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Tilapia hepcidin (TH)2-3 as a transgene in transgenic fish enhances resistance to *Vibrio vulnificus* infection and causes variations in immune-related genes after infection by different bacterial species

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ABSTRACT

Hepcidin is an antimicrobial peptide (AMP) secreted by the liver during inflammation that plays a central role in mammalian iron homeostasis. But the function of hepcidin in fish is still not completely understood. We recently described three different hepcidins (named tilapia hepcidin (TH)1-5, TH2-2, and TH2-3) from tilapia *Oreochromis mossambicus*, the cDNA sequences were determined, the predicted peptides were synthesized, and the TH2-3 peptide showed antimicrobial activity against several bacteria. We hypothesized that TH2-3 may have a biological function like an AMP in fishes and can be used as a transgene to boost resistance against bacterial infection. To examine the antimicrobial effects of TH2-3, we produced and engineered the overexpression of TH2-3 in zebrafish (*Danio rerio*) and the convict cichlid (*Archocentrus nigrofasciatus*). The microinjected plasmid also contained a green fluorescent protein (GFP) which was used as an indicator to trace germline transmission. In vivo, transgenic TH2-3 fish (of the F3 generation) were challenged with *Vibrio vulnificus* (204) and *Streptococcus agalactiae* (SA). Results showed significant clearance of bacterial numbers of *V. vulnificus* (204) but not of *S. agalactiae* in transgenic TH2-3 fish. A gene expression study using a real-time RT-PCR revealed that transgenic TH2-3 zebrafish showed increased endogenous expressions of Myd88, tumor necrosis factor-α, and TRAM1 in vivo. After transgenic TH2-3 zebrafish were infected with *V. vulnificus* (204), interleukin (IL)-10, IL-26, lysozyme, toll-like receptor (TLR)-4a, and Myd88 were upregulated, but IL-1β (at 12–24 h) and IL-15 (at 1–12 h) were downregulated post-infection. After transgenic TH2-3 zebrafish were infected with *S. agalactiae*, IL-1β (at 1–24 h), IL-15 (at 6 h), IL-22 (at 1–6 h), and TLR3 (at 1–24 h) were downregulated, but TLR4a (at 6–12 h) and c3b (at 12 h) were upregulated post-infection. Our findings identify the TH2-3 transgene in transgenic fish as an active component of the host response to bacterial pathogens. These results suggest that using TH2-3 as a transgene in zebrafish can effectively inhibit bacterial growth, specifically the *V. vulnificus* (204) strain for up to 24 h.

1. Introduction

Hepcidin, a small peptide hormone made in the liver that is also called liver-expressed antimicrobial peptide (AMP) (LEAP)-1, was originally identified by two research groups [1,2]. It is a conserved 25-amino acid peptide produced in the liver and detectable in blood and urine in humans. In addition to its direct antimicrobial activity in vitro, human hepcidin exerts antibacterial and antifungal activities at concentrations of 10–30 μM [1,2]. Synthetic fish hepcidin peptides were also shown to be bactericidal and fungicidal [3,4]. Those above-described results suggest that hepcidin may play an important role in host defense against infections, and it may be possible to develop it as an anti-infective drug. Recently, hepcidin was demonstrated to regulate iron homeostasis and likely acts on iron metabolism by limiting intestinal iron absorption and release from macrophages [5], which suggests that hepcidin is instrumental in regulating normal homeostasis of iron levels in humans. Overexpression of hepcidin from hepatic adenomas was found in a patient with refractory anemia, and mutations in the hepcidin gene were present in severe cases of juvenile hemochromatosis (JH) [6,7].

Hepcidin genes and peptides were identified in a number of mammalian, amphibian, and fish species [4,8–13]. The structures and sequences of hepcidin genes are conserved between mammals and fish. Our previous work demonstrated that three hepcidin-like AMPs (named tilapia hepcidin (TH)1-5, TH2-2, and TH2-3) exist in

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tilapia (*Oreochromis mossambicus*). The minimal inhibitory concentration (MIC) results suggested that tilapia-synthesized TH1-5 and TH2-3 peptides possess antimicrobial activity at 50–100 μg/ml against gram-negative bacteria [4]. In another report, synthetic bass hepcidin was found to inhibit gram-negative bacteria and fungi at concentrations of 10–100 μM [3]. Weak antimicrobial activities may be due to the disulfide bond not forming when the TH peptides are synthesized. Most AMPs are cationic peptides, and the disulfide bond plays an important role in the antimicrobial activity by supporting the tertiary structure [14].

In fact, it has long been known that multiple mechanisms contribute to the development of gene functions in the setting of transgenic animal technology. The technology for producing transgenic animals exists for a variety of vertebrate and invertebrate species [15]. In a transgenic model, overexpression of hepcidin induced iron-restricted anemia similar to anemia associated with inflammation [16]. Another report indicated severe iron-deficient anemia in transgenic mice expressing liver hepcidin [17]. Thus, embryonic hepcidin transgene expression decreasing transferring receptor 1 messenger (m)RNA levels in mouse placenta suggests that hepcidin's action on the placenta is mostly through transcriptional downregulation of the iron uptake machinery [18]. However, in contrast to the severe iron-deficient anemia characterized in hepcidin 1 transgenic mice, hepcidin 2 transgenic mice develop normally similarly to non-transgenic mice [19]. Taken together, these data from a transgenic mouse model provide important insights into anemia associated with inflammation. But these animal models still do not elucidate the specific role of hepcidin in fish, and an open question about whether fish hepcidin has any antimicrobial activity in vivo remains. The relation of hepcidin to the immune response comes from a study of the regulation of hepcidin gene expression following inflammatory stimuli in transgenic fish.

The molecular and biological functions of fish hepcidin mostly remain unknown. The antimicrobial activity of fish hepcidin may lend itself to potential applications in aquaculture species against bacterial and viral infections [20]. Herein, we describe the generation of a muscle-specific mylz2 promoter-driven TH2-3 gene in transgenic zebrafish (*Danio rerio*) and convict cichlid (*Archocentrus nigrofasciatus*) that conferred resistance to bacterial infections. To assess whether this transgenic TH2-3 zebrafish model faithfully reproduces all of the features of the immune responses after a bacterial infection, we investigated the impact of TH2-3 overexpression on muscle after a bacterial infection compared to wild-type (WT) groups after a bacterial infection by qualitative reverse-transcription polymerase chain reaction (qRT-PCR). We inferred that TH2-3 may induce immune-related gene expressions, resulting in significant clearance of bacterial numbers of *Vibrio vulnificus* but not *Streptococcus agalactiae* in transgenic TH2-3 fish. Our results attempt to shed light on fish hepcidin's specific functions for future applications.

