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Notch1a can widely mediate innate immune responses in zebrafish larvae infected with *Vibrio parahaemolyticus*

Ce Ji[a,b,c], Xinya Guo[a,b,c], Xuehong Dong[a,b,c], Jianfeng Ren[a,b,c], Yao Zu[a,b,c], Weiming Li[d], Qinghua Zhang[a,b,c,*]

[a] Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Ministry of Education, Shanghai Ocean University, Shanghai, 201306, China
[b] International Research Center for Marine Biosciences, Ministry of Science and Technology, Shanghai Ocean University, Shanghai, 201306, China
[c] Key Laboratory of Freshwater Aquatic Genetic Resources, Ministry of Agriculture, Shanghai Ocean University, Shanghai, 201306, China
[d] Department of Fisheries and Wildlife, Michigan State University, East Lansing, MI 48824, USA

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ABSTRACT

The Notch signaling pathway is known to regulate innate immunity by influencing macrophage function and interacting with the Toll-like receptor (TLR) signaling pathway. However, the comprehensive role of the Notch signaling pathway in the innate immune response remains unknown. To assess the function of Notch1a in immunity, we examined the innate immune responses to *Vibrio parahaemolyticus* strain Vp13 of wild-type (WT) and *notch1a*$^{-/-}$ zebrafish larvae generated using the clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/Cas9) system. The median lethal dose (LD_{50}) of *V. parahaemolyticus* was significantly lower in *notch1a*$^{-/-}$ larvae than in WT larvae 3 days post fertilization (dpf). Transcriptome data analysis revealed 359 significantly differentially expressed genes (DEGs), including 246 significantly down-regulated genes and 113 significantly up-regulated genes, in WT infected groups compared with WT control groups. In contrast, 986 significantly DEGs were found in *notch1a*$^{-/-}$ infected groups compared with *notch1a*$^{-/-}$ control groups, of which 82 genes were significantly down-regulated and 904 genes were significantly up-regulated. These DEGs belonged to the tumor necrosis factor (TNF), complement, nuclear factor kappa B (NF-κB), cathepsin, interleukin (IL), chemokine, serpin peptidase inhibitor, matrix metallopeptidase, innate immune cells, pattern recognition receptor (PRR), and other cytokine families. Our results indicate that Notch1a plays roles in inhibiting many immunity-related genes and could comprehensively mediate the innate immune response by regulating TLRs, nucleotide-binding-oligomerization-domain-like receptors (NLRs), lectins, complement, ILs, chemokines, TNF, cathepsin, and serpin. Further studies are required to understand the specific mechanism of Notch1a in innate immunity.

1. Introduction

The innate immune system is the first line of host defense against infection. This system recognizes pathogens and triggers proinflammatory responses through a number of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) [1]. Subsequently, several intracellular signaling pathways are activated to regulate gene expression and synthesis of a range of cytokines, including proinflammatory and chemotactic cytokines, and antimicrobial peptides [2]. The main PRRs are toll-like receptors (TLRs), nucleotide-binding-oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), scavenger receptors and C-type lectin receptors (CLRs) [3]. Furthermore, the complement system plays a crucial role in innate defenses through proteolysis and production of potent proinflammatory molecules [4].

The Notch signaling pathway, which was originally identified as a mediator of cell fate and pattern formation during development in invertebrates, has been demonstrated to regulate immune cell development and function [5]. Results of a previous study showed that the Notch signaling pathway regulates macrophage function by interacting with the TLR signaling pathway and activating nuclear factor kappa B (NF-κB), thereby altering expression patterns of genes involved in proinflammatory responses [6]. Specifically, activation of Notch

* Corresponding author. Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Ministry of Education, Shanghai Ocean University, Shanghai, 201306, China.
E-mail address: qhzhang@shou.edu.cn (Q. Zhang).

