Fish & Shellfish Immunology 54 (2016) 179–187

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Matrix metalloproteinase-9 plays a role in protecting zebrafish from lethal infection with *Listeria monocytogenes* by enhancing macrophage migration

Ying Shan, Yikai Zhang, Xunhui Zhuo, Xiaoliang Li, Jinrong Peng, Weihuan Fang*

Institute of Preventive Veterinary Medicine, College of Animal Sciences, Zhejiang University, Hangzhou 310058, CHINA

ARTICLE INFO

Article history:
Received 25 February 2016
Received in revised form
19 March 2016
Accepted 7 April 2016
Available online 8 April 2016

Keywords:
Matrix metalloproteinase-9
Macrophage
Migration
Listeria monocytogenes
Germ-free zebrafish
Microarray

ABSTRACT

Zebrafish could serve as an alternative animal model for pathogenic bacteria in multiple infectious routes. Our previous study showed that immersion infection in zebrafish with *Listeria monocytogenes* did not cause lethality but induced transient expression of several immune response genes. We used an Affymetrix gene chip to examine the expression profiles of genes of zebrafish immersion-infected with *L. monocytogenes*. A total of 239 genes were up-regulated and 56 genes down-regulated compared with uninfected fish. Highest expression (>20-fold) was seen with the *mmp-9* gene encoding the matrix metalloproteinase-9 (Mmp-9) known to degrade the extracellular matrix proteins. By morpholino knockdown of *mmp-9*, we found that the morphants showed rapid death with much higher bacterial load after intravenous or intraventricular (brain ventricle) infection with *L. monocytogenes*. Macrophages in *mmp-9*-knockdown morphants had significant defect in migrating to the brain cavity upon intraventricular infection. Decreased migration of murine macrophages with knockdown of *mmp-9* and *cd44* was also seen in transwell inserts with 8-μm pore polycarbonate membrane, as compared with the scrambled RNA. These findings suggest that Mmp-9 is a protective molecule against infection by *L. monocytogenes* by engaging in migration of zebrafish macrophages to the site of infection via a non-proteolytic role. Further work is required on the molecular mechanisms governing Mmp-9-driven macrophage migration in zebrafish.

1. Introduction

Zebrafish, *Danio rerio*, has been widely used as a novel vertebrate animal model to study human infectious diseases for easiness of experimental settings due to its small body dimension but with large clutch size of its embryos [1]. Approaches of genetic manipulation and high resolution imaging is readily utilized on zebrafish embryos for close monitoring of host-pathogen interactions *in vivo* [2]. We have found that the germ-free zebrafish embryos at 26 hpf are particularly useful to examine the innate immune responses and trafficking of phagocytes to infections by *Listeria monocytogenes* [3]. Other bacterial pathogens tested in the zebrafish model includes *Salmonella typhimurium*, *Vibrio anguillarum*, *Staphylococcus aureus* and *Mycobacteriu marinum* [4–8].

Listeria monocytogenes has been well described as a model intracellular pathogen to study its interaction with host cells [9,10]. By microarray-based transcriptional analysis, Cohen et al. (2000) found that the genes encoding proinflammatory cytokines and those related to signal transduction and metabolism were up-regulated in cultured human promyelocytic THP1 cells infected with *L. monocytogenes* [11]. Microarray analyses on Caco-2 cells revealed strong induction of NF-κB signaling and interferon-responsive genes upon *L. monocytogenes* infection [12]. Various pathogens other than *Listeria* have been tested in zebrafish for genome-wide transcriptional profiling [5,13,14]. Transcriptomic profiling on *Salmonella*-infected zebrafish revealed some consistent innate immune responsive genes [5]. Several common infection-responsive genes were up-regulated in *Streptococcus suis* infected adult zebrafish [14]. Static immersion with *Edwardsiella tarda* showed little overlap on induced genes as compared to intravenous injection [13]. However, these studies were performed on conventionally reared zebrafish and could thus be interfered by microbes in the living environments and gut microbiota. Germ-free zebrafish were seldomly used for transcriptomic analysis [15].

* Corresponding author.
E-mail address: whfang@zju.edu.cn (W. Fang).

http://dx.doi.org/10.1016/j.fsi.2016.04.003
1050-4648/

In our initial work with transcriptomic profiles of germ-free zebrafish larvae immersion-infected with *L. monocytogenes*, *mmp-9* was the most upregulated gene. The gene encodes matrix metalloproteinase 9. Matrix metalloproteinases (MMPs) have important functions in extracellular matrix (ECM) degradation and tissue repair [16]. Mmp-9 has been found to contribute not only to gelatinase activity, but also to leukocyte migration, thus participating in mammalian inflammation and immunity [17,18]. Mmp-9 in zebrafish is expressed notably in the head-kidney and in peritoneal and peripheral blood leucocytes, indicating its role in immune responses [19]. In *Mycobacterium marium* infected zebrafish, Mmp-9 was found to enhance recruitment of macrophages and contributed to granuloma formation and bacterial growth [20].

In this study, morpholino antisense oligonucleotides were used to investigate the functions of zebrafish Mmp-9 in response to infection by *L. monocytogenes*. Macrophage migration, fish survival and bacterial burden were examined in fish receiving intravenous or intraventricular inoculation. We found that silencing of *mmp-9* reduced the number of migrated macrophages to brain ventricles, increased the bacterial burden in infected fishes and led to rapid death, suggesting that Mmp-9 plays an important role in protecting zebrafish against *L. monocytogenes* infection by engaging in macrophage migration.

2. Materials and methods

2.1. Bacterial strains and culture condition

Listeria monocytogenes strain EGDe and *Listeria innocua* strain ATCC33090 were used for infection experiments. *L. monocytogenes* EGDe expressing green fluorescent protein (EGDe-gfp) constructed in our laboratory [3] was used to observe the bacteria *in vivo*. The bacterial strains were routinely grown in brain heart infusion (BHI, Oxoid, UK) medium at 37 °C with shaking at 150 rpm.