2. Materials and methods

2.1. Generation of muscle-specific mylz2 promoter-driven TH2-3 transgenic fish

A 273-base pair (bp) fragment of TH2-3 was amplified from a tilapia complementary (c)DNA library using the forward (5′-cta cgaattccgccaccatgaagacgttcagtgttgcagttgc-3′) and reverse primers (5′-ccccccgggtcaatgatgatgatgatgatggaacctgcagcagaagccgcag-3′). The product was gel-purified and subcloned into a pTLR cloning vector using EcoRI and XmaI restriction sites [21]. A fragment of the BGH polyadenylation sequence (pA) was amplified from the pcDNA3.1 vector (Invitrogen, Madison, WI, USA) using the primers, 5′-tgacccgggctgtgccttctagttgccagccat-3′ and 5′-gtggcggccgcccatagagcccaccgcatccccagc-3′. The product was gel-purified and subcloned into the pTLR cloning vector after TH2-3 using NotI and XmaI restriction sites. The mylz2 promoter fragment was amplified from the pm2.5K-DsRed vector [21] using the primers 5′-cttctcgagatgctgtgaagtattctctacttatc-3′, 5′-ggcggctagcgtagtgtcctgtacttgaggggct-3′, 5′-gtataagcttatgctgtgaagtattctctacttatc-3′, and 5′-cgaattcgtagtgtcctgtacttgaggggct-3′. The two fragments were gel-purified and subcloned into the pTLR cloning vector (which already possessed the TH2-3 and BGH pA sequences) by XhoI and NheI or HindIII and EcoRI restriction sites, respectively. A 702-bp fragment of enhanced green fluorescent protein (EGFP) was amplified from the pEGFP-N1 vector (Clontech Laboratories, Mountain View, CA, USA) using the forward (5′-ctacgctagccgccaccatggtgagcaagggcg-3′) and reverse primers (5′-agtggatccagagtgatcccggcggcggtcacg-3′). The product was gel-purified and subcloned into the NheI and BamHI restriction sites in the pTLR cloning vector (which already possessed the mylz2 promoter, TH2-3, and BGH pA sequences). A 213-bp fragment of SV40 early mRNA polyadenylation signal (SV40 pA) was amplified from the pEGFP-N1 vector using the forward (5′-tctggatccactctagatcataatcagccatac-3′) and reverse primers (5′-gcataagcttatacattgatgagtttggacaaacc-3′). The product was gel-purified and subcloned into the HindIII and BamHI restriction sites in the pTLR cloning vector (which already possessed the mylz2 promoter, TH2-3, EGFP, and BGH pA sequences), and the final constructed plasmid was named the pTLR-Mylz-EGFP-Mylz-TH2-3 vector. DNA sequence analysis confirmed that every step of the construct was the correct sequence. The L200- and R150-flanking region was a transposon arm for transposon excision events (Fig. 1). We introduced two polyA signals (BGH pA and SV40 pA) into the expression vector because the pEGFP-N1 vector contained the enhanced green fluorescent protein (EGFP) gene ligated with an SV40 early mRNA polyadenylation signal. The TH2-3 ligated with BGH pA followed sequences constructed with the pcDNA3.1 vector.

Plasmid DNA of pTLR-Mylz-EGFP-Mylz-TH2-3 (Fig. 1) and transposase mRNA were injected into the one-cell stage of ~1000 zebrafish or convict cichlid eggs following our previous report [21]; after injection, the eggs were placed in a 28 °C incubator. The whole body of zebrafish or convict cichlid was examined for the presence of green fluorescent color expression by fluorescence microscopy using an FITC filter every 120 min (IX71; Olympus, Tokyo, Japan). We obtained several transgenic zebrafish and convict cichlids, and chose only one transgenic zebrafish or convict cichlid to mate with a wild-type (WT) zebrafish or convict cichlid, respectively. To obtain sufficient numbers of transgenic zebrafish and convict cichlid for bacterial challenge and subsequent studies, we followed the mating, breeding, and propagation procedures in our previous report [21].

2.2. Bacterial challenge analysis in transgenic fish

The F3 transgenic zebrafish (about 4 cm in body length) and convict cichlid (about 6 cm in body length) were placed communally in 500-L aquaria for the bacterial challenge. *V. vulnificus* (204; from Dr. Chun-Yao Chen, Department of Life Science, Tzu Chi University, Hualien, Taiwan) was cultured in trypticase soy broth and agar at 28 °C. *S. agalactiae* (SA; from Dr. Stone S.-C. Chen, Department of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan) was cultivated in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) overnight at 28 °C with rotary shaking (150 rev/min). Stock cultures were kept at −70 °C in 25% glycerol. Ten microliters of *V. vulnificus* (strain 204; at 1×10^6 colony-forming units (CFU)/ml) or *S. agalactiae* (strain SA; at 1×10^7 CFU/ml) was injected into the caudal peduncle of transgenic zebrafish and WT zebrafish. The same bacterial number

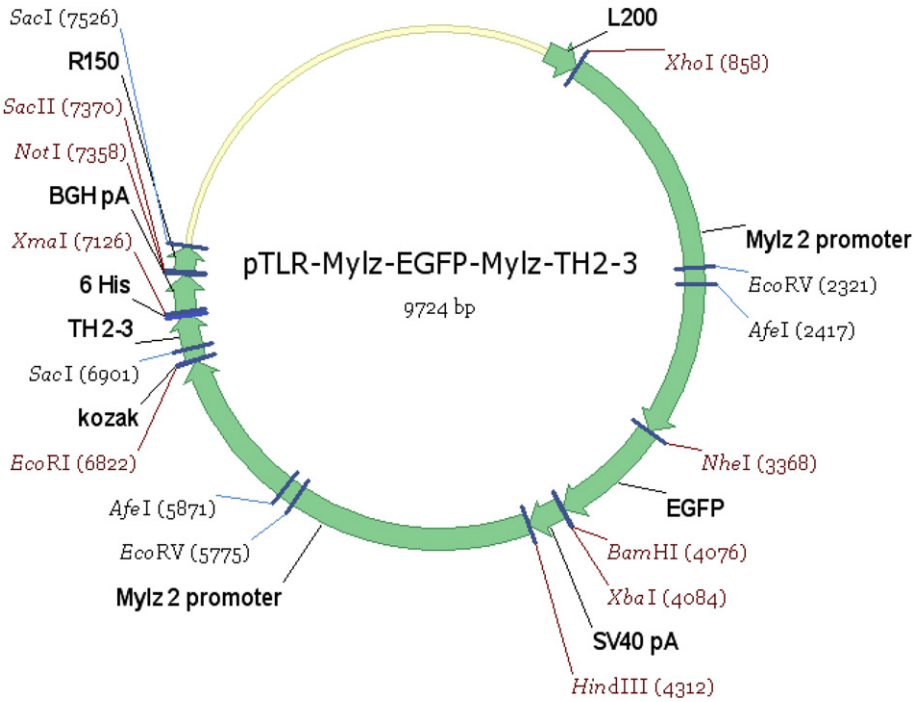

Fig. 1. Schematic illustration of tilapia hepcidin (TH)2-3 expression cassettes. The expression vector was reconstructed from the pTLR cloning vector [21]. pTLR-Mylz-EGFP-Mylz-TH2-3, which contains an Mylz2 promoter fused to the EGFP reporter gene, was used in this vector. The Mylz2 promoter fused to the TH2-3 gene was also used in this vector. The positions of the Kozak (The Kozak consensus sequence plays a major role in initiating the translation process), 6 His (for ease of protein purification a polyhistidine tail, -HHHHHH, was fused onto the C-terminus), and polyadenylation site are shown. The restriction enzyme sites are shown in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

