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Congenital asplenia due to a *tlx1* mutation reduces resistance to *Aeromonas hydrophila* infection in zebrafish

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ABSTRACT

It is documented that *tlx1*, an orphan homeobox gene, plays critical roles in the regulation of early spleen developmental in mammalian species. However, there is no direct evidence supporting the functions of *tlx1* in non-mammalian species, especially in fish. In this study, we demonstrated that *tlx1* is expressed in the splenic primordia as early as 52 hours post-fertilization (hpf) in zebrafish. A *tlx1*$^{-/-}$ homozygous mutant line was generated via CRISPR/Cas9 to elucidate the roles of *tlx1* in spleen development in zebrafish. In the *tlx1*$^{-/-}$ background, *tlx1*$^{-/-}$ cells persisted in the splenic primordia until 52 hpf but were no longer detectable after 53 hpf, suggesting perturbation of early spleen development. The zebrafish also exhibited congenital asplenia caused by the *tlx1* mutation. Asplenic zebrafish can survive and breed normally under standard laboratory conditions, but the survival rate of animals infected with *Aeromonas hydrophila* was significantly lower than that of wild-type (WT) zebrafish. In asplenic zebrafish, the mononuclear phagocyte system was partially impaired, as demonstrated by retarded *b7r* expression and reduced *ccr2* expression after injection with an inactivated *A. hydrophila* vaccine. Furthermore, the expression of *MHCII/IgM* was significantly reduced in the congenitally asplenic fish compared with that of the WT zebrafish. Taken together, our data suggest that *tlx1* is a crucial regulator of spleen development in fish, as it is in mammals. We have also provided a new perspective for studying the role of the spleen during pathogen challenge in fish.

1. Introduction

In mammalian species, the spleen is one the most important sites for the production of lymphocytes, which play central roles in immune responses [1–3]. In mice, several candidate transcription factors, including *nkx2-3*, *nkx3-2*, *pbx1*, *sox11*, *wt1*, and *tcf21*, expressed in the spleen primordium are essential for spleen development, and mice lacking the functions of these proteins exhibit spleen agenesis or hypoplasia accompanied by other organogenesis defects [4–9]. In contrast, *tlx1* (also known as *hox11*) is expressed in the trabeculae and capsule of the spleen in the developing mice embryo, and its mutation results in isolated asplenia without other abnormalities [10–14]. Based on these observations, *tlx1* is considered the earliest known genetic marker for cells committed to forming the spleen in mice. However, the study of *tlx1* in non-mammalian species has been limited to molecular cloning and mRNA expression analyses. Previous findings demonstrated that the presumptive chicken *tlx1* orthologue exhibits a similar expression pattern with that of mouse *tlx1* [15]. Interestingly, *tlx1* is not expressed in the developing spleen in *Xenopus*, suggesting it is not involved in spleen development in this genus [16]. In zebrafish, *tlx1* is detected in the pharyngeal arches and neurons by 24 hours post-fertilization (hpf) and asymmetrically on the left side of the developing anterior gut in a position corresponding to the putative splenic primordium by 5 days post-fertilization (dpf). Thus, *tlx1* has been considered as a marker of the early spleen in zebrafish [17]. Beyond the characterization of the expression patterns, there have been no reports that provided direct evidence for the functions of *tlx1* in the developing spleen in non-mammalian species, especially in fish. In addition, whether *tlx1* acts as a marker of the early spleen in zebrafish needs further scrutiny.

As one of the largest lymphoid and immunocompetent organs in fish, the spleen is enriched with several immune cells, including

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monocytes, lymphocytes, macrophages, and granulocytes, which participate in phagocytosis and antigen capture [18–21]. The spleen is also recognized as an essential immune organ involved in both innate and adaptive immunity in fish [22,23]. Thus, the spleen is considered a suitable organ for studying immune responses in fish. However, its role in disease resistance is not well understood in fish. It is well documented that surgical splenectomy or congenital asplenia increases susceptibility to infection by encapsulated bacterial in humans and rodents [24–27]. In contrast to mammals, rudimentary spleens appeared to regrow following surgical splenectomy in rainbow trout, and a reduction in spleen size had no significant effects on the relative survival rate after challenge with *Flavobacterium psychrophilum* compared with that of control fish [28]. Due to other technological limitations, research on the roles of the spleen in fish immunity have largely focused on its roles in host-pathogen interactions after pathogen infection, and information on the phenotypes of fish with congenital asplenia under pathogen challenge is scarce.

In recent years, zebrafish (*Danio rerio*) has become a popular model for basic research, and it is an accepted model system for studying infectious disease pathogenesis for the purpose of improving the health and welfare of humans and fish aquaculture [29–31]. Experimental infections using zebrafish have been conducted with various bacteria, including *Aeromona hydrophila* [32–34]. In the present study, we characterized the spatiotemporal expression of *tlx1* in detail at different developmental stages in zebrafish embryos using whole-mount *in situ* hybridization (WISH). We also validated the functions of *tlx1* by knocking it out using CRISPR/Cas9 technology. In addition, the immune response against this bacterial pathogen was investigated in both WT and *tlx1*$^{-/-}$ mutant zebrafish after *A. hydrophila* infection. To the best of our knowledge, this is the first study to functionally characterize *tlx1* in a non-mammalian vertebrate.