2.2. Zebrafish husbandry and generation of germ-free embryos

The zebrafish line AB was maintained in the Developmental Biology Laboratory at Zhejiang University. Adult fishes were raised in standard zebrafish units (Aisheng, Beijing, China) at 28 °C under a constant light cycle of 14-hr on/10-hr off. Germ-free embryos were generated with the method as previously described [21]. Natural breeding eggs were collected immediately after hatching and transferred to a sterile dish with sterilized egg water containing antibiotics (ampicillin and kanamycin). Embryos were monitored every 2 h and unfertilized eggs were removed.

2.3. Bacterial infection by immersion and micro-injection

Overnight bacterial cultures were washed with sterile egg water and adjusted to OD_{600nm} at 0.6 (10^9 CFU/mL). Infection was performed, depending on the type of experiments, by immersion infection or by micro-injection via yolk sac, brain ventricle or blood island as previously described [22,23]. Naturally hatched germ-free fish 5 dpf (when mouth and gut are functional) were used for static immersion with 1×10^8 CFU/mL egg water of *L. monocytogenes* or *L. innocua*, for 24 hs and then used to extract total RNA for transcriptomic analysis. Intravenous or intraventricular infection was described in detail in a previous study [3]. Briefly, fish of 26 hpf were dechorionated carefully before injection, an inoculum of 100 CFU bacterial cell per fish were micro-injected into caudal vein or brain ventricle. In all cases, injection or immersion with egg water was used as mock-infected control.

2.4. RNA extraction and quantitative reverse transcription PCR

Twenty embryos of each group (*L. monocytogenes*-, *L. innocua*- or mock-infected group) were pooled and collected by centrifugation at 13,800× *g* for 5 min and stored at −80 °C for later use. Embryos were homogenized in 500 μL lysis buffer and total RNA was extracted using the Uniq-10 TRNzol total RNA Extraction and Purification Kit instruction (Tiangen, Beijing, China). DNaseI (Promega, USA) was used to remove residual genomic DNA at 37 °C for 1 h before cDNA synthesis. Reverse transcriptase (TOYOBO, Japan) was used for cDNA synthesis. Quantitative PCR was then performed in a 20-μl reaction mixture containing SYBR quantitative PCR mix (TOYOBO, Japan) to confirm the microarray results with specific primer pairs (Table S1) using an iCycler iQ5 real-time PCR detection system (Bio-Rad, USA). The housekeeping gene *β-actin* was used as internal control for normalization of transcriptional levels of the target genes.

2.5. Microarray hybridization, gene ontology and cluster analysis

The RNA samples were sent to CapitalBio Beijing, China for microarray hybridization on Affymetrix Zebrafish Genome Array containing 14,900 transcripts (Affymetrix) with three replicate chips for each RNA sample. All manipulations were performed as described in the Technical Manual (Affymetrix). Hybridization data were analyzed using GeneChip Operating Software (GCOS 1.4). The images were first assessed by visual inspection and then analyzed to generate raw data files saved as CEL files using the default setting of GCOS 1.4. A global scaling factor of 500 was used to normalize different chips. The differentially expressed genes were selected using SAM (Significant Analysis of Microarray) software 3.0, based on $|\text{Score(d)}| \geq 2$ when compared with mock-infected control.

Categorization of biological processes, molecular functions and cellular components were analyzed using Gene Ontology project (http://www.geneontology.org). Cluster analysis was run on the GeneCluster3.0 software. All significant GO enrichments and gene clusters were valued by corrected *P*-value < 0.05.

2.6. Silencing of mmp-9 by specific morpholino oligonucleotides

Zebrafish *mmp-9* translation-blocking morpholino (Mmp9-MO, 5′-CGCCAGG ACTCCAAGTCTCATTTTG-3′) and off-target control human β-globin antisense morpholino (Control-MO, 5′-CCTCTTACCTCAGTTACAATTTATA-3′) were purchased from Gene Tools. Concentration of 500, 250, 125, 50 mM morpholinos were microinjected into yolk sac of the zebrafish embryos at one-cell stage. The fish were sacrificed at 10 dpf and total protein samples prepared for Western blotting.

2.7. Infection with GFP-expressing listeria and live embryo imaging

Zebrafish larvae were infected by injection with the EGDe-gfp strain via brain ventricles or blood island and observed microscopically at 6 hpi (brain infection) or at 24 hpi (intravenous). The GFP-expressing bacteria were monitored and photographed *in vivo* by laser confocal microscope IX81-FV1000 (Olympus, Japan).

2.8. Bacterial burden assay

Eight embryos in each group were anesthetized at 6 and 24 hs after intravenous injection. Each embryo was then rinsed and homogenized in 1 mL sterile egg water. Serial dilutions in PBS (10 mM, pH 7.4) of the homogenates were plated on PALCAM agar (*Listeria* selective medium) (Luqiao, Beijing, China). The colonies were enumerated after incubation at 37 °C for 24 h. The results were

presented as mean $\log_{10}$ CFU ± SE per group.

2.9. Cell culture and small interfering RNA (siRNA) transfection

The murine RAW264.7 macrophage cells were grown to 60% confluency at 37 °C and 5% CO_2 in 24-well tissue culture plates (Corning, USA) containing RMPI 1640 medium supplemented with 10% fetal bovine serum (Gibco). The siRNAs (15 pmol) were delivered into cells by transfecting with Lipofectamine 2000 reagent (Invitrogen). Gene silencing proceeded for 24 hs for migration assays described below. The specific siRNA and scrambled control siRNA were purchased from GenePharma (Shanghai, China): Mmp-9 sense (5′-GACGGCAAAUUUGGUUUCUTT-3′) and Mmp-9 antisense (5′-AGAAACCAAAUUUGCCGUCTT-3′); CD44 sense (5′-CUCCCACUAUGACACAUAUTT-3′) and CD44 antisense (5′-AUAUGUGUCAUAGUGGGAGTT-3′); Control sense (5′-UUCUCCGAACGUGUCACGUTT-3′) and (5′-ACGUGACACGUUCGGAGAA TT-3′).