for bacterial challenge was also injected into transgenic and WT convict cichlid. We cut a piece of the tail to the caudal peduncle of fish and placed it into culture buffer (trypticase soy broth or BHI broth) for 60 min at 37 °C. The piece of each tail to the caudal peduncle of fish was the same size, and it was macerated using clippers. The solution was serially diluted and transferred to TSB plates for different times for culture at 37 °C. Bacterial colonies were incubated and counted at 6, 12, and 24 h following our previous report with no modification [21]. Each trial included 20 fish for each experimental group. Statistical analysis used a *t*-test to compare between two groups. Multiple group comparisons were examined using analysis of variance (ANOVA) in the SPSS software (SPSS, Chicago, IL, USA). Differences were defined as significant at $p < 0.05$ and <0.01.

2.3. Detection of immune-related gene expressions by real-time polymerase chain reaction (PCR)

Total RNA was isolated from the whole body of transgenic TH2-3 zebrafish and WT zebrafish following our previous publication [21]. In addition, RNA, isolated from transgenic TH2-3 zebrafish and WT zebrafish, was collected after injecting bacteria (204 and SA). Levels of mRNA expressions of IL-1β, -10, -15, -21, -22, and -26, complement component c3b, lysozyme, TLR1, -3, and -4a, TRAM1, Myd88, nuclear factor (NF)-κB activating protein-like, TNF-α, and elongation factor 1-α were determined by a quantitative (q)PCR. Elongation factor 1-α was used as a reference normalizer gene. The specific PCR primers used are shown in Table 1. The PCR was performed using SYBR Green PCR reagents and an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). Five fish were examined for all conditions, and the PCR was initiated after activation of the AmpliTaq Gold enzyme (Applied Biosystems) in a reaction mixture by heating for 10 min at 95 °C. All genes were amplified by 40 cycles of 15 s at 95 °C followed by 60 s at 60 °C. Statistical analysis used a *t*-test to compare between groups. Multiple group comparisons were examined using ANOVA in the SPSS software. Differences were defined as significant at $p < 0.05$ and <0.01.

3. Results

3.1. Generation of mylz2-TH2-3 transgenic fish

A schematic of the construction is given in Fig. 1. After microinjecting the plasmid with transposase [21], 20 independent transgenic zebrafish founders (F0) and four independent transgenic convict cichlid founders (F0) were obtained. We chose one transgenic fish to mate with WT fish to produce offspring that carried the GFP gene and TH2-3 fragment in the genome. Expression of TH2-3 mRNA in transgenic fish was detected by RT-PCR (Supplementary Fig. 1). Fig. 2a shows the 14-day-old transgenic zebrafish line that contained green fluorescent color expression; these were raised to the F3 generation under microscopic observation. However, expression levels showed the strongest expression pattern in the convict cichlid F3 generation, and one transgenic convict cichlid (Fig. 2b) line was selected for further experiments.

3.2. Suppression of V. vulnificus but not S. agalactiae growth in transgenic TH2-3 fish

To determine to what extent bacterial loading was inhibited by transgenic TH2-3 fish, we injected *V. vulnificus* (204) and *S. agalactiae* (SA) into the caudal peduncle of WT fish and transgenic TH2-3 fish, and the consequent bacterial numbers were determined. Numbers of *V. vulnificus* were counted in the caudal peduncle of transgenic and WT fish at 6, 12, and 24 h after an injection as shown

Table 1
Primer sequences, and gene names and functions listed in this paper.

Gene name	PCR primers	Gene function
Elongation factor 1α (EF-1a)	AACAGCTGATCGTTGGAGTCAA TTGATGTATGCGCTGACTTCCT	This gene encodes an isoform of the alpha subunit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome
Toll-like receptor-1 (TLR1)	CAGAGCGAATGGTGCCACTAT GTGGCAGAGGCTCCAGAAGA	which recognizes triacyl lipopeptides
Toll-like receptor-3 (TLR3)	TGGAGCATCACAGGGATAAAGA TGATGCCCATGCCTGTAAGA	which recognizes single-stranded (ss) RNA virus, double-stranded (ds) RNA virus, and respiratory syncytial virus
Lysozyme (Lysozyme)	GATTTGAGGGATTCTCCATTGG CCGTAGTCCTTCCCCGTATCA	which catalyzes the hydrolysis of certain mucopolysaccharides of bacterial cell walls
Complement component c3b (C3b)	CGTCTCCGTACACCATCCATT GGCGTCTCATCAGGATTTGTTAC	an acute-phase protein
Interleukin-15 (IL 15)	ATGTCATTGGAACTCAGAGGTTTG CTGTTCTGGATGTCCTGCTTGA	which stimulates growth of intestinal epithelium, T cells, and NK cells
Interleukin-21 (IL 21)	AATCATTCATCGTGGACAGTGTGT AACGTTCGGCTGTTGACCAT	which induces proliferation of B, T, and NK cells
Interleukin-22 (IL 22)	CATCGAGGAACAACGGTGTACA CACGAGCACAGCAAAGCAAT	a proinflammatory cytokine
Interleukin-26 (IL 26)	AATCCGGCTGCCTATGATCA TTTCCCCAATATCCGATGGA	which imparts mucosal and cutaneous immunity
Nuclear factor-κB (NF-κB)	AGAGAGCGCTTGCGTCCTT TTGCCTTTGGTTTTTCGGTAA	a ubiquitous transcription factor
Translocation associated membrane protein 1 (TRAM1)	AGAAGGCCAAGAAAGAAGACATTC CCCAACCGGTTCAGATTCAG	which influences glycosylation and is stimulatory or required for the translocation of secretory proteins
Tumor necrosis factor-α (TNF-α)	AAGGAGAGTTGCCTTTACCG ATTGCCCTGGGTCTTATGG	a multifunctional proinflammatory cytokine
Interleukin-1β (IL-1β)	TGGACTTCGCAGCACAAAATG CACTTCACGCTCTTGGATGA	a proinflammatory cytokine
Interleukin-10 (IL 10)	TCACGTCATGAACGAGATCC CCTCTTGCATTTCACCATATCC	a potent suppressant of macrophage functions
Toll-like receptor-4a (TLR4-a)	TGTCAAGATGCCACATCAGA TCCACAAGAACAAGCCTTTG	which recognizes LPS, mannan, and glycoinositolphospholipids
Myeloid differentiation primary response gene 88 (Myd88)	TCCGAAAGAAACTGGGTCTG TCGTCATCTAAAATTTCTTTGAGC	which plays a central role in signaling through most TLRs as well as signaling mediated through receptors for IL-1 and IL-18

in Fig. 3a and c. Transgenic TH2-3 fish showed a statistically significant reduction in the numbers of bacterial cells 24 h after an injection of *V. vulnificus* in both the zebrafish and convict cichlid. Similar results were not observed for *S. agalactiae*-injected transgenic fish; the bacterial number increased in transgenic fish and WT fish at 6–24 h in the zebrafish and convict cichlid (Fig. 3b and d).