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signaling inhibits NF-κB transcription, and the Notch1 and Notch2 intracellular domains can suppress production of TLR-triggered proinflammatory cytokines such as tumor necrosis factor (TNF) α and interleukin (IL)-6 [7]. Furthermore, Notch-PBPJ promotes the synthesis of IRF8 protein, which regulates the TLR-induced inflammatory polarization of macrophages [8]. However, most studies have been performed in mammalian macrophages, and a global understanding of the function of the Notch signaling pathway in the innate immune response is still lacking.

Zebrafish (*Danio rerio*) possess four Notch receptors (Notch1a, Notch1b, Notch2, and Notch3) that are highly homologous to those of mammals, and the phenotype of Notch1a mutant zebrafish is consistent with that of Notch1 mutant mice [9]. Furthermore, zebrafish are a good model for studying infectious diseases, including human diseases [10–12]. This species is particularly advantageous for studying innate immunity because its adaptive immune system does not develop until 3 weeks post fertilization [13]. Zebrafish larvae have been widely used to study immunity and inflammation [14] following infection with bacteria [15–17], fungi [18,19], and viruses [20,21]. In our previous study, we showed that the *Vibrio parahaemolyticus* strain Vp13, a Gram-negative bacterium, can infect zebrafish larvae and activate the innate immune response [22].

In this study, we hypothesized that, in addition to regulating TLR, the Notch signaling pathway plays various other roles in mediating innate immunity. We found that loss of *notch1a* led to low tolerance and resistance to infection in larvae 3 days post fertilization (dpf), indicating that the Notch signaling pathway is an important regulator of innate immunity. We also confirmed that the Notch signaling pathway negatively regulates the TLR signaling pathway. In addition, the Notch signaling pathway regulated the expression of genes involved in other PRR signaling pathways (i.e., the NLR and CLR signaling pathways), innate immune cells, cathepsin and serpin family members.

2. Materials and methods

2.1. Vibrio parahaemolyticus culture and viable counts

Pathogenic *V. parahaemolyticus* strain Vp13 isolated from infected *Litopenaeus vannamei* was a gift from Dr. Yong Zhao at the College of Food Science and Technology, Shanghai Ocean University, Shanghai, China. Bacteria were incubated to the mid-logarithmic stage at 28 °C in trypticase soy broth (TSB) containing 3% NaCl and serially diluted with sterile water to a concentration of 10^{-6}. Each diluted solution was plated onto thiosulfate citrate bile salts sucrose (TCBS) agar culture medium and incubated for approximately 24 h at 28 °C before the number of colony-forming units (CFUs) were counted.

2.2. Zebrafish maintenance, notch1a mutant zebrafish generation and infection

Wild-type (WT) adult zebrafish (strain AB) were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China. Zebrafish were handled according to the procedures of the Institutional Animal Care and Use Committee of Shanghai Ocean University, Shanghai, China and maintained according to standard protocols (http://zfin.org). Research methods were approved by the Shanghai Ocean University Experimentation Ethics Review Committee (SHOU-DW-2016-002).

Notch1a mutant zebrafish were generated using the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system according to our US invention patent (application number: 16422975).

Embryos were grown at 28.5 °C in egg water (60 μg/ml Instant ocean sea salts) [23]. WT embryos were spawned by adult zebrafish that were the siblings of zebrafish used to generate *notch1a* mutants, and *notch1a*$^{-/-}$ embryos were selected by crossing *notch1a*$^{+/-}$ adult zebrafish. WT and *notch1a*$^{-/-}$ larvae were challenged with Vp13 by microinjection at 3 dpf as previously described [24] with minor modifications. To determine the median lethal dose (LD_{50}) of Vp13 in WT and *notch1a*$^{-/-}$ larvae, five suspensions of Vp13 in phosphate-buffered saline (PBS) were prepared at concentrations of 0, 40, 200, 1000, and 5000 CFU/nL and 1 nL Vp13 suspended in PBS was microinjected. Each group contained three replicate samples and each sample contained 15 larvae.