2.10. Western blotting

Cells or fishes were homogenized in 50 μL RIPA lysis buffer (with 1 mM PMSF) (Beyotime, Shanghai, China). The lysates were collected and protein concentration was quantified by using a bicinchoninic acid assay kit. Proteins were separated on 10% SDS-PAGE gels and electrotransferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The blots were blocked for 1 h in Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% nonfat milk and then probed overnight at 4 °C with rabbit polyclonal anti-Mmp-9 IgG at 1:800 dilution (1.25 μg/mL) (Cat#AV33090, Sigma) and rabbit monoclonal anti-β-actin IgG at 1:2000 dilution (Cat#R1207-1, Hua'an, Hangzhou, China). Blots were then washed with TBST, incubated for 1 h with goat anti-rabbit HRP-labeled antibodies (Sangon Biotech, Shanghai, China), and visualized using West Pico chemiluminescent substrate (Thermo, Marina, CA) under the conditions recommended by the manufacturer. Images were captured in a Gel 3100 chemiluminescent imaging system (Sage creation, Beijing, China).

2.11. In vitro cell migration assay

The cell migration assay was performed as described elsewhere [24,25]. RAW264.7 macrophages (with or without knockdown of *mmp-9* and *cd44*) were resuspended at a density of 3.4×10^5 cells/mL in low serum medium (RMPI 1640 with 1% FBS). A total of 500 μL cells were seeded in the transwell inserts with 8-μm pore polycarbonate membrane (Millipore). One-mL of high serum medium (RMPI 1640 with 10% FBS) was added to each of the lower chambers of the 24-well plate. The inserts were removed after incubation for 24 hr at 37 °C and 5% CO_2. Cells on the top side were removed with cotton swabs and the inserts were then subjected to fixation with 4% poly-paraformaldehyde. The migrated cells on the bottom side were then permeabilized with 0.5% Triton X-100 and stained with 2-(4-Amidinophenyl)-6- indolecarbamidine dihydrochloride (DAPI, Sigma). The migrated cells were imaged using the confocal microscope IX81-FV1000 (Olympus, Japan) and 4 random fields for each insert were selected for cell quantification. The experiment was repeated three times, each with triplicate inserts.

2.12. In vitro phagocytosis assay

Overnight bacterial cultures were harvested by centrifugation (1500× *g* for 10 min), resuspended in 10 mM PBS (pH 7.4) and OD_{600} was adjusted to 0.2. The RAW264.7 cells with or without *mmp-9*-knockdown were grown to confluence at 37 °C and 5% CO_2 for 24 hs after transfection (about 2×10^5) in 24-well plates (Corning, USA) and infected for 1 h with *L. monocytogenes* EGDe at MOI of 10:1. Cells were washed with PBS after 1-h infection and incubated for another hour in RMPI 1640 medium containing 10% FBS and 200 μg/ml gentamycin. At the indicated times, the cells were lysed and 10-fold diluted for plating on BHI agar. The agar plates were incubated overnight at 37 °C for colony counting. Phagocytosis was calculated as the ratio of recovered colonies after gentamicin treatment to those inoculated.

2.13. Statistical analysis

All results were presented as mean ± SEM of triplicate experiments and subjected to one-way analysis of variance.

2.14. Conformity to the relevant regulatory standards

Experiments with zebrafish were approved by the Laboratory Animal Management Committee of Zhejiang University (Approval No. 2013038).

3. Results

3.1. Microarray analysis and confirmation by quantitative real-time PCR

As static immersion of different *Listeria* strains could not cause mortality of zebrafish embryos, we performed microarray on pools of 20 zebrafish after immersion with *L. monocytogenes* EGDe. Fish infected with *L. innocua* or those mock-infected were included to find specific genes induced by the pathogenic *L. monocytogenes*. Comparison with mock-infected control reveals up-regulation of 239 genes (fold change >2.0) in EGDe infected fish. There were only 25 genes up-regulated in *L. innocua* infected fish. Down-regulation (fold change <0.5) was seen with 56 and 4 genes in EGDe-infected and *L. innocua*-infected fish, respectively (Fig. 1A). EGDe infection led to up-regulation of 102 genes, as compared with *L. innocua*. Fig. 1 also shows part of the microarray results as fold changes in EGDe infected fish, as compared with mock-infection (Fig. 1B) or with *L. innocua* infected fish (Fig. 1C). The gene *mmp9* exhibited highest induction in EGDe infected fish. The expression levels of some genes by the microarray method were verified by qRT-PCR (Fig. 2). More details of the genes, induced and suppressed, are presented in Tables S2 and S3.

3.2. Gene ontology and cluster analysis

Gene ontology analysis revealed that most of the 239 up-regulated genes in EGDe infected fish relative to mock-infected fish were classified into functional categories of cellular components (CC), biological processes (BP) and molecular functions (MF) with 53% induced genes belonging to BP, followed by MF (25%) and CC (22%). The main GO categories for up-regulated genes were GO:0005576 related to extracellular region (Fig. S1A). In the 102 up-regulated genes from EGDe infected fishes relative to *L. innocua* infected counterparts, most differentially expressed genes were classified into CC (45%), followed by MF (38%) and BP (17%). The main GO category for the up-regulated genes was GO:0050,896 involved in response to stimulus, while others were regulation of actin filament-based process, multi-organism process, proteolysis, cell junction, extracellular region, binding, peptidase activity and enzyme regulator activity (Fig. S1B). More details of other GO categories are presented in Tables S4 and S5.