3.3. Different immune-related gene expressions in transgenic TH2-3 zebrafish with V. vulnificus and S. agalactiae infection

Transgenic TH2-3 fish showed decreased bacterial survival after injection with *V. vulnificus* but no significant decrease in bacterial survival was found with *S. agalactiae*. We next asked whether the

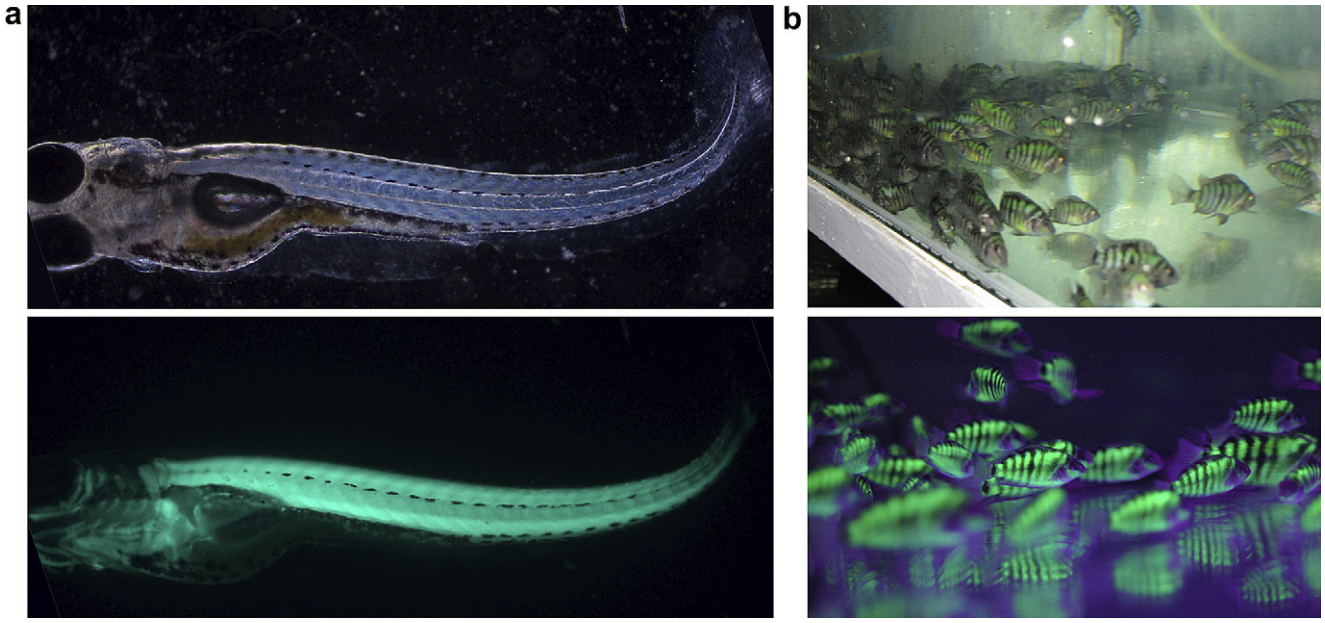

Fig. 2. Transgenic tilapia hepcidin (TH)2-3 (a) 14-day-old zebrafish (*Danio rerio*) containing green fluorescent color expression were retained and raised to the F3 generation under microscopic observation. A bright-field photograph and fluorescence image of the same zebrafish. All photographs were taken with a 45× lens on an Olympus IX70 microscope. (b) Bright-field and fluorescence images of the convict cichlid (*Archocentrus nigrofasciatus*) (F3 generation). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

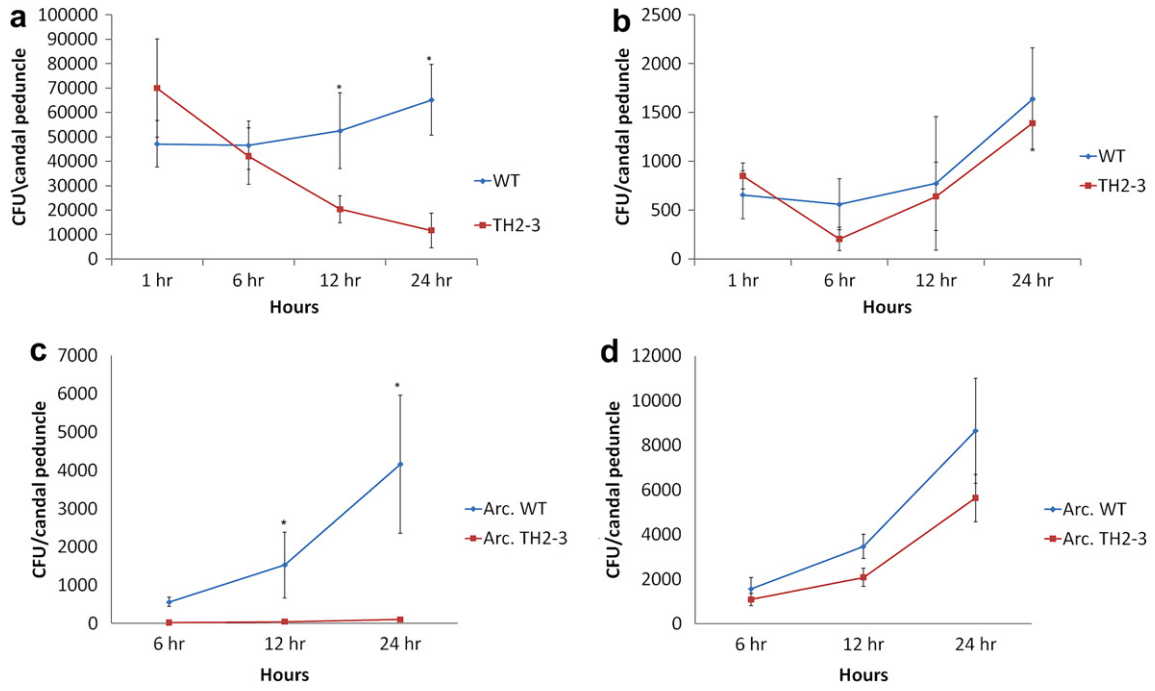

Fig. 3. In vivo bactericidal properties of transgenic tilapia hepcidin (TH)2-3 zebrafish (*Danio rerio*) injected with (a) *Vibrio vulnificus* (204) and (b) *Streptococcus agalactiae* (SA) or transgenic TH2-3 convict cichlid (*Archocentrus nigrofasciatus*) injected with (c) *V. vulnificus* (204) and (d) *S. agalactiae* (SA) compared to non-transgenic fish (wild-type; WT). Colony counts are shown as the mean and standard error of the mean. Values with an asterisk significantly differ ($*p < 0.05$).