Based on the LD_{50}, we chose to infect WT and *notch1a*$^{-/-}$ larvae with 500 CFU/nL Vp13 and analyzed the survival curves. WT and *notch1a*$^{-/-}$ larvae were then microinjected with 1 nL of their respective LD_{50} of Vp13 suspended in PBS for 2, 4, 8, 12, or 24 h, whereas larvae in control groups were injected with 1 nL PBS alone. Each time point included three replicate samples in every group. All samples were flash-frozen in liquid nitrogen and stored at -80 °C until RNA extraction.

2.3. Transcriptome analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Random hexamers were used to synthesize first-strand cDNA, followed by synthesis of the complementary strand. Samples of WT control (WT PBS), WT infected (WT Vp), *notch1a*$^{-/-}$ control (*notch1a*$^{-/-}$ PBS), and *notch1a*$^{-/-}$ infected (*notch1a*$^{-/-}$ Vp) larvae were chosen for transcriptome sequencing 2 h post infection (hpi). Paired-end sequencing was carried out using an Illumina HiSeq 4000 (Illumina, San Diego, CA, USA). Mapping and enrichment were performed as previously described [22].

2.4. Analysis of differentially expressed genes by quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

cDNA synthesis was performed in 20 μL reaction mixtures containing 1 μg RNA and HiScript III SuperMix for qPCR (+gDNA wiper) (Vazyme Biotech Co.,Ltd, Nanjing, Jiangsu, China) according to the manufacturer's instructions. RT-qPCR was performed using a Roche 480 real-time PCR detection system (Roche, Basel, Switzerland) according to the manufacturer's instructions. Results were normalized to the zebrafish *ef1a* gene [25], whose expression showed no change over time throughout the course of infection. Results were analyzed using the $2^{-\triangle\triangle Ct}$ method [26]. Each group contained three replicate samples (10 zebrafish larvae per sample). Primer sequences are listed in Table 1.

2.5. Statistical analysis

Statistical analyses were performed using Excel (Microsoft, Redmond, WA, USA), SPSS (IBM Corp., Armonk, NY, USA) and GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Column statistics were performed on datasets to check for normal distribution.

Table 1
Primer sequence used for RT-qPCR analysis.

Genes	Primer sequence (5′–3′)	Accession No.
ef1a	CTGGAGGCCAGCTCAAACAT ATCAAGAAGAGTAGTACCGCTAGCATTAC	ENSDART00000023156
tnfb	TTGAAGATGTTGAAGGAGATG CAAGGTAAATGGTGCTGTAGG	NM_001024447.1
il1b	TGGACTTCGCAGCACAAAATG GTTCACTTCACGCTCTTGGATG	NM_212844.2
ctslb	CTGGATTCTGAGCAGTCATACC CAGCAGCAACAGCATTCATTAG	NM_131198.1
c3a.3	GATGGAGTAGGAGGAGTCAACA TATCCGCCTGCTGCTCATT	NM_001037236.2
nfkbiaa	ATAGCAAGCCTGAAGGAGACA CCTCTGCCTCGTGAATGATG	NM_213184.2
il13ra1	AAGCAACTGTGGACGAGGAT CAAGAGACACTATCAGGCAACA	NM_001110837.1