Genes in immunity-related GO categories were performed with cluster analysis. Of the nine clusters, up-regulation was seen with genes related to tight junction, signaling, immune cells, interferons,

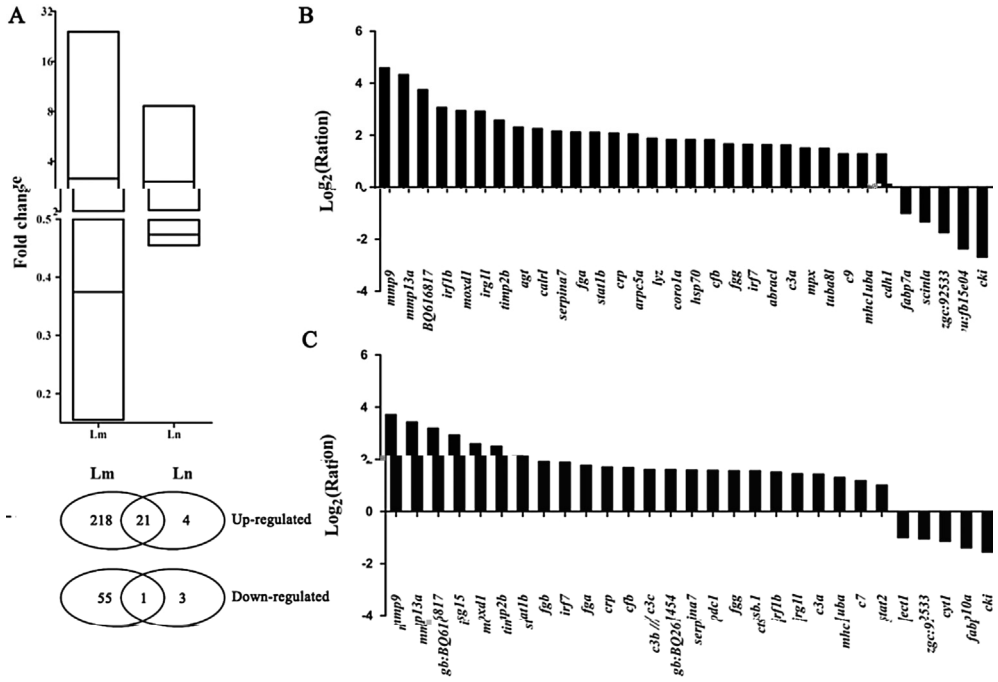

Fig. 1. Expression profiles of genes of germ-free zebrafish immersion-infected with *Listeria monocytogenes* (Lm) and *Listeria innuoca* (Ln). (A) A venn diagram showing total numbers of genes that were significantly ($p \leq 10^{-4}$) up- or down-regulated (fold change ≥ 2 or ≤ -0.5) compared with the mock control group. (B) Part of the differentially expressed genes in fish infected with *L. monocytogenes* shown as fold changes compared to mock control. (C) Part of the differentially expressed genes in fish infected with *L. monocytogenes* shown as fold changes relative to *L. innocua* infection. The most up-regulated genes was *mmp-9* in response to *Listeria* infections.

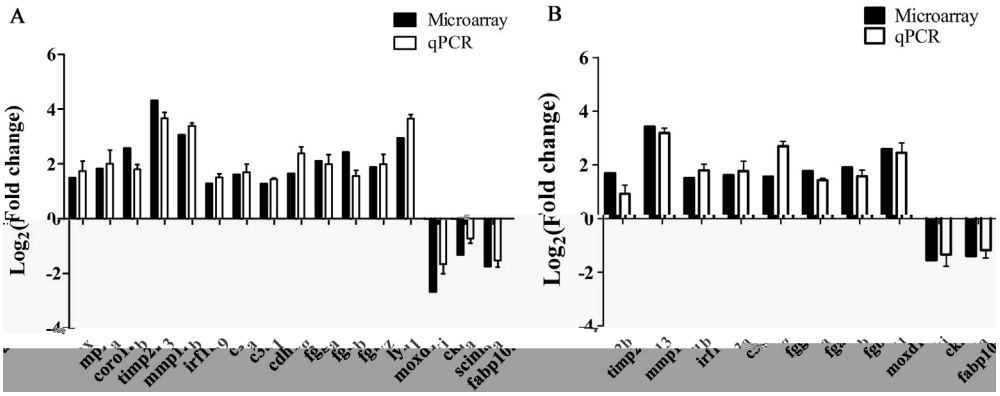

Fig. 2. Analysis of selected genes from microarray results by quantitative PCR (qPCR). Selected genes were analyzed on the same RNA samples previously used in microarray profiling. The qPCR data were normalized to β-actin. Values are the means ± SEM of three independent experiments. (A) Differentially expressed genes in *Listeria monocytogenes* infected fish relative to mock control. (B) Differentially expressed genes in fish infected with *L. monocytogenes* shown as fold changes relative to *L. innocua* infection.

receptors, heat shock proteins (Hsp), fibrinogen, complement and actin. High induction in EGDe infected fish was seen with genes stat1b, moxd1, irf7 and c9-2 (Fig. 3) that encode signal transducer and activator of transcription 1b, monooxygenase, interferon regulatory factor 7 and complement component 9, respectively.

3.3. Mmp-9 morphants were more sensitive to infection as a result of defect in migration of macrophage

Initial experiments showed that the silencing effect could last for 10 days at a concentration of 500 mM morpholino (Fig. S2). Immersion infection of the *mmp-9* morphants with EGDe led to gastrointestinal bleeding (Fig. 4), while the control morphants and uninfected fish did not show any abnormality. Since immersion infection did not cause death to the *mmp-9* morphants, we performed intravenous infection on the 26 hpf fish. The *mmp-9* morphants showed rapid death within 3 days after infection, more rapid than the control morphants that presented progressive mortality in 6 days (Fig. 5A). Significantly higher bacterial load was seen in the *mmp-9* morphants than in the control morphants (Log_{10} CFU as 5.04 ± 0.22 *vs* 3.94 ± 0.19, $P < 0.01$) (Fig. 5B) at 24 hpi. Whole fish imaging also showed more GFP-expressing listerial cells in the *mmp-9* knockdown fish than those receiving control morpholino (Fig. 5C). These results imply that Mmp-9 contributes to protection against listeria infection by controlling bacterial load.