two kinds of bacterial infection could stimulate expressions of immune-related genes in vivo in WT and transgenic TH2-3 zebrafish, and if those genes were transcriptionally regulated. We performed real-time RT-PCR analysis of transgenic TH2-3 zebrafish, WT zebrafish, WT zebrafish injected with *V. vulnificus*, WT zebrafish injected with *S. agalactiae*, transgenic TH2-3 zebrafish injected with *V. vulnificus*, and transgenic TH2-3 zebrafish injected with *S. agalactiae*. Results were normalized to elongation factor 1-α. We found that WT zebrafish produced 4.00-, 3.33-, 2.59-, 2.94-, and 1.90-fold significantly higher mRNA expressions of IL-21, IL-22, IL-26, TLR1, and TLR3 compared to transgenic TH2-3 zebrafish (Fig. 4). IL-10 and IL-15 were also expressed at higher levels in WT than in transgenic fish. TRAM1, TNF-α, and Myd88 were expressed at lower levels in WT than in transgenic fish (Fig. 4). The results suggest that the TH2-3 transgene suppressed the overexpression of IL-10, IL-15, IL-21, IL-22, and IL-26 immune-related cytokine molecules compared to WT zebrafish when the fish had received no bacterial injection. Comparing the levels in *Vibrio*-infected transgenic and WT fish showed that lysozyme, Myd88, TNF-α, IL-10, IL-21, IL-26, TLR4a, and TRAM1 were all upregulated, IL22, IL-1b, and IL15 were all downregulated, and the others were unchanged. Comparing the levels in *S. agalactiae*-infected transgenic and *S. agalactiae*-infected WT fish showed that TLR4a was upregulated, TLR3 and IL-1b were downregulated, and the others were unchanged.

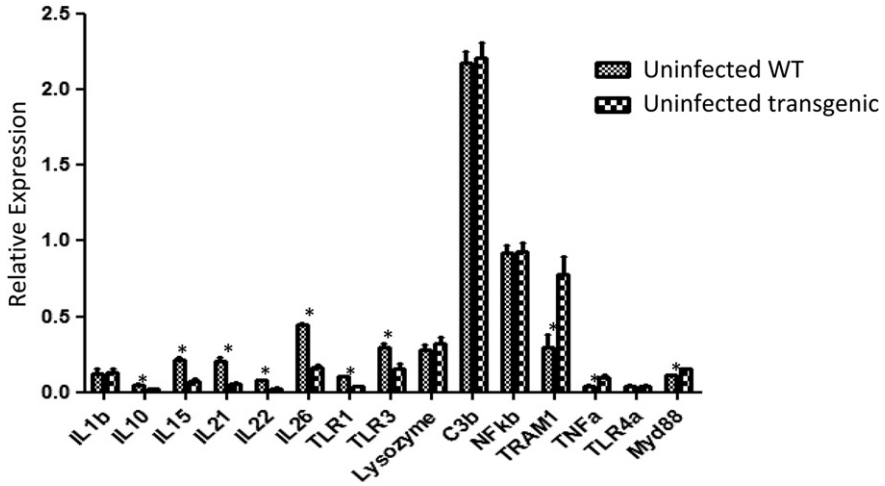

Fig. 4. Quantitative PCR analysis of cytokine gene expressions with no bacterial infection in transgenic and non-transgenic zebrafish (*Danio rerio*). Expressions of immune-related genes of interleukin (IL)-1β (IL1b), IL-10 (IL10), IL-15 (IL15), IL-21 (IL21), IL-22 (IL22), IL-26 (IL26), complement component c3b (C3b), lysozyme, toll-like receptor (TLR)1, TLR3, TLR4a, TRAM1, Myd88, nuclear factor (NF)-κB activating protein-like (NFkb), and tumor necrosis factor (TNF)-α were examined. These results are representative of five independent experiments, and significance was set at (*) $p < 0.01$.

At 12 and 24 h after a systemic infection by an injection of *V. vulnificus* (204), IL-1β had strongly increased in WT zebrafish compared to transgenic TH2-3 zebrafish, and a time-dependent increasing effect was shown (Fig. 5). In contrast, transgenic TH2-3 zebrafish showed significant downregulation of IL-22 gene expression at 1–12 h after injection of *V. vulnificus*, while other genes showed no significant differences (Fig. 5). Expressions of lysozyme, Myd88, and TRAM1 progressively decreased after the *V. vulnificus* (204) injection in WT zebrafish at 1–24 h, but significant differences in high expression levels were shown in transgenic TH2-3 zebrafish. However, high expression levels of IL-10, IL-26, TLR4a, and TNF-α were seen at 1, 6, 12, and 24 h after the bacterial injection in transgenic TH2-3 fish compared to WT fish; at 1 and 6 h for IL-21; and at 1 h for TLR3 (Fig. 5). These results suggest that the TH2-3 transgene inhibited proinflammatory cytokine (IL-1β and IL-22) expressions compared to WT zebrafish after a *V. vulnificus* (204) injection.

Different bacterial infections are recognized by different TLRs, and activation of different signaling pathways leads to transcriptional activation of inflammatory response genes. To determine whether the transgenic TH2-3 zebrafish response of immune-related genes differed with a *V. vulnificus* (204) injection, we performed a qRT-PCR analysis after the transgenic TH2-3 zebrafish and WT zebrafish were injected with *S. agalactiae* (SA) (Fig. 6). Upon exposure to this gram-positive pathogen, expressions of cytokines in WT zebrafish showed significant inductions: IL-22 gene expression at 1 and 6 h compared to transgenic TH2-3 zebrafish; and at 1, 6, 12, and 24 h for TLR3. However, high expression of TLR4a showed significant differences in transgenic TH2-3 zebrafish compared to WT zebrafish at 6 and 12 h. These results suggest that transgenic TH2-3 zebrafish did not induce IL-22 or TLR3 overexpression when the zebrafish received an *S. agalactiae* (SA) injection.