2. Materials and methods

2.1. Animals

The AB strain of zebrafish was used in this study. Zebrafish were maintained in flow-through aquaria at 28 ± 0.5 °C with a photoperiod of 14 h light and 10 h dark. Fish were fed twice daily with newly hatched brine shrimp (Brine Shrimp Direct). To inhibit pigmentation, the embryos were treated with 0.003% PTU (N-phenylthiourea) (Sigma, USA) after 24 hpf. The embryos were fixed in 4% PFA (paraformaldehyde) in 1 × PBS (phosphate-buffered saline) and stored at −20 °C in 100% methanol for further analysis. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Committee for Laboratory Animal Experimentation at Southwest University, China.

2.2. Whole-mount in situ hybridization

For the WISH analysis, the open reading frame (ORF) of *tlx1* (NM_170765) was amplified from zebrafish larva 48 hpf and subcloned into the pGEMT-easy vector (Promega, USA). Digoxigenin-labelled probes were generated, and *in situ* hybridization was performed as previously reported [35]. To confirm the results, the WISH experiments were repeated in triplicate. Representative images were obtained using a LEICA DM 3000 microscope (Leica, Germany).

2.3. Generation of tlx1$^{-/-}$ mutant lines and validation

To elucidate the functions of *tlx1* during early spleen development in zebrafish, CRISPR/Cas9 targeting sites in *tlx1* were designed according to the protocols in a previous report [36]. Briefly, the gRNA and Cas9 mRNA (gRNA 25–200 pg, Cas9 mRNA 300–500 pg) were co-injected into one-cell stage zebrafish embryos. Two days after injection, genomic DNA (gDNA) was isolated from 8 to 10 pooled larvae with normal morphology, and genomic fragments spanning the *tlx1* targeting site were amplified using a pair of gene-specific primers (*tlx1*-F1/R1) adjacent to the target site (Suppl. Fig. S1A). The target genomic region was amplified via limited cycles of PCR and subcloned into pMD19-T (Takara, Japan). The mutation was analysed by PCR or by sequencing. Subsequently, F0 mutant animals were raised to adulthood and outcrossed with WT fish to generate heterozygous F1 offspring. The heterozygous F1 fish were genotyped via a fin clip assay, and the individuals with frameshift or nonsense sequence alterations were selected. Male and female siblings of the F1 generation carrying the same mutation were mated to generate homozygous F2 mutant animals. Genotyping of the F2 generation was carried out via heteroduplex motility assays with polyacrylamide gel electrophoresis (PAGE), as previously reported [37]. To confirm the mutation at the mRNA level, we performed RT-PCR analysis of the *tlx1* mRNA by using a deletion-specific primer (*tlx1*-F2/R2). To assess the potential off-target effects, the web tool CHOPCHOP (https://chopchop.rc.fas.harvard.edu/index.php) was used to predict possible off-target sites based on the *tlx1* gRNA sequence.

2.4. Preparation of whole-mount abdominal organs

After euthanasia with 300 ng/ml MS-222, to prepare whole-mounts of abdominal organs, organs were carefully removed from WT and *tlx1*$^{-/-}$ mutants (10 fish per group) and fixed in 4% PFA for 5 min. Representative images were obtained using a Nikon SMZ800 stereoscope (Nikon, Japan).

2.5. Pathogenic A. hydrophila infection

A. hydrophila strains isolated from diseased crucian carp (*Carassius auratus*) were cultivated in our previous work. The strains were grown in LB medium for 14 h at 30 °C with shaking at 170 rpm. Bacterial cells were harvested via centrifugation at 5000 rpm for 5 min and then resuspended in PBS, and the final concentration was determined via a turbidity meter (Qiwei, China). The bacterial cells were then serially diluted in PBS for injection into zebrafish. WT and *tlx1*$^{-/-}$ zebrafish (6–8 mouths old) were fasted for 24 h prior to the experiments and for 24 h following injection, after which twice-daily feeding was resumed. Before the intraperitoneal (i.p.) injections, the zebrafish were anaesthetized with MS-222. Duplicate groups of adult WT and *tlx1*$^{-/-}$ zebrafish (8 fish per group) were injected intraperitoneally with 10 μl of the serial dilutions of *A. hydrophila*, which ranged from 1×10^4 to 1×10^8 CFU/ml. Control animals were injected with PBS. Mortality was recorded every 6 h after the bacterial challenge for 2 days. The survival rates of the *tlx1*$^{-/-}$ mutants and WT zebrafish were compared in each injection group.

To confirm that fish are not dying from endotoxin shock, bacteriological analyses were carried out on kidneys form dead fish using LB-agar plates. After incubation at 30 °C for 24 h, Single colonies were selected and grown in LB medium. The identification of the isolated bacteria was confirmed by sequencing the small subunit of 16S rRNA. The complete 16S rRNA gene was amplified using primers F27 and R1492. The sequence of isolated bacteria and *Aeromonas* reference sequences obtained from GenBank were aligned using Clustal W. The neighbor-joining method was used to construct the phylogenetic tree by MEGA5.0. The credibility of the branching was tested using bootstrap resampling with 1000 pseudo replicates.

2.6. Vaccination and sampling

A. hydrophila cells were inactivated via addition of 0.3% formalin to the culture, followed by incubation at 4 °C for 48 h with gentle agitation. Completely inactivated bacterial cells were washed thrice with

PBS. For the vaccination, triplicate groups of 40 WT and *tlx1*$^{-/-}$ adult zebrafish were injected with 10 µl of inactivated bacterial cells (1×10^8 CFU/ml). After euthanasia with 300 ng/ml MS-222, complete kidneys were sampled at 0, 2, and 6 h and 10 days following intraperitoneal injection, with duplicates of 10 fish in each treatment group. The samples were stored at −80 °C until RNA extraction.