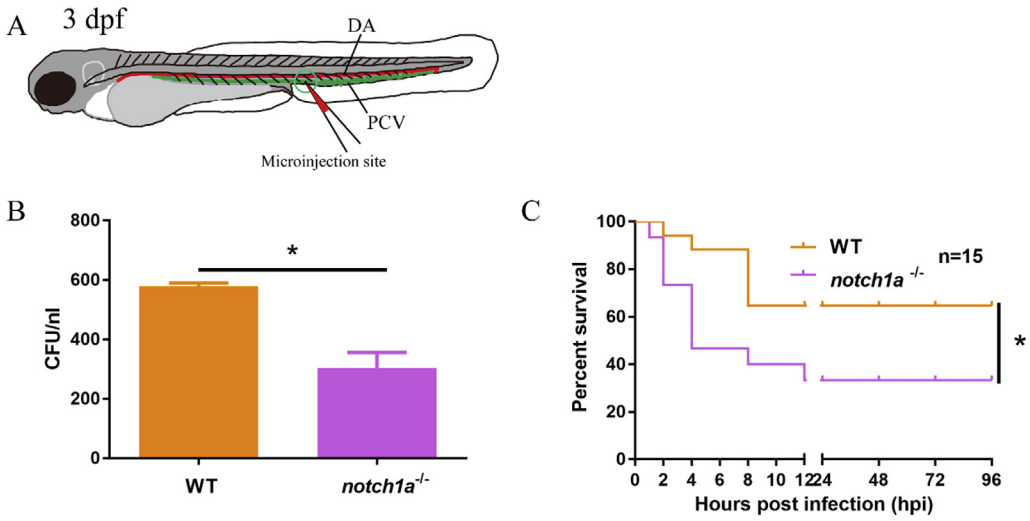

Fig. 1. Microinjection infection of WT and *notch1a*$^{-/-}$. (A) Microinjection site model of 3 dpf larvae. Dorsal aorta (DA), posterior cardinal vein (PCV); (B) Median lethal dose (LD_{50}) of wild-type (WT) and *notch1a*$^{-/-}$ larvae infected with *Vibrio parahaemolyticus* strain Vp13 for 96 h. (C) Statistics of survival curve of WT and *notch1a* $^{-/-}$ larvae infected with 500 CFU Vp13. Each group contained three independent replicates and each replicate included 15 larvae. LD_{50} was calculated using Bliss's method. *p < 0.05.

T-tests and one-way analysis of variance (ANOVA) were performed, and multiple comparisons were corrected using least-significant difference (LSD) and Bonferroni procedures. Values are expressed as mean ± standard error.

3. Results

3.1. Median lethal dose of Vibrio parahaemolyticus Vp13 in WT and notch1a$^{-/-}$ larvae and survival curve analysis

The caudal vein injection route was selected to infect WT and *notch1a*$^{-/-}$ larvae (Fig. 1A). The LD_{50} values of *V. parahaemolyticus* Vp13 in WT and *notch1a*$^{-/-}$ larvae infected for 96 h were calculated using Bliss's method [27]. The LD_{50} in *notch1a*$^{-/-}$ larvae was significantly lower than that in WT larvae (Fig. 1B). Survival curve analysis revealed that a dose of 500 CFU Vp13 led to a lower survival percentage in *notch1a*$^{-/-}$ larvae (Fig. 1C). Respective LD_{50} values were then used to infect WT and *notch1a*$^{-/-}$ larvae for subsequent experiments.

3.2. Identification of differentially expressed genes

Comparison of gene expression levels between WT Vp and WT PBS revealed 359 significantly DEGs, including 246 significantly down-regulated genes and 113 significantly up-regulated genes (Fig. 2A). A total of 986 significantly DEGs were found in *notch1a*$^{-/-}$ Vp compared with *notch1a*$^{-/-}$ PBS, of which 82 genes were significantly down-regulated, and 904 genes were significantly up-regulated (Fig. 2B). More changes in gene expression in response to infection were observed in *notch1a* mutants, and most DEGs were up-regulated. This is consistent with previous reports that the Notch signaling pathway inhibits the innate immune response.