Brain ventricle is lack of macrophages in 26 hpf fish and could serve as an appropriate infection site to study migration of macrophages across the blood-brain barrier. To investigate how Mmp-9 functioned in controlling bacterial load, we employed intraventricular infection to examine migration of macrophages to the brain

Fig. 3. Cluster analysis of differentially expressed genes by two-dimensional hierarchical clustering method (average link, cosine correlation). Up-regulated genes are depicted in red color and down-regulated genes, in green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

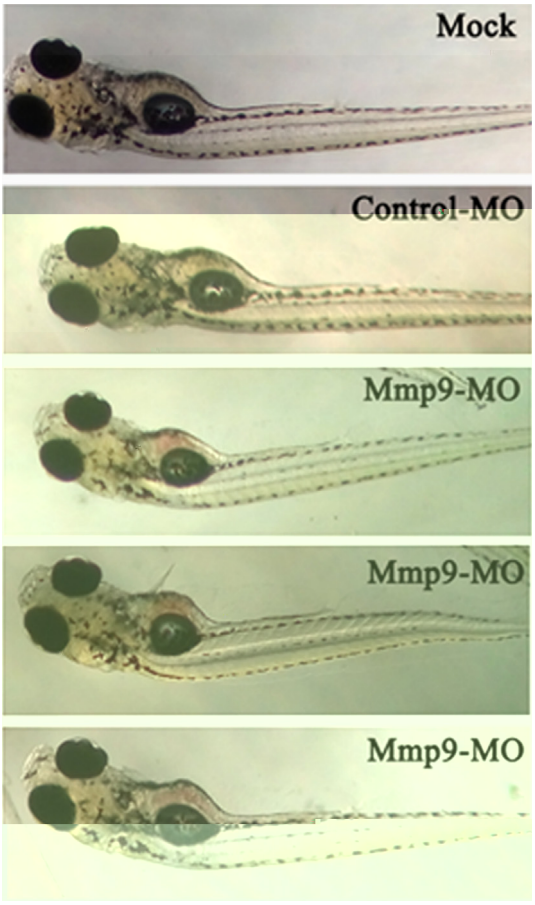

Fig. 4. Mmp-9 morphants (Mmp9-MO) showed gastrointestinal bleeding after immersion infection with *Listeria monocytogenes* but no abnormality in fish receiving control morpholino (Control-MO).

cavity, a prerequisite for clearing of the invading microorganism. Migration of phagocytes to the brain ventricle was markedly impaired in *mmp-9* morphants as compared with control morphants upon intraventricular infection with EGDe (cell numbers as 3 ± 1 *vs* 17 ± 2, $P < 0.001$) (Fig. 6A and C). Fish with *mmp-9* knockdown seemed to have significant defect in phagocytosis as shown by listeria-containing macrophages at 3.1% in the *mmp-9* morphants versus 27.5% in those treated with control morpholino ($P < 0.001$) (Fig. 6B). In fish receiving control morpholino, macrophages were observed only at the injection site (Fig. 6C). The Mmp-9 morphants showed more rapid death than the control ones (7 *vs* 10 days) after intraventricular infection (Fig. 6D).

3.4. Knockdown of mmp-9 or cd44 decreased migration of murine macrophages

To further investigate the role of Mmp-9 in macrophage migration, we also performed *in vitro* migration assay of murine macrophages. Western blotting showed that Mmp-9 expression was markedly reduced in cells transfected with *mmp-9* specific siRNA (Fig. S2). Migration of murine macrophages with *mmp-9* knockdown through the transwell insert membranes was significantly impaired, as compared with the scrambled RNA (15 ± 2 *vs* 62 ± 3, $P < 0.001$) (Fig. 7A). However, silencing *mmp-9* did not impair the phagocytic ability *in vitro* (Fig. 7B). Mammalian MMP-9 is reported to be involved in migration of cancer cells via interaction with CD44 (Bauvois, 2012) (Chetty et al., 2012). To investigate the role of CD44 in macrophage migration, we also performed *in vitro* migration assay on *cd44*-knockdown murine macrophages. Silencing of *cd44* significantly decreased migration of macrophages, as compared with the scrambled RNA (28 ± 5 *vs* 69 ± 5, $P < 0.01$) (Fig. 8). These findings suggest that Mmp-9 might be involved in macrophage cell shaping and motility through interaction with CD44 other than proteolysis because the transwell insert membrane is made of inert material with fixed pore size.

4. Discussion

Zebrafish, either embryonic or adult, has been widely used as infection models to examine the transcriptional responses of immunity-related genes to different bacterial pathogens [5,13,14,26]. MMPs, particularly Mmp-9, are often found up-regulated significantly in bacteria-infected, chemical exposed or wounding zebrafish [27–30]. Our earlier report also indicated that transcription of *mmp-9* was up-regulated in immersion-infected