2.7. Real-time PCR analysis

Total RNA was extracted using the RNAiso Plus kit (Takara, Japan). The reverse transcription was performed with the GoScript™ Reverse Transcription System (Promega, USA) following the manufacturer's instructions. All real-time PCRs were carried out in a CFX96 Real-Time PCR Detection System (Bio-Rad, USA) in 20 µl reactions containing 10 µl of SsoFast™ EvaGreen® SuperMix (Bio-Rad, USA), 1 µl of diluted cDNA or nuclease-free water as a negative control, 8 µl of nuclease-free water, and 0.5 µl of 10 µM stock solutions of each primer. The PCRs were initiated by denaturation at 95 °C for 30 s, followed by 40 amplification cycles of 95 °C for 5 s and 60 °C for 5 s. The melting curve for the PCR products was generated by heating from 65 to 95 °C in 0.5 °C increments with a 5 s dwell time, and a plate read was performed at each temperature. The relative expression levels of the target genes were evaluated using formula $R = 2^{-\Delta\Delta Ct}$ with *ef1α* as a reference gene to normalize the expression values. Three biological replicates were performed for each sample. The primer sequences used for the PCRs are listed in Supplemental Table 1.

2.8. Statistical analysis

Data are expressed as the mean ± SD. Student's *t*-test and one-way ANOVA followed by the Duncan post hoc test were performed to determine statistical significance at $P < 0.05$.

3. Results

3.1. Expression of tlx1 during embryonic development

The WISH analysis revealed that *tlx1* was mainly expressed in the pharyngeal arches and segmentally distributed in the hindbrain and cerebellum throughout embryonic development until 6 dpf (Fig. 1). Furthermore, the signal for the *tlx1* transcript was initially faint, but it could be clearly detected asymmetrically on the left side of the developing anterior gut as early as 52 hpf (Fig. 1B, b) (red arrowhead). The signal became progressively more prominent (Fig. 1B–F, b-f).

3.2. Establishment of tlx1 mutant zebrafish lines

CRISPR/Cas9 targeting sites were selected within the first exon of *tlx1*, immediately downstream of the translation initiation site (Fig. 2A). PCR amplification of genomic DNA isolated from pooled injected embryos indicated the high frequency of somatic mutation, and sequencing results confirmed that mutations correctly occurred at the target site (Suppl. Fig. S1B). Frameshifts due to a 26-bp deletion and an 8-bp insertion in the target site were identified in the F2 generation via Sanger sequencing of a homozygous *tlx1* mutant line, and these mutations resulted in the expression of a truncated *tlx1* protein (Fig. 2A and B). A heteroduplex motility assay showed that heterozygous *tlx1*$^{+/-}$ individuals possessed both heteroduplex and homoduplex amplicons, while the *tlx1*$^{-/-}$ and *tlx1*$^{+/+}$ individuals showed only single band profiles representing only homoduplex amplicons (Fig. 2C). RT-PCR analysis using deletion-specific primers showed no detection of *tlx1* mRNAs in the *tlx1*$^{-/-}$ mutants 72 hpf (Fig. 2D). An off-target site was predicted at chr18:30992580 in the zebrafish genome (Suppl. Fig. S2A). A PCR primer (*tlx1*-off-target-F/R) was designed to detect whether deletions or insertions existed in the possible off-target genomic fragment (Suppl. Fig. S2B). PCR amplification of genomic DNA showed that the resulting ~518 bp amplicons from WT and *tlx1*$^{-/-}$ zebrafish were identical, suggesting that no off-target effects occurred at chr18:30992580 (Suppl. Fig. S2C).

3.3. The fate of tlx1$^{-/-}$ cells on the left side of the anterior gut in zebrafish

Via WISH, *tlx1*$^{+/+}$ cells can be detected in the arches, hindbrain and cerebellum between 51 and 68 hpf (black arrowhead) and on the left side of the anterior gut after 52 hpf (red arrowhead) in WT fish (Fig. 3A–D, 3a-d). No discernible differences were observed in the distribution patterns of *tlx1*$^{+/+}$ and *tlx1*$^{-/-}$ cells between WT and *tlx1*$^{-/-}$ mutants in any of the above regions after 51 hpf except for on the left side of the anterior gut (Fig. 3E-H,3e-h). However, the signal from the *tlx1*$^{-/-}$ cells on the left side of the anterior gut persisted but decreased 52 hpf (Fig. 3F, f) and was no longer detectable after 53 hpf in the *tlx1*$^{-/-}$ mutants in contrast to the observations in WT animals (Fig. 3G–H, 3g-h).

3.4. Tlx1 mutation causes congenital asplenia in zebrafish

Whole-mount preparations of abdominal organs show the spleen located in the peritoneal cavity, and it appears as a fully developed dark red organ adjacent to the liver lobes in adult WT zebrafish. Analysis of *tlx1*$^{-/-}$ mutants revealed that the entire spleen was absent, suggesting that *tlx1* mutation causes congenital asplenia in zebrafish (Fig. 4).

