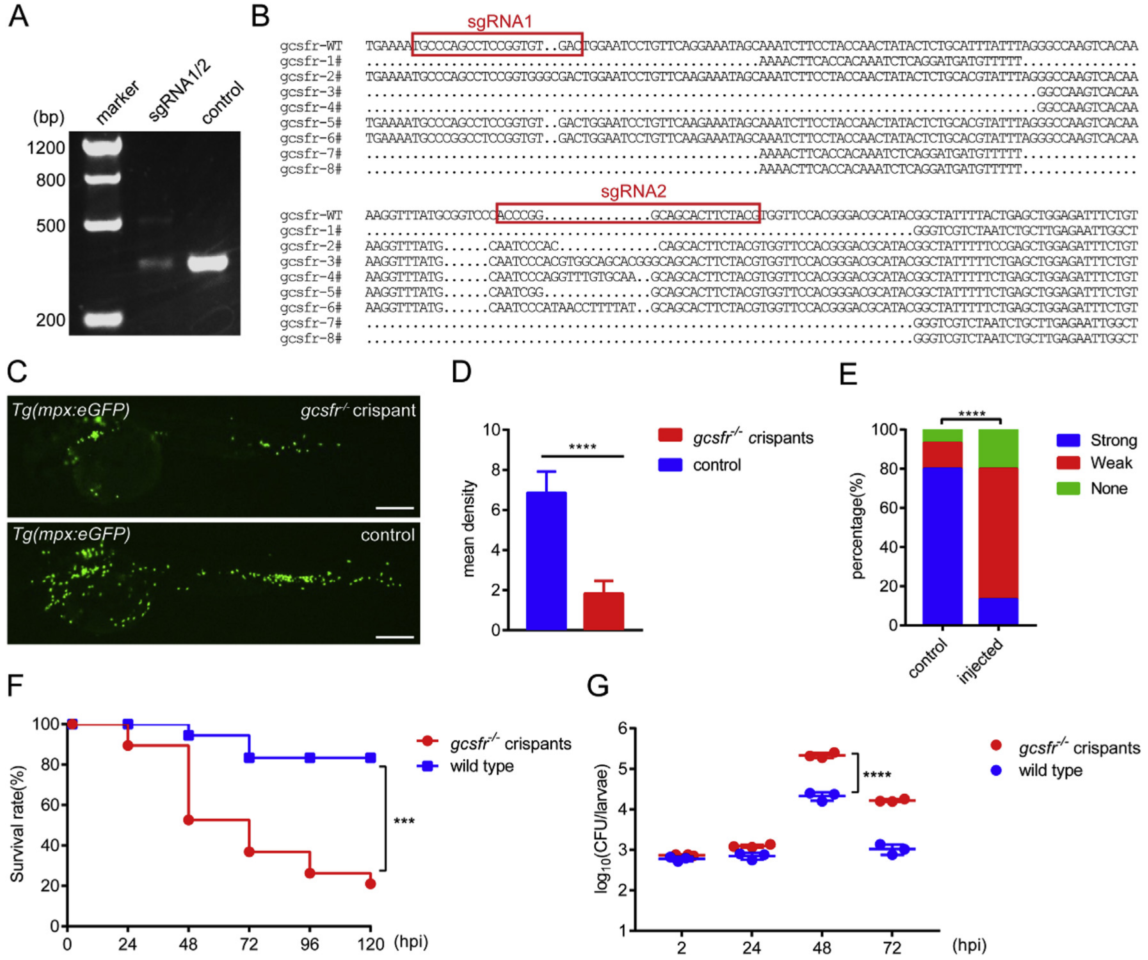

Fig. 4. Neutrophils play critical role in *E. piscicida* clearance. (A and B) Embryos microinjection with two-guide Cas9 RNP mix targeting *gcsfr* were collected at 48 hpf. Genotyping of *gcsfr* amplicons was analyzed by PCR **(A)**, and amplicons were cloned for sequencing **(B)**. **(C)** Lateral images of 2 dpf *Tg(mpx:eGFP)* zebrafish larvae after injected with two-guide Cas9 RNP mix targeting *gcsfr*. None-injected larvae were showing as control. Scale bar, 100 μm. **(D)** Quantitative analysis of fluorescence intensity from *gcsfr*$^{-/-}$ crispants and wild type zebrafish larvae. 10 zebrafish larvae were randomly selected to quantify fluorescence intensity. Mean density was calculated by the measured parameters (ratio of RawIntDen and Area) from ImageJ. $****p < 0.0001$. **(E)** The ratio of positive phenotype after injected with two-guide Cas9 RNP mix targeting *gcsfr*. Strong (strong fluorescent) indicates negative phenotype; Weak (weak fluorescent) indicates positive phenotype; None (no fluorescent) indicates no labeled fluorescent. n = 20; $****p < 0.0001$. **(F and G)** Percent survival (n = 30) **(F)** and bacterial loads **(G)** of 5 dpf *gcsfr*$^{-/-}$ crispants and wild type larvae infected with EIB202 at indicated time-points. Data are presented as mean ± SD of three independent experiments. $***p < 0.001$; $****p < 0.0001$.

promote the neutrophils' recruitment and ROS release to kill the pathogens [51]. In our study, we found a robust expression of TNF-α and IL-1β during the early phage of *E. piscicida* infection, which suggest that the inflammasome and MAPK pathways might activate in response to infection in zebrafish. However, the anti-inflammatory cytokine IL-10 could counterbalance the excessive inflammation [52]. Previous study has revealed that IL-10 could protect the host from systemic infection in a *Staphylococcus aureus* infection [53], but in our study, the transcription of IL-10 was not affected in response to *E. piscicida* infection, which need to be further studied. Furthermore, GCSFa and GCSFb are two paralogs of GCSF in zebrafish, which is relative to neutrophils proliferation and differentiation [30,31], and the CXCL8 and MMP9 are the chemokines implicated in neutrophils recruitment during bacterial infection [54]. In our study, although we observed an unexpected dynamic change of CXCL8 and MMP9 in response to *E. piscicida* infection, the coordinated mechanisms about these cytokines and chemokines in regulating the function of neutrophils *in vivo* still needs to be explored.