4. Discussion

Hepcidin functions as a key regulator of iron homeostasis and anemia associated with inflammation. To our knowledge, this study represents the first demonstration of the overexpression of a fish hepcidin in transgenic fish which enhanced resistance to a *V. vulnificus* infection. Hepcidin was recently shown to regulate cellular iron efflux in vitro by binding to ferroportin and causing its internalization and degradation [22], and those results suggested that a lipopolysaccharide (LPS) injection can induce the rapid development of hypoferremia. In tilapia, three cDNAs encoding putative hepcidin peptides (TH1-5, TH2-2, and TH2-3) were previously isolated and characterized [4]. Treatment with poly I:poly C showed that TH1-5 was highly expressed, while TH2-3 was markedly expressed by LPS treatment. In tilapia, hepcidin research results suggested that hepcidin 1–5 can inhibit *Enterococcus faecium* (MIC: 100 μg/ml), but TH2-3 had no activity against gram-positive pathogens in tested bacterial strains [4]. The synthetic hepcidin peptide studied from *Pseudosciaena crocea* demonstrated a rather wide spectrum of antimicrobial activity in vitro against the bacteria and fungi tested, and showed particularly strong activity against *Micrococcus luteus*, *Staphylococcus aureus*, and *S. epidermidis* [22]. Described above, the synthesized hepcidin from *P. crocea* showed antimicrobial activity against gram-positive pathogens and the synthesized tilapia TH2-3 showed antimicrobial activity against gram-negative pathogens. Therefore, direct evidence that hepcidin functions as an antibacterial peptide in vivo has not been demonstrated in fish.

Transgenic TH2-3 fish described here provide a new model for studying the effects of the overexpression of TH2-3 in resisting bacterial infection and inducing immune-related gene expressions after bacterial infection. Expression of the TH2-3 transgene in our model decreased *V. vulnificus* but not *S. agalactiae* growth in both the transgenic TH2-3 zebrafish and convict cichlid. The lack of protection could have been due to the fact that we did not test many gram-positive pathogens in transgenic TH2-3 fish for the antimicrobial activity study. But we observed variations in immune-related genes after injecting a gram-positive pathogen in transgenic TH2-3 fish compared to WT, which suggested that TH2-3 may be involved in immune gene expression after gram-positive bacterial challenge.

After the discovery of hepcidin in 2000 [1], its key role in iron homeostasis was realized. Injection of hepcidin into mice resulted in a dramatic drop in serum iron within 60 min, and the effect of a single dose was apparent for up to 72 h [23,24]. Chronic overexpression of hepcidin causes iron-restricted anemia and microcytic, hypochromic anemia in mice and humans. Hepcidin also partially restrained the export of stored iron from hepatocytes in mice carrying hepcidin-overproducing tumors [25]. However, the importance of iron for microorganisms is well known, and the host's iron is a participating factor in a number of experimental bacterial infections [26,27]. Many animal models were used to demonstrate increased susceptibility to bacterial infections after an injection of iron-containing compounds. Hence, the injection of iron into mice increased both the lethality and rapidity of *V. vulnificus* infections, and iron treatment greatly reduced the host survival time after *V. vulnificus* infections at inoculations below 10^9

Fig. 5. Quantitative PCR analysis of cytokine gene expressions after *Vibrio vulnificus* (204) infection in transgenic tilapia hepcidin (TH)2-3 and non-transgenic zebrafish (*Danio rerio*). Expressions of immune-related genes of interleukin (IL)-1β (IL1b), IL-10 (IL10), IL-15 (IL15), IL-21 (IL21), IL-22 (IL22), IL-26 (IL26), complement component c3b (C3b), lysozyme, toll-like receptor (TLR)1, TLR3, TLR4a, TRAM1, Myd88, nuclear factor (NF)-κB activating protein-like (NFkb), and tumor necrosis factor (TNF)-α were determined at 1, 6, 12, and 24 h after the bacterial injection. These results are representative of three independent experiments, and significance was set at (*) $p < 0.01$.