3.5. Congenital asplenia reduces the survival rate after A. hydrophila challenge

As shown in Fig. 5A, both WT and *tlx1*$^{-/-}$ mutants died within 1 day post-injection (p.i.) when injected with *A. hydrophila* concentrations of 1×10^7 and 1×10^8 CFU/ml. WT fish that were intraperitoneally injected with 1×10^6 CFU/ml of *A. hydrophila* showed a mortality of rate of 75%, while the mortality rate of the *tlx1*$^{-/-}$ mutants was 87.5%. The mortality of the WT and *tlx1*$^{-/-}$ mutants decreased to 62.5% and 75% when the fish were injected with 1×10^5 CFU/ml of *A. hydrophila*. Furthermore, injection with 1×10^4 CFU/ml of *A. hydrophila* resulted in an increase in the mortality rate to 62.5% in the *tlx1*$^{-/-}$ mutants compared with 37.5% in the WT animals. Both WT and *tlx1*$^{-/-}$ fish injected with PBS as a negative control exhibited 100% survival.

External clinical signs of an experimental *Aeromonas* infection (distended visceral cavity and abdominal haemorrhages) could be observed in the dead fish after *A. hydrophila* challenge (Fig. 5B). To further confirm the infection, the identification of the isolated bacteria was confirmed by sequencing the small subunit of 16S rRNA gene. The results showed the 16S rRNA gene sequence from the isolated bacteria showed a homology of 99% from the same region belonging to the Genus *Aeromonas* by using the online BLAST search alignment tool (data not shown). The phylogenetic analysis revealed that the isolated bacteria was clustered into one clade with *Aeromonas hydrophila* (Fig. 5C).

3.6. Expression levels of immunity-related genes after vaccination

To further explore the mechanism by which the congenital asplenia caused by *tlx1*$^{-/-}$ mutation affected the immune system, the expression levels of several important immune-related genes (i.e., *b7r*, *ccr2*, *MHCII* and *IgM*) in the kidney were analysed. The expression of *b7r*, which was used as a marker of mononuclear phagocytes, was significantly reduced at 0 h in the *tlx1*$^{-/-}$ mutants compared with its level in WT animals. After vaccination, the *b7r* expression reached its highest level 2 h post vaccination (p.v.) in WT animals, while in *tlx1*$^{-/-}$ mutants, it showed a retarded response following vaccination and

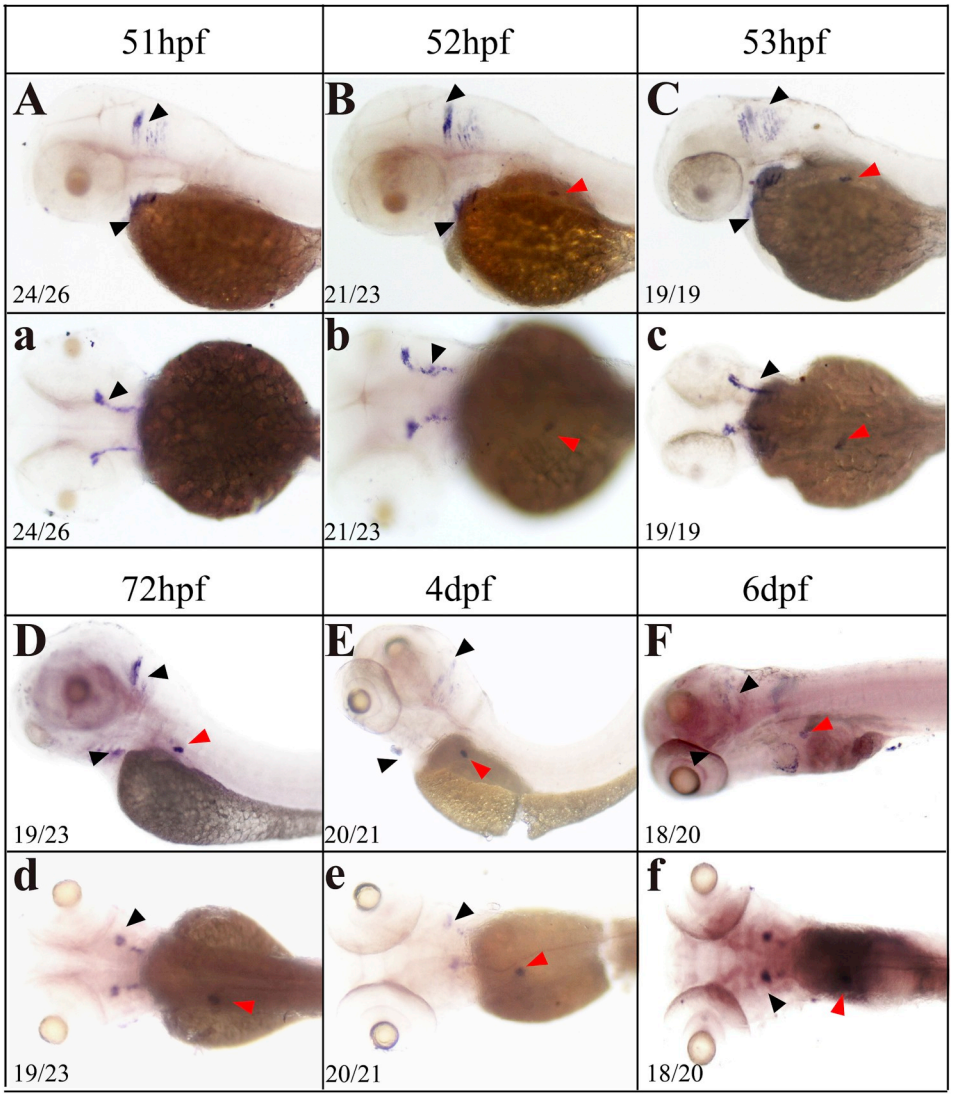

Fig. 1. Expression pattern of *tlx1* during embryonic development assessed via whole-mount *in situ* hybridization. The photographs in A-F and a-f show lateral and dorsal views with the anterior end to the left. The expression of *tlx1* was detected in the presumptive pharyngeal arches, neurons, hindbrain and cerebellum (black arrowhead) between 51 hpf and 4 dpf (A-E, a-e). At 52 hpf, *tlx1* was found to be expressed asymmetrically on the left side of the developing anterior gut (B, b) (red arrowhead). Neuronal expression of *tlx1* decreased overall, while its expression on the left side of the anterior gut (red arrowhead) increased after 52 hpf (B–F, b-f).