Neutrophils are the critical inflammatory cells in mucosal immune system, which execute the function of host defense in different ways, such as secreting granule protein, producing ROS to promote inflammatory reaction [55,56], releasing NETs [24,57,58] to trap and kill bacteria, or directly phagocytizing pathogens [59,60]. With the development of different transgenic zebrafish lines for immune cells [61,62], neutrophils action could be observed in specific transgenic larvae, and we could use them to focus on the neutrophils recruitment and migration after infection or injury [63–65]. Although the *in vitro* experiments showed that neutrophils from zebrafish kidney could release NETs after stimulation [23], analyzing the formation of NETs remains to be clarified *in vivo*. In our work, the neutrophils action was observed during the infection of *E. piscicida in vivo*, along with a significant number of neutrophils decreased in the late stage of infection, but the mechanisms of neutrophils reduction remain unclear. We expected that the neutrophils might be recruited and release NETs, resulting in cell death, to kill the bacteria *in vivo*. Thus, an effective *in vivo* model to analyze the NETs formation in zebrafish larvae would be ideal.

Besides, unlike the research of pathogen infection in adult zebrafish [48,66,67], the larvae are more suitable to study the mechanism of mucosal immunity in response to infection, because of their unique development advantages [68,69] and readily genetic manipulation [70]. Combined with CRISPR/Cas9 system, gene editing in zebrafish is increasingly used to investigate the pathway molecular and develop targeted therapies [71–73]. Recently, a method to generate mosaic F_0

crispants has been applied for rapid function identification and high-throughput screening [35,74,75], which make the zebrafish as a superior model to accelerate the understanding of functional molecular in immune cells, and our work take this advantage to provide an available method to knock down the neutrophils, and give a clue for better analyzing the function of myeloid cells during bacterial infection *in vivo*.

In summary, we established an effective *E. piscicida* immersion infection model in zebrafish larvae to analyze the intestine mucosal immune responses. Based on this model, we explored the dynamics of *E. piscicida* infection and preliminary revealed the critical role of neutrophils in bacterial clearance to mediate immune homeostasis, which will provide us a better clue for understanding the action of innate immune cells during infection *in vivo*.

Authors contribution

D.Y. conceived the study; Z.W. conducted the majority of experiments with help from L.L. and W.C.; Y.Z. and Q.L. provided expert advice and critical review of the manuscript. D.Y. and Z.W. wrote the manuscript; all authors discussed the results and commented on the manuscript.

Conflicts of interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.02.008.

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