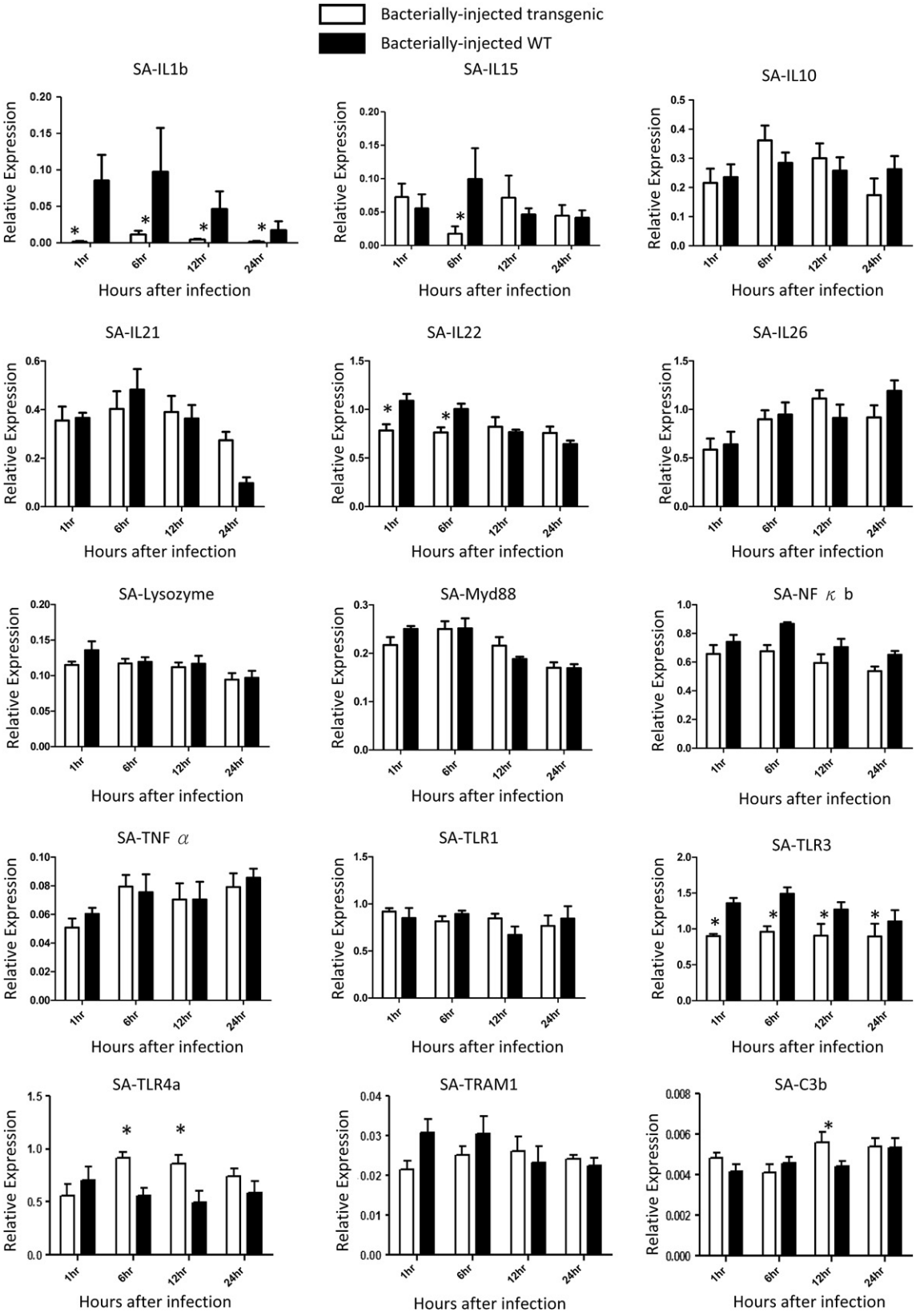

Fig. 6. Quantitative PCR analysis of cytokine gene expressions after a *Streptococcus agalactiae* (SA) infection in transgenic tilapia hepcidin (TH)2-3 zebrafish (*Danio rerio*) and non-transgenic zebrafish. Expressions of immune-related genes of interleukin (IL)-1β (IL1b), IL-10 (IL10), IL-15 (IL15), IL-21 (IL21), IL-22 (IL22), IL-26 (IL26), complement component c3b (C3b), lysozyme, toll-like receptor (TLR)1, TLR3, TLR4a, TRAM1, Myd88, nuclear factor (NF)-κB activating protein-like (NFkb), and tumor necrosis factor (TNF)-α were determined at 1, 6, 12, and 24 h after the bacterial injection. The results are representative of three independent experiments, and significance was set at (*) $p < 0.01$.

cells [28]. Results of those and more-recent studies consistently demonstrated that excessive iron plays a major role in the pathogenesis of *V. vulnificus* infection, indicating that iron is an important growth factor for pathogenic bacteria [29]. Our results in transgenic TH2-3 fish demonstrated that hepcidin was expressed in the muscle and showed significant suppression of *V. vulnificus* infection. It was presumed that this regulation evolved as a host defense strategy in transgenic TH2-3 fish to limit iron availability to *V. vulnificus*. In another transgenic model, generation of liver-specific and tetracycline-regulated Hepc1 transgenic mice induced iron-restricted anemia [16]. Such models used a liver-specific promoter to drive hepcidin expression in the liver; it consequently reduced systemic iron by reducing intestinal uptake. Although hepcidin mRNA was detected in several tissues [2,13,30], under basal conditions, hepcidin is known to predominantly be synthesized by the liver and secreted in the serum [11,31]. This does not preclude the possibility that other as yet unknown mechanisms contribute to hepcidin expression in muscles in our transgenic TH2-3 fish. For example, *Borrelia burgdorferi*-infected mice produced hepcidin in both liver and spleen. Both intact and sonicated *B. burgdorferi* induced hepcidin expression by cultured mouse bone marrow macrophages, suggesting that splenic macrophages appear to be an important contributor to serum hepcidin production [32]. Based on these observations, it was proposed that hepcidin is the "store regulator" [18], which infers that hepcidin TH2-3 expression in muscles and secretion into blood vessels in transgenic TH2-3 fish involve controlling intestinal iron absorption and iron recycling by macrophages.

In this study, we confirmed that TH2-3 was mostly expressed in muscles by fluorescent protein expression. Intriguingly, our transgenic TH2-3 fish did not inhibit *S. agalactiae* growth after an injection of 10 μl of *S. agalactiae* (at 1×10^7 CFU/ml) into the caudal peduncle of transgenic fish. A previous report stated that hepcidin is found in the genus, *Morone*, and is activated by organisms such as *S. iniae* (a gram-positive species). Synthetic hybrid striped bass (*Morone chrysops* x *M. saxatilis*) hepcidin was active in vitro against gram-negative pathogens and fungi but showed no activity against gram-positive pathogens and a yeast strain [3]. This paradox may explain why hepcidin plays only a small part in the response to different bacteria and suggests that hepcidin was derived from an "evolutionary oversight" [33]. Although the majority of existing studies focused on the susceptibility of gram-negative bacteria and our results indicated that the tilapia TH2-3 gene does not have a potent effect of suppressing gram-positive bacteria, more experiments are still needed to understand the functions of these three hepcidins from tilapia using other gram-positive bacterial infection tests and producing TH1-5 and TH2-2 transgenic fish.

The synthetic TH2-3 peptide was found to have antibacterial activity against a few bacteria. The weak antimicrobial activities may have been due to the disulfide bond not forming when we synthesized the TH2-3 hepcidin peptide [4]. The TH2-3 transgene in transgenic fish may present proper folding of its structure and act as an AMP in the innate immune response of transgenic fish with a bacterial infection. Although it is likely that hepcidins have antimicrobial activity, another function is its iron-regulatory activity. Mouse hepcidins 1 and 2 both are responsive to iron, but only mouse hepcidin 1 functions in iron regulation, while mouse hepcidin 2 is not iron deficient [19]. Overexpression of mouse hepcidin 1 in transgenic mice led to a severe iron-deficiency phenotype [17]. Functional iron-regulatory activity was also demonstrated for zebrafish hepcidin [8,34]. In this study, we showed that TH2-3 transgenic zebrafish had an enhanced ability to resist *V. vulnificus* compared to control non-transgenic zebrafish. We also investigated the immune-related gene expressions involved in the resistance to *V. vulnificus* and *S. agalactiae* infection in these transgenic zebrafish. There were significant differences in immune-related gene expressions of IL-10, IL-15, IL-21, IL-22, IL-26, TRAM1, TNF-α, Myd88, TLR1, and TLR3 before the injection of bacteria between transgenic and control WT zebrafish. Cytokines and iron availability have so far been identified as regulators of hepcidin expression. Our results presented here indicate that tilapia hepcidin TH2-3 exerted regulatory effects on TH2-3 transgenic fish by IL-10, IL-15, IL-21, IL-22, IL-26, TRAM1, TNF-α, Myd88, TLR1, and TLR3 mRNA expressions. To date, other studies of hepcidin regulation have provided few insights into the aberrant expression of hepcidin in adenoma patients [6]. As a result of those findings, elevated hepcidin expression was proposed to be the underlying cause of anemia with chronic inflammation [35] and may also cause decreased cytokine production.

As mentioned above, the roles in immunity against gram-negative and -positive pathogens of each of these cytokines are described below. IL-10 is a potent immunosuppressive cytokine that provides a mechanism of counter-regulation, and in diverse models of infection, IL-10 is critical for the maintenance of an immune balance on inhibitory effects of proinflammatory cytokine production [36,37]. However, in vivo, IL-10 suppresses the killing of phagocytosed bacteria, and neutralization of endogenous IL-10 led to enhanced survival in murine models of *Klebsiella pneumoniae*, *S. pneumoniae*, and *Mycobacterium avium* infections, suggesting that it could have potent anti-inflammatory activities in vivo against gram-positive and -negative bacterial infections [38]. IL-15, a cytokine produced by monocytes, shares many biological activities with IL-2 [39]. To date, elevated levels of IL-15 were found in vivo only during inflammatory diseases such as melioidosis and autoimmune disorders [40]. The elevated levels of IL-15 in melioidosis suggest that IL-15 may play a role in the pathogenesis of inflammatory processes during gram-negative infection. On the other hand, the overexpression of IL-15 isoforms revealed IL-15 to be an inhibitor of pneumococcus-induced apoptosis in pulmonary epithelial cells suggesting that IL-15 is a beneficial cytokine in pulmonary host defense against gram-positive bacterial infections [41]. IL-21 is produced by activated $CD4^+$ T cells, which regulates the growth and functional properties of T cells, B cells, NK cells, and dendritic cells. The administration of IL-21 to mice enhances the influx of immune cells into inflamed tissues and the severity of immune-mediated diseases, suggesting that IL-21 may play an important role in the initiation and perpetuation of chronic inflammatory processes such as *Helicobacter pylori* and *M. tuberculosis* infections [42,43]. IL-22 expression is found in activated T cells and, at lower levels, in activated natural killer cells [44]. Emerging evidence supports the concept that IL-22 mediates mucosal host defense against gram-negative bacterial pneumonia [45], but it is still not clear what role it plays in gram-positive bacterial pathogen infections. IL-26 is primarily generated by monocytes and memory T cells. It plays an important role in the transformation of human T cells after their infection by herpes virus saimiri [46]. The roles of IL-26 in gram-positive and -negative bacterial pathogen infections are also not understand.