remained barely detectable throughout the different time points. *Ccr2* expression was also reduced in the *tlx1*$^{-/-}$ mutants, and it remained at relatively low expression levels after vaccination compared with its levels in WT animals. The expression of *MHCII* showed a decreased trend at 2 and 6 h p.v.in both WT and *tlx1*$^{-/-}$ mutants, but it was significantly upregulated 10 days post vaccination (d p.v.) and exhibited a higher expression level in WT animals compared with its level in the *tlx1*$^{-/-}$ mutants. *IgM* showed upregulation, and its expression reached the highest level 10 d p.v. in both WT animals and the *tlx1*$^{-/-}$ mutants, while its expression 10 d p.v. was significantly reduced in the *tlx1*$^{-/-}$ mutants compared with its expression in WT animals (Fig. 6).

4. Discussion

In this study, a homozygous mutation of the *tlx1* gene was successfully constructed in zebrafish via CRISPR/Cas9 gene editing. Subsequently, its roles in spleen development were extensively investigated. To our knowledge, this was the second animal model used to examine the effects of *tlx1* mutation, since *tlx1*-deficient mice were first generated more than 24 years ago. Our report is the first to present findings related to the roles of the spleen in disease resistance during pathogen challenge in an asplenic fish model.

It is well documented that *tlx1* is expressed in the splenic primordia during mouse embryonic development, and mice with *tlx1* mutations are asplenic [10,12–14]. Except for in *Xenopus*, its expression pattern is relatively well conserved in the developing spleen during embryonic development in various species, suggesting that its function in spleen development might be evolutionarily conserved. In our present study, *tlx1* expression was detected in the pharyngeal arches and neurons as well as on the left side of the developing anterior gut, which is in agreement with a previous report [17]. Furthermore, the zebrafish with the *tlx1* mutation exhibited asplenia, suggesting that *tlx1* is functionally conserved in spleen development. The conserved expression patterns and conserved functions in spleen development suggest that like its orthologue in mice, *tlx1* is expressed in the splenic primordia at the very early stages of spleen development in zebrafish. Due to the similar expression pattern to that observed in mice, *tlx1* was considered to act as a marker of early spleen in zebrafish in a previous study [17]. Thus, the splenic primordium in zebrafish was considered to appear 5 dpf during the ontogeny of the immune system [20]. Our results strongly support that *tlx1* can act as a marker of the early spleen in zebrafish by defining its functional roles during early spleen development. Furthermore, our results represent novel and important observations that the zebrafish splenic primordium begins to develop as early as 52 hpf,