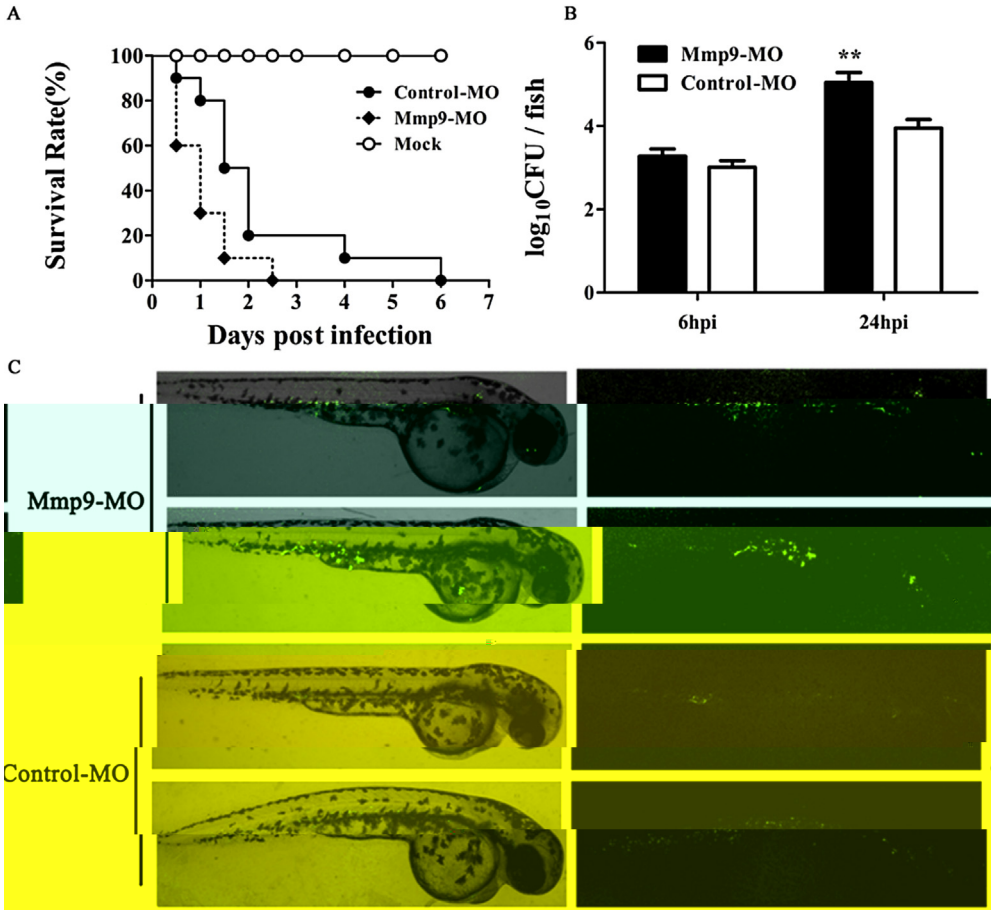

Fig. 5. Mmp-9 morphants (Mmp9-MO) showed more rapid death and higher bacterial load than the control counterparts (Control-MO) in response to intravenous infection with *Listeria monocytogenes*. (A) Survival curves (n = 20 per treatment). (B) Bacterial load with ($P < 0.01$) at 24 hpi (n = 8 per treatment). (C) Fluorescent imaging of GFP-expressing *Listeria monocytogenes* showing significantly higher bacterial load in the Mmp-9 morphants at 24 hpi.

zebrafish [3]. However, few studies so far have investigated the role of Mmp-9 in infection with functions other than proteolytic activity to extracellular matrix. Here we further reveal that Mmp9 is involved in migration of macrophages cross the blood-brain barrier of zebrafish independent of its proteolytic activity.

Phagocytic cells are the second line of defense by migration to the sites of infection to destroy the invading pathogens. This has been attributed to the proteolytic activity of MMPs (including Mmp-9) to degrade extracellular matrix proteins such as collagens, thus promoting migration of macrophages [31,32]. We found that migration of macrophages across the blood-brain barrier was markedly impaired in Mmp-9 morphants intraventricularly infected with *L. monocytogenes*. The increased number of bacterial load and lethality indicating that Mmp-9 might recruit macrophages to defense the bacteria invasion. With *M. marinum* infection, Mmp-9 was found to enhance recruitment of macrophages and contributed to granuloma formation and bacterial growth [20,33]. The morphants also displayed a decreased number of migrated macrophages in intraventricular infection but not as significantly as in our study. Besides the different bacterial species used, the microbial status in the conventional zebrafish and their living environment might contribute to distinct responses from the germ-free counterparts. There was also significant reduction of macrophage migration across the inert polycarbonate membrane with fixed pore-size when the *mmp-9* gene was silenced. These results indicate that Mmp-9 also plays a part in macrophage migration, besides its established role in proteolysis, in fighting against invading bacteria. The reduced phagocytic activity, shown as decreased number of macrophages containing phagocytosed bacteria in *mmp-9* kncokdown fish, might be due to significant reduction of migrating macrophages to the brain cavity (i.e. site of infection), thus having less chance to be observed. This is because silencing of *mmp-9* in murine macrophages did not have any negative impact on phagocytosis. Still we can not rule out the possibility that Mmp-9 operates in fish macrophages differently from its mammalian counterparts, or the molecules governing phagocytosis in fish might be different from mammalians.

Recent studies demonstrate that MMP-9 is involved in migration of cancer cells via interaction with CD44 [34,35]. In mammals, CD44 is a transmembrane adhesion molecule serving as a receptor for phagocytes, thus contributing to ingestion and clearance of engulfed particles [36]. CD44 is also expressed in zebrafish [37]. Mmp-9 was up-regulated in fish immune organs such as the head kidney during the initial phase of inflammation and down-regulated during the later phase, suggesting migration of cells from the head kidney to the focus of inflammation [38]. In our work with *cd44*-knockdown, we also observed reduced migration of the murine macrophages. Therefore, it is possible that cell motility, not only of murine macrophages *in vitro* but also of zebrafish macrophages *in vivo*, might result from the interaction of Mmp-9 with CD44 independent of its proteolytic activity. Increased bacterial burden and shortened survival period of the *mmp-9* morphants relative to their controls could be due to the bi-functional (proteolytic and motility-enhancing) roles of Mmp-9 that favor