To understand the roles that Notch1a plays in the innate immune response, fold changes calculated from fragments per kilobase of exon model per million mapped reads (FPKM) for each gene were compared between WT PBS and WT Vp (1 vs 2), WT PBS and *notch1a*$^{-/-}$ PBS (1 vs 3), WT Vp and *notch1a*$^{-/-}$ Vp (2 vs 4), and *notch1a*$^{-/-}$ PBS and *notch1a*$^{-/-}$ Vp (3 vs 4). DEGs in the different groups and overlapping DEGs are shown in the Venn diagram in Fig. 3. Fifty genes in the red boundary were differentially expressed in comparisons of 1 vs 2, 2 vs 4 and 3 vs 4 but not 1 vs 3, suggesting that these genes were not differentially expressed in *notch1a*$^{-/-}$ larvae in the absence of infection. Additionally, 180 genes in the green boundary were differentially expressed only in comparisons 2 vs 4 and 3 vs 4 (Supplementary Table 1), suggesting that these genes may be involved in the innate immune response and may be regulated by Notch1a.

To understand the function of Notch1a in innate immune responses, the expression patterns of the 50 DEGs in both the WT Vp and *notch1a*$^{-/-}$ Vp groups were analyzed (Fig. 4). The genes *mpeg1.2*, *itln3*, *tnip2*, *cxcl18b*, *cxcl8a*, *nfkbiaa*, *tnfaip2b*, *il1b*, *cxcr3.3*, *ccl35.1*, *il34*, *irak3*, *ncf1*, *rel*, *cfb*, *steap4*, *wu:fb18f06*, *neo1b*, *CR762483.1*, *si:ch211-218g23.6*, *fbxo28*, *si:dkey-97i18.5*, *cnksr3*, *tifa*, *si:dkey-19d21.2*, *sele*, *tmem9*, *si:dkey-31e10.5*, *ncalda*, *fam207a*, *dnmt3bb.3*, *vps26b*, *emp2*, *plac8.1*, *glmnb*, *ace2*, *si:ch73-185c24.2*, *aqp9b*, *si:dkeyp-57d7.4*, *serpinf2a*, *si:ch1073-469d17.2*, *pla2g12b*, *lmbr1*, *noxo1a*, *ddx19*, *arr3b*, and *rab3aa* were up-regulated, whereas the gene *ctss2.1* was down-regulated in both infected groups compared with their respective control groups. In contrast, the genes *appa* and *necab2* were down-regulated in the WT Vp group but up-regulated in the *notch1a*$^{-/-}$ Vp group. These genes were not differentially expressed in the *notch*$^{-/-}$ PBS group compared with the WT PBS group. These results indicate that the 50 genes that were differentially expressed in response to infection in both WT and *notch1a*$^{-/-}$ larvae are important in the innate immune response. The opposite expression pattern of the genes *appa* and *necab2* indicates that these two genes may function downstream of Notch1a to defend against infection.

DEGs that were included in at least one of the 1 vs 2, 1 vs 3, 2 vs 4, or 3 vs 4 comparisons were selected for deeper analysis, including innate immunity-related genes belonging to the TNF, complement, NF-κB, cathepsin, IL, chemokine, serpin peptidase inhibitor, matrix metallopeptidase, innate immune cells, PRR-related, and other cytokine families. The FPKM values of these genes were then compared in WT PBS, WT Vp, *notch1a*$^{-/-}$ PBS, and *notch1a*$^{-/-}$ Vp larvae (Fig. 5). The TNF-related genes *traf2b*, *tnfa*, *tnfrsfa*, *tnfb*, and *tnfaip2b* were all up-regulated in *notch1a*$^{-/-}$ Vp larvae compared with WT PBS or *notch1a*$^{-/-}$ PBS larvae, whereas the gene *tnfrsfa* was significantly up-regulated in *notch1a*$^{-/-}$ Vp compared with WT Vp larvae (Fig. 5A). The complement-related genes *c3a.1*, *c3a.3*, *cfhl4*, *cfhl5*, and *cfb* were all up-regulated in *notch1a*$^{-/-}$ Vp compared with WT Vp larvae, but the gene *cfd* was down-regulated in *notch1a*$^{-/-}$ Vp compared with WT Vp larvae (Fig. 5B). The NF-κB-related gene *nakp* was up-regulated, whereas the gene *nfkbiaa* was down-regulated, in *notch1a*$^{-/-}$ Vp compared with WT Vp larvae (Fig. 5C). The cathepsin-related genes *ctsll*, *ctsbb*, *ctsc*, and *cts12* were all down-regulated in *notch1a*$^{-/-}$ Vp compared with WT Vp larvae, and these genes were all down-regulated in *notch1a*$^{-/-}$ PBS compared with WT PBS larvae. In contrast, the gene *ctslb* was up-regulated in *notch1a*$^{-/-}$ Vp compared with WT Vp larvae and in *notch1a*$^{-/-}$ PBS compared with WT PBS larvae (Fig. 5D). The IL-related gene *il13ra1* was up-regulated in *notch1a*$^{-/-}$ Vp compared with WT Vp larvae, but the gene *il1b* was down-regulated in *notch1a*$^{-/-}$ Vp compared with WT Vp larvae (Fig. 5E). The chemokine-related genes *crfb6* and *ccl35.1* were up-regulated in *notch1a*$^{-/-}$ Vp compared with WT Vp larvae (Fig. 5F). The serpin