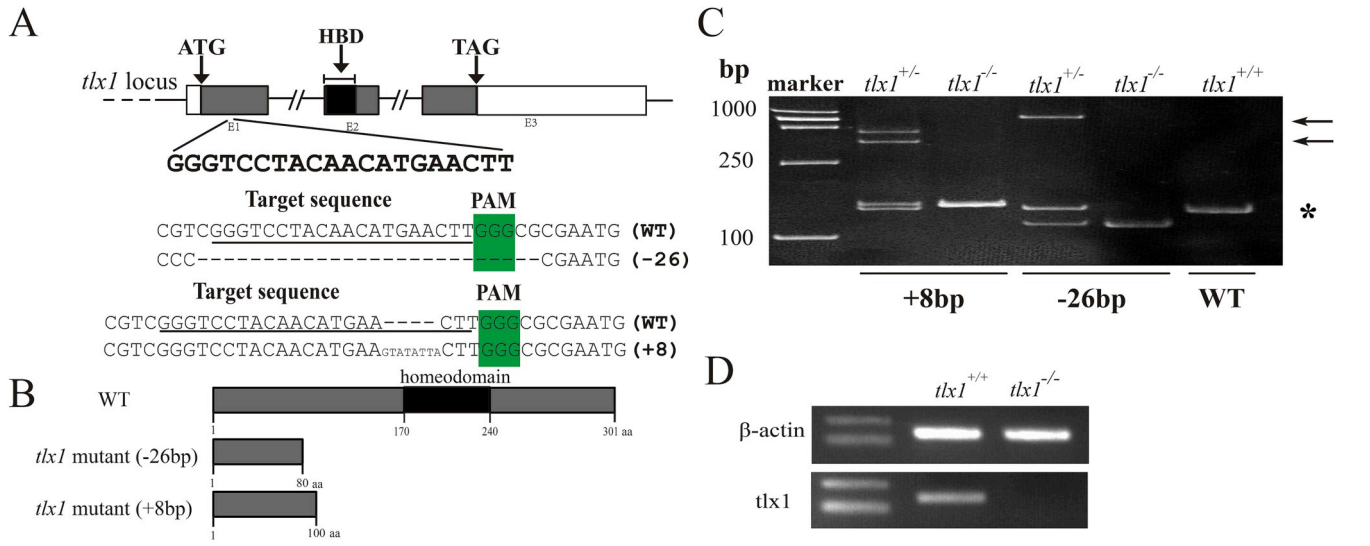

Fig. 2. Targeted disruption of the zebrafish *tlx1* gene and establishment of the *tlx1*$^{-/-}$ mutant line. CRISPR/Cas9 were used to target the first exon of the *tlx1* gene, and animals in the F2 generation bearing the *tlx1*$^{-/-}$ mutation were identified via Sanger sequencing. Frameshifts caused by a 26 bp deletion and an 8 bp insertion in the target site were identified in animals in the F2 generation via Sanger sequencing (A). The homeodomain (HBD) is marked by a black box (A). Schematic protein structures of *tlx1* from the WT and mutant zebrafish based on sequencing results (B). The genotype of the F2 animals was identified via a heteroduplex motility analysis of PCR amplicons. Homoduplexes, indicated by asterisks, were detected in the WT (*tlx1*$^{+/+}$) animals and homozygous mutant (*tlx1*$^{-/-}$) animals, while both homoduplexes and heteroduplexes, marked by arrows, were found in the heterozygous mutants (*tlx1*$^{+/-}$) (C). RT-PCR analysis of *tlx1* mRNA via deletion-specific primers in WT (*tlx1*$^{+/+}$) animals and homozygous mutants (*tlx1*$^{-/-}$) (D).

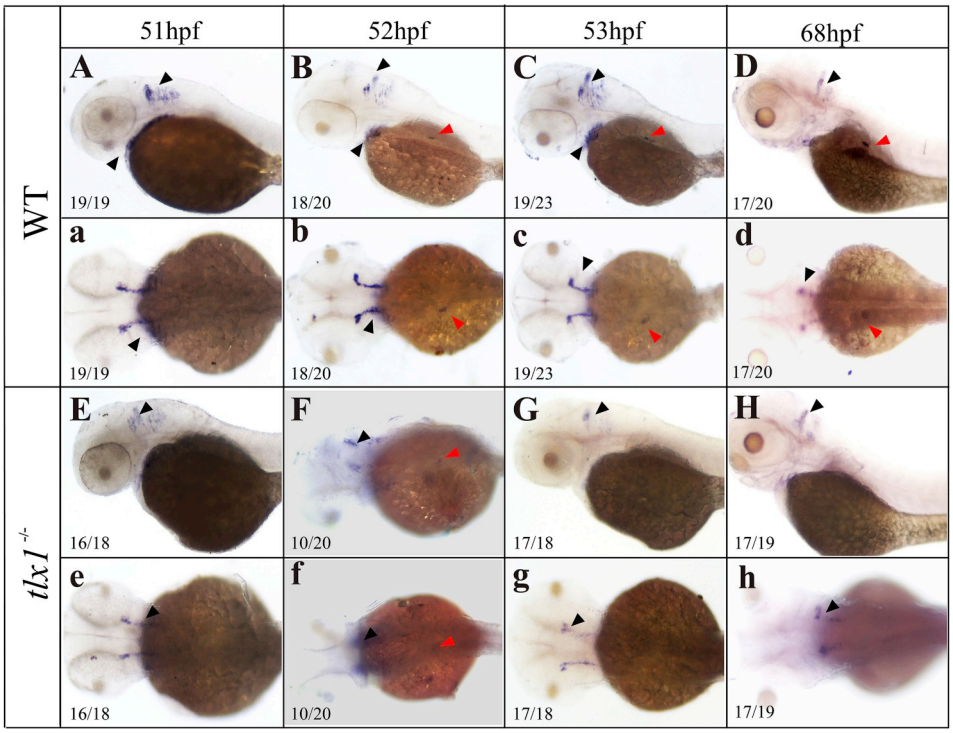

Fig. 3. *Tlx1* is essential for the cell fate of *tlx1*$^{-/-}$ cells on the left side of the anterior gut in zebrafish. The photographs in A-H and a-h show lateral and dorsal views with the anterior end to the left. *Tlx1*$^{+/+}$ cells were detected in the arches, hindbrain and cerebellum between 51 hpf and 68 hpf (black arrowhead) and on the left side of the developing anterior gut after 52 hpf (red arrowhead) in WT zebrafish (A-D, a-d). Like in WT zebrafish, *tlx1*$^{-/-}$ cells could be found in the arches, hindbrain and cerebellum of *tlx1*$^{-/-}$ homologous mutants (black arrowhead), while no positive signal from *tlx1*$^{-/-}$ cells was detected on the left side of the anterior gut after 53 hpf (E-H, e-h).

as indicated by the *tlx1* expression in the splenic primordium. A previous study revealed that the splenic primordium initially forms normally in *tlx1*$^{-/-}$ mice but then undergoes rapid atrophy after its initial development [10]. In present study, *tlx1*$^{-/-}$ cells persisted but then disappeared 52 hpf in the splenic primordia, and they underwent extensive removal after 53 hpf, suggesting that *tlx1* is not required to initiate spleen development but that it is essential for the cell fate decision in the splenic primordia during early spleen development in zebrafish.