Human hepatocyte were treated for 24 h in the presence and absence of 10% FCS, with serum-free medium, ferric ammonium citrate, iron-saturated ferritin, lipopolysaccharide, 12.5% monocyte medium + LPS, and 12.5% monocyte medium conditioned by monocytes exposed to LPS that the results suggested hepcidin upregulated the IL-6, IL-1α, and IL-1β inflammatory cytokines [47,48]. Therefore, elucidation of immune-related molecule variations involved in *V. vulnificus* and *S. agalactiae* infections in transgenic TH2-3 zebrafish that induced TNF-α and immune-related gene productions may lead to alternative strategies to treat sepsis caused by these bacteria. In our study, we found different variations in immune-related molecules after infection of transgenic TH2-3

zebrafish with *V. vulnificus* and *S. agalactiae*. Higher expressions of IL-10, IL-26, lysozyme, Myd88, TNF-α, TLR4, and TRAM1 were seen in transgenic TH2-3 zebrafish than in control WT zebrafish at 1–24 h after an injection of *V. vulnificus*, but lower expressions of IL-1β (at 12 and 24 h), IL-15 (at 1–12 h), and IL-22 (at 1–24 h) were found. Investigations of the expressions of IL-10 and IL-26 showed significantly higher levels in transgenic epinecidin-1 fish 12 h after a bacterial injection compared to WT zebrafish [21] that was similar to results of our study. Other research results suggested that IL-10 suppresses macrophage and DC functions, thereby limiting Th1 and Th2 effector responses [49]. Thus, IL-22 and other structurally related cytokines (IL-19, IL-20, IL-24, IL-26, IL-28αβ, and IL-29) join IL-22 as part of the IL-10 cytokine family. Our results showed that IL-22 and IL-26 had higher expressions in transgenic TH2-3 zebrafish after infection suggesting that IL-22 and IL-26 are important immune mediators produced by transgenic TH2-3 zebrafish muscles. Using a *V. vulnificus* injection in mouse showed that TLR4 signaling is Myd88 dependent and plays a key role in TNF-α production by mouse blood and splenocytes. In contrast to a TLR4 or TNF-α deficiency, an Myd88 deficiency is deleterious to mice infected with *V. vulnificus*. These results show that the harmful TNF-α response is strongly attenuated in the absence of Myd88, and Myd88 deficiency enhances the resistance of mice to sepsis due to polymicrobial infection [50].

Similarly, divergence in these immune-related gene expressions was observed with a *S. agalactiae* infection in transgenic TH2-3 zebrafish. It only decreased the expressions of IL-1β (at 1–24 h), IL-22 (at 1–6 h), and TLR3 (at 1–24 h) in transgenic TH2-3 zebrafish after a *S. agalactiae* infection. One recent study showed that after infection with *S. agalactiae*, marked changes were observed in levels of IL-1β produced by resident macrophages in mammary glands. Nevertheless, the growth of *S. agalactiae* was uncontrolled until 24 h post-infection [51]. Our results are in agreement with studies reporting an increase in the levels of the IL-1β produced as a consequence of *S. agalactiae* infection and indicate the results of growth of *S. agalactiae* was uncontrolled, similar to our data. Another study showed that group B streptococcus (GBS) potently activates inflammatory macrophage genes via Myd88. But only Myd88, not the TLR adapter proteins MAL/TIRAP, TRIF, or TRAM, essentially mediates the cytokine (TNF and IL-6) and chemokine (RANTES) responses to whole GBS organisms, although MAL, TRIF, and TRAM were shown to mediate the responses to other gram-positive and -negative bacteria [52]. In a C3b study, the strain of *S. agalactiae* that served as a test organism was shown to be an activator of an alternative pathway, triggering the deposition of C3 in bovine milk suggesting C3b's participation in the classical pathway for inhibiting mastitis-causing *S. agalactiae* [53]. But whether IL-22, IL-15, and TLR3 play roles in fish immunity against gram-positive pathogen infections needs more study in the future.

Collectively, complete inhibition of TNF-α release in transgenic TH2-3 zebrafish can be achieved by blocking an upstream reaction leading to immune-related gene activation. In fact, recent data indicated that mitogen-activated protein kinase (MAPK) and NF-κB are involved in TNF-α responses to group B streptococci [54]. This reaction is shared by the TLR and IL-1R families and includes Myd88 [55]. In addition, in normal mice, a targeted local immune response destroys low subcutaneous group B streptococci (*S. agalactiae*) inoculums without measurable systemic inflammatory activity in the blood [56]. Our studies and that described above demonstrate that hepcidin may serve as a differential regulator to mediate innate immunity after various bacterial infections.

V. vulnificus is a pathogenic bacterium that causes fatal septicemia in humans [57]. Our data indicated that transgenic TH2-3 zebrafish could more-effectively suppress bacterial numbers after a *V. vulnificus* infection. The excellent antimicrobial ability of transgenic TH2-3 zebrafish may have been due to enhanced IL-10, IL-26, lysozyme, Myd88, TNF-α, TLR4a, and TRAM1 gene expressions. Although the main cause of elevated hepcidin expression was proposed to be the underlying anemia of chronic inflammation [35], inflammation might also have caused increased cytokine production. Unfortunately, no group has succeeded in producing transgenic fish containing other hepcidins for comparison of antibacterial functions with our results thus far, so it is not yet feasible to measure hepcidin's functions in other fish. However, our transgenic TH2-3 fish may be useful tools for studying iron disorders or anemia of chronic diseases in the future.

In summary, we report the production of fluorescent fish containing TH2-3; zebrafish and convict cichlid featured resistance to *V. vulnificus* infection and variations in immune-related gene expressions after different bacterial infection. Our results may prove to be a crucial part of efforts to understand teleost innate immunity and to apply that knowledge to control microbial infections in aquaculture species using a variety of antimicrobial peptides.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsi.2010.05.001.

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