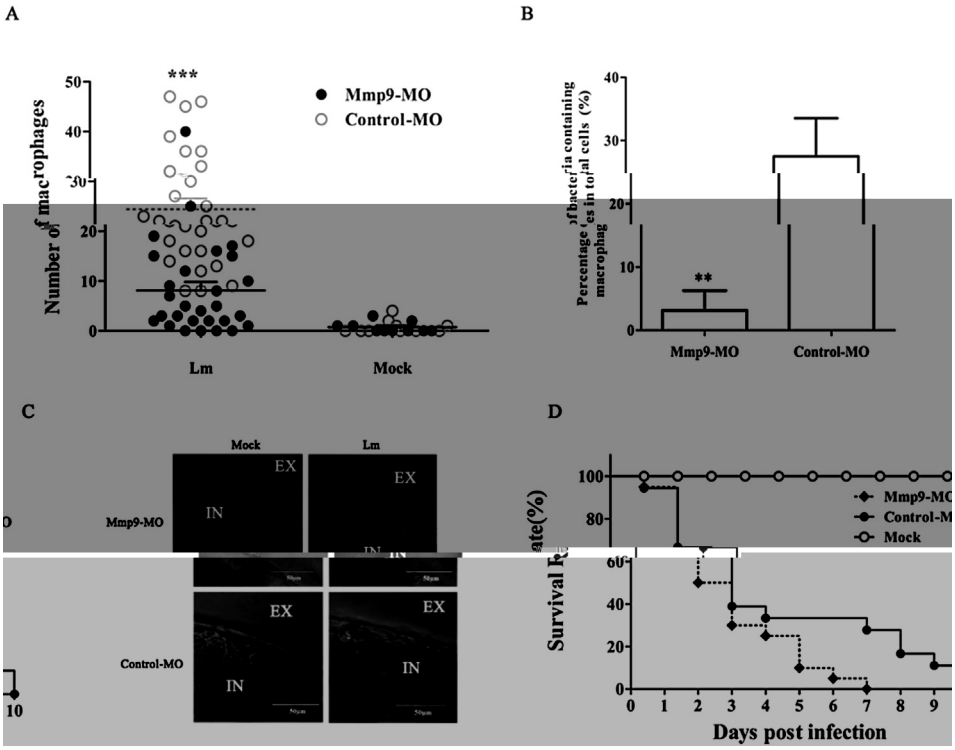

Fig. 6. Mmp-9 morphants exhibited significant defect in migration of macrophages across the brain cavity as well as rapid death in response to intraventricular infection with *Listeria monocytogenes* expressing green fluorescent protein. (A) Macrophages in Mmp-9 morphants (Mmp9-MO) failed to migrate efficiently to the brain ventricle relative to their control counterparts (Control-MO) 6 hpi after intraventricular infection with *Listeria monocytogenes* and *Listeria innocua* (n = 30 per treatment). (B) Mmp-9 morphants had significant lower percentage of phagocytes containing the GFP-expressing bacteria in total migrated cells. Macrophages of individual fish migrated to the brain cavity at 6 hpi, 10 fish per group, were counted and those containing more than one GFP-expressing bacterial cells were recorded. (C) Microscopic images (600×) showing migration of macrophages at the sites of infection in Control-MO but remained resident in Mmp9-MO at 6 hpi ('EX' and 'IN' represent exterior and interior of the brain cavity). (D) Mmp-9 morphants showed more rapid death than controls presented (n = 20 per treatment).

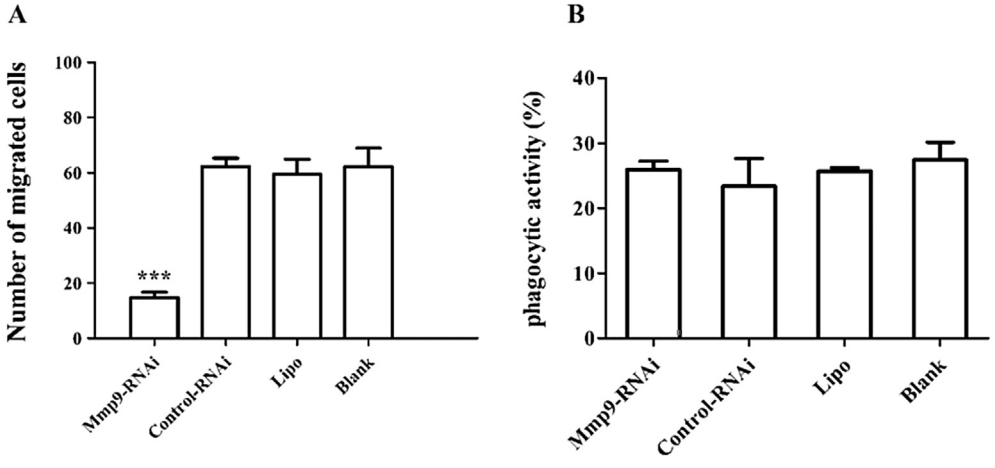

Fig. 7. Silencing of *mmp-9* decreased migration of murine macrophages through the 8-μm pore polycarbonate membrane without compromising their phagocytic ability. (A) Quantification of migrated macrophages expressed as means ± SEM of migrated macrophages of three independent experiments, each with four random fields per treatment. Mmp9-RNAi means *mmp-9* specific interferene RNA; Control RNAi, scrambled RNA; Liposome treatment, Lipo; and untreated control, Blank. (B) Silencing of *mmp-9* did not impair phagocytic ability of murine macrophages. Data are expressed as means ± SEM of three independent experiments.

migration of macrophages to the foci of infection in fighting against the invading bacteria.

Transcriptomic analysis of bacteria-infected zebrafish has led to indepth characterization of up-regulated genes related to innate immunity, such as ISG15 and Mmp-13a [39,40]. Recombinant *Sciaenops ocellatus* ISG15 was able to enhance the respiratory burst activity and bactericidal activity of macrophages, indicating its role in host defense against bacterial infection [39]. Mmp-13a is reported as an early host protective factor during mycobacterial infection in zebrafish [40]. In addition to *mmp-9* characterized in this study, we also found other up-regulated genes related to innate immunity that require further investigation, such as *stat1b*, *moxd1*,

irf7 and *c9-2*. While zebrafish is a good model for both mammals and marine fish [41,42], the versatility of the zebrafish model to other species should be properly addressed by approaches of comparative genomics and functional studies. We believe that the research findings from such model are particularly applicable to marine fish because of their close anatomic and physiologic similarities. The model is cost-effective because of the easiness in manipulation of the genetic system and experimental settings to produce germ-free zebrafish and live imaging.

In summary, Mmp-9 is significantly induced in response to infection by *L. monocytogenes* and involved in migration of zebrafish macrophages to the site of infection to exert their phagocytic activity. We suppose that Mmp-9 plays a non-proteolytic role in facilitating macrophage migration in zebrafish. Further research is required to decipher the molecular mechanisms by which Mmp-9 deploys to signal the macrophage migration in zebrafish.

Acknowledgements

This work is funded in part by the Zhejiang Provincial Department of Science and Technology (2014C02003) and the Ministry of Science and Technology of the People's Republic of China (2013BAD19B09-2).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2016.04.003.

References

[1] C. Sullivan, C.H. Kim, Zebrafish as a model for infectious disease and immune function, Fish. Shellfish Immun. 25 (2008) 341–350.

[2] D.M. Tobin, R.C. May, R.T. Wheeler, Zebrafish: a see-through host and a fluorescent toolbox to probe host–pathogen interaction, PLoS Pathog. 8 (2012) e1002349.