Previous studies have been shown that surgically splenectomized or congenitally asplenic humans and rodents can live normal lives; however, splenectomy increases susceptibility to bacterial infections [25,38–40]. In contrast, spleen size reduction via surgical splenectomy in rainbow trout had no significant effect on the relative survival rate after challenge with *F. psychrophilum* [28]. Thus, we wondered whether congenital asplenia could affect disease resistance in zebrafish. In the

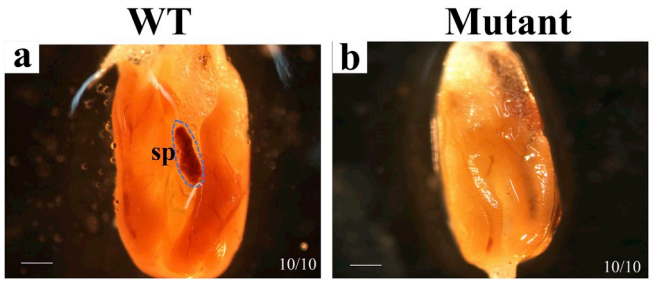

Fig. 4. Loss of *tlx1* causes asplenia in zebrafish. Comparative whole-mount preparations of abdominal organs showed lack of a spleen in *tlx1*$^{-/-}$ mutant (b) compared with WT (a).

present study, asplenic zebrafish could survive and breed normally under standard laboratory conditions (data not shown), indicating that the spleen is not an essential organ for fish survival, similar to the mammalian observations. However, after *A. hydrophila* challenge, the mortality rate of the asplenic zebrafish was substantially and significantly increased compared with that of the control, implying that congenital asplenia decreased disease resistance in zebrafish, probably at least partially due to immune system impairment.

To further compare the changes in immune responses in WT and asplenic zebrafish, the expression levels of several important immune-related genes were quantified after vaccination with inactivated *A. hydrophila*. The mononuclear phagocyte system (MPS), an important innate immune component that consists of monocytes, tissue macrophages and dendritic cells, has been characterized in zebrafish [41]. *B7r*, a marker of the MPS, was used to monitor the process of antigen uptake in the immune organs in previous studies [42]. In this study, the *b7r* levels were reduced, and it failed to respond to vaccination in the congenital asplenia mutants. Meanwhile, the expression level of *ccr2,* which is associated with the recruitment of monocytes, was significantly reduced in the congenitally asplenic zebrafish, suggesting that the MPS might be partially impaired. A previous study revealed that although the monocytes are produced by the bone marrow, undifferentiated monocytes reside in the spleen and splenic monocytes, rather than the bone marrow-derived monocytes, increase their motility and accumulate in injured tissue in response to injury [43]. Our study suggested that the fish spleen might function as a reservoir for mononuclear phagocytes, similarly to that in mammals. After pathogen infection, these mononuclear phagocytes exited the spleen and moved to the infected tissue, as indicated by upregulation of *b7r* in WT animals and loss of *b7r* expression in the congenitally asplenic zebrafish. In fish, antigen presenting cells ingest, process and present antigens for T cells via *MHCII*. IgM, the main component of systemic immunity, is the first antibody produced during the immune response, and it provides a crucial line of defence in the immune system. In this study, *MHCII* showed a reduced expression level in the congenitally asplenic mutants. Furthermore, the expression of *IgM* in the congenitally asplenic fish was significantly lower than that in WT fish after vaccination, suggesting that antigen processing and presentation/antibody secretion might be retarded due to the congenital asplenia. All of these results indicate that congenital asplenia reduces resistance to *A. hydrophila* infection in zebrafish. However, further analysis is likely to be required to fully understand the effects of congenital asplenia on immunity in fish.

In summary, using CRISPR/Cas9-generated *tlx1* mutations, the present study provides, for the first time, proof that *tlx1* is functionally conserved during spleen development. *Tlx1* mutation resulted in congenital asplenia in zebrafish. In addition, we demonstrated that the splenic primordium begins to develop as early as 52 hpf in zebrafish. Moreover, the immune system is partially impaired in congenitally asplenic zebrafish. Our data reinforce the importance of the fish spleen in resistance to pathogen infection and provide a new perspective for studying the role of the spleen in fish immunity.