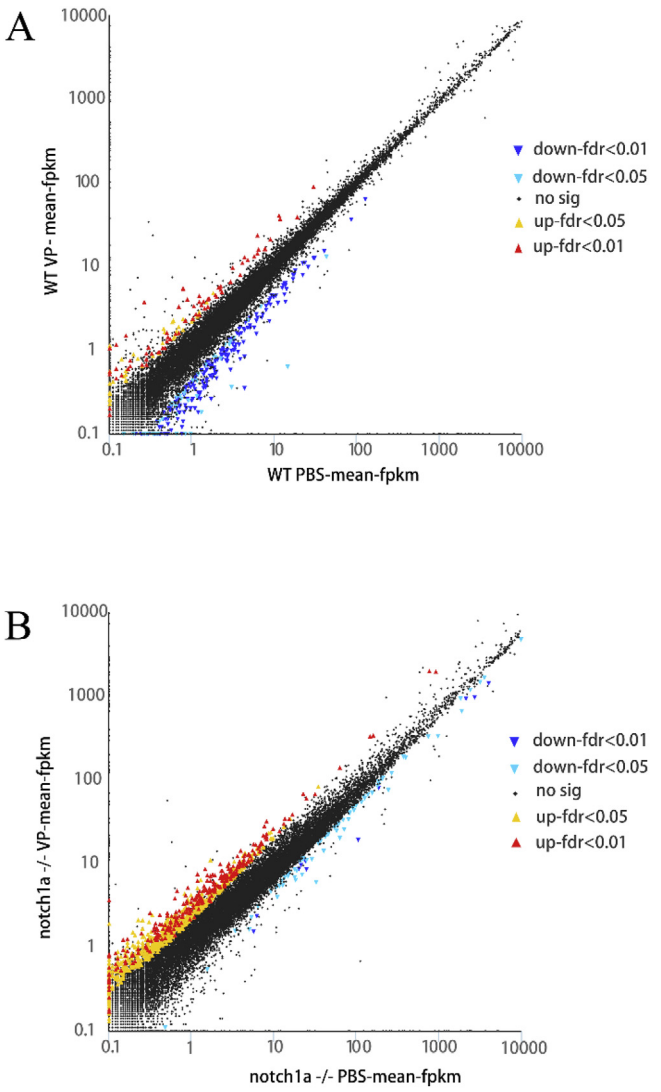

Fig. 2. Scatter plots of differentially expressed genes (DEGs) showing differences in gene expression between control and infected groups. Blue and cyan triangles represent significantly down-regulated genes; red and orange triangles represent significantly up-regulated genes in infected groups; and black triangles represent genes that were unchanged in infected groups compared with control groups. (A) DEGs in WT Vp compared with WT PBS groups; (B) DEGs in *notch1a*$^{-/-}$ Vp compared with *notch1a*$^{-/-}$ PBS groups. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