[3] Y. Shan, C. Fang, C.Y. Cheng, Y. Wang, J.R. Peng, W.H. Fang, Immersion infection of germ-free zebrafish with Listeria monocytogenes induces transient expression of innate immune response genes, Front. Microbiol. 6 (2015) 373.

[4] T.K. Prajsnar, V.T. Cunliffe, S.J. Foster, S.A. Renshaw, A novel vertebrate model of Staphylococcus aureus infection reveals phagocyte-dependent resistance of zebrafish to non-host specialized pathogens, Cell Microbiol. 10 (2008) 2312–2325.

[5] O.W. Stockhammer, A. Zakrzewska, Z. Hegedus, H.P. Spaink, A.H. Meijer, Transcriptome profiling and functional analyses of the zebrafish embryonic innate immune response to salmonella infection, J. Immunol. 182 (2009) 5641–5653.

[6] R. O'Toole, J. von Hofsten, R. Rosqvist, P.E. Olsson, H. Wolf-Watz, Visualisation of zebrafish infection by GFP-labelled Vibrio anguillarum, Microb. Pathog. 37 (2004) 41–46.

[7] M.G. Prouty, N.E. Correa, L.P. Barker, P. Jagadeeswaran, K.E. Klose, Zebrafish-Mycobacterium marinum model for mycobacterial pathogenesis, Fems Microbiol. Lett. 225 (2003) 177–182.

[8] L.M. van Leeuwen, M. van der Kuip, S.A. Youssef, A. de Bruin, W. Bitter, A.M. van Furth, et al., Modeling tuberculous meningitis in zebrafish using *Mycobacterium marinum*, Dis. Model Mech. 7 (2014) 1111–1122.

[9] A. Lebreton, F. Stavru, P. Cossart, Organelle targeting during bacterial infection: insights from Listeria, Trends Cell Biol. 25 (2015) 330–338.

[10] P. Cossart, Illuminating the landscape of host–pathogen interactions with the bacterium Listeria monocytogenes, Proc. Natl. Acad. Sci. 108 (2011) 19484–19491.

[11] P. Cohen, M. Bouaboula, M. Bellis, V. Baron, O. Jbilo, C. Poinot-Chazel, et al., Monitoring cellular responses to Listeria monocytogenes with oligonucleotide arrays, J. Biol. Chem. 275 (2000) 11181–11190.

[12] D.N. Baldwin, V. Vanchinathan, P.O. Brown, J.A. Theriot, A gene-expression program reflecting the innate immune response of cultured intestinal epithelial cells to infection by Listeria monocytogenes, Genome Biol. 4 (2003) R2.

[13] J.J. van Soest, O.W. Stockhammer, A. Ordas, G.V. Bloemberg, H.P. Spaink, A.H. Meijer, Comparison of static immersion and intravenous injection systems for exposure of zebrafish embryos to the natural pathogen Edwardsiella tarda, Bmc Immunol. 12 (2011) 58.

[14] Z.F. Wu, W. Zhang, Y. Lu, C.P. Lu, Transcriptome profiling of zebrafish infected with Streptococcus suis, Microb. Pathog. 48 (2010) 178–187.

[15] Z. Kanwal, A. Zakrzewska, J. den Hertog, H.P. Spaink, M.J. Schaaf, A.H. Meijer, Defiwith immune27 resp2-477.[*(and)27yte-

[32] C. Legrand, C. Gilles, J.-M. Zahm, M. Polette, A.-C. Buisson, H. Kaplan, et al., Airway epithelial cell migration dynamics: MMP-9 role in cell–extracellular matrix remodeling, J. Cell Biol. 146 (1999) 517–529.
[33] H.E. Volkman, T. Pozos, J. Zheng, J.F. Rawls, L. Ramakrishnan, The Mycobacterium marinum RD1 locus promotes virulence and macrophage aggregation into tuberculous granulomas by enhancing induction of host matrix metalloproteinase 9 in proximal epithelial cells, J. Immunol. 182 (2009) 133, 19.
[34] B. Bauvois, New facets of matrix metalloproteinases MMP-2 and MMP-9 as cell surface transducers: outside-in signaling and relationship to tumor progression, Biochimica Biophysica Acta (BBA)-Reviews Cancer 1825 (2012) 29–36.
[35] C. Chetty, S.K. Vanamala, C.S. Gondi, D.H. Dinh, M. Gujrati, J.S. Rao, MMP-9 induces CD44 cleavage and CD44 mediated cell migration in glioblastoma xenograft cells, Cell. Signal. 24 (2012) 549–559.
[36] E. Vachon, R. Martin, J. Plumb, V. Kwok, R.W. Vandivier, M. Glogauer, et al., CD44 is a phagocytic receptor, Blood 107 (2006) 4149–4158.
[37] T. Xu, J. Zhao, D. Yin, Q. Zhao, B. Dong, High-throughput RNA sequencing reveals the effects of 2, 2′, 4, 4′-tetrabromodiphenyl ether on retina and bone development of zebrafish larvae, Bmc Genomics 16 (2015) 1.
[38] M. Chadzinska, P. Baginski, E. Kolaczkowska, H.F. Savelkoul, B. Lidy Verburg-van Kemenade, Expression profiles of matrix metalloproteinase 9 in teleost fish provide evidence for its active role in initiation and resolution of inflammation, Immunology 125 (2008) 601–610.
[39] Liu C-s, Y. Sun, M. Zhang, L. Sun, Identification and analysis of a Sciaenops ocellatus ISG15 homologue that is involved in host immune defense against bacterial infection, Fish. Shellfish Immun. 29 (2010) 167–174.
[40] E.S. Seilie, Matrix Metalloproteinase 13a: An early host protective factor during mycobacterial infection, 2015.
[41] R. Dahm, R. Geisler, Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species, Mar. Biotechnol. 8 (2006) 329–345.
[42] G.J. Lieschke, P.D. Currie, Animal models of human disease: zebrafish swim into view, Nat. Rev. Genet. 8 (2007) 353–367.