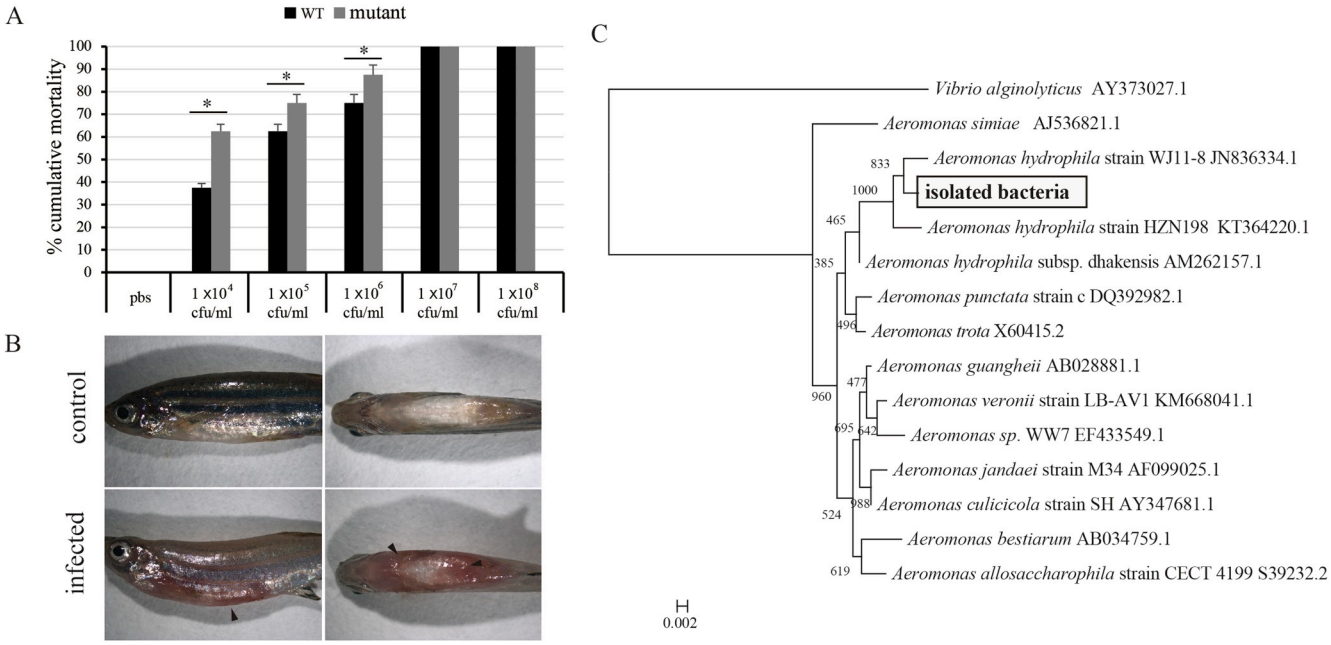

Fig. 5. Congenital asplenia reduces the survival rate after *A. hydrophila* challenge in zebrafish. Average cumulative percent mortality in adult zebrafish inoculated i.p. with different doses of viable bacteria for 2 d. Fish injected with PBS were used as negative controls (A). Aspect of fish showing the external clinical signs of an experimental *Aeromonas* infection mainly characterized by distended visceral cavity and abdominal haemorrhages (B). Phylogenetic tree showing isolated bacteria (in the box) was clustered into one clade with *Aeromonas hydrophila* (C). The (*) symbol indicates significant differences ($p < 0.05$).

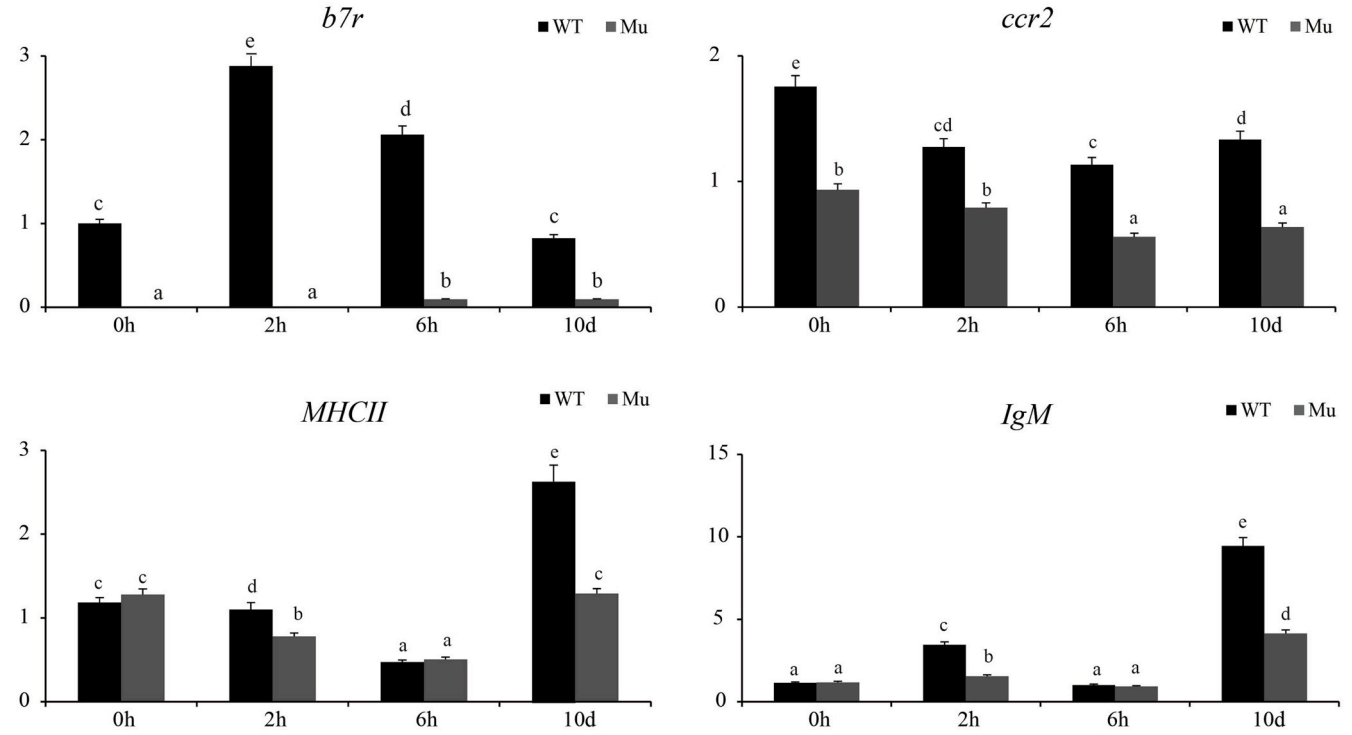

Fig. 6. Expression profiles of immune-related genes after vaccination in whole kidneys. The expression levels of each gene were normalized to the *ef1α* mRNA level. WT animals and *tlx1*$^{-/-}$ mutants sampled at 0 h were used as controls. The data were normalized to the expression level of WT-0h. The data are reported as the mean ± SEM. Different letters (a, b, c, etc.) indicate significant differences ($p < 0.05$).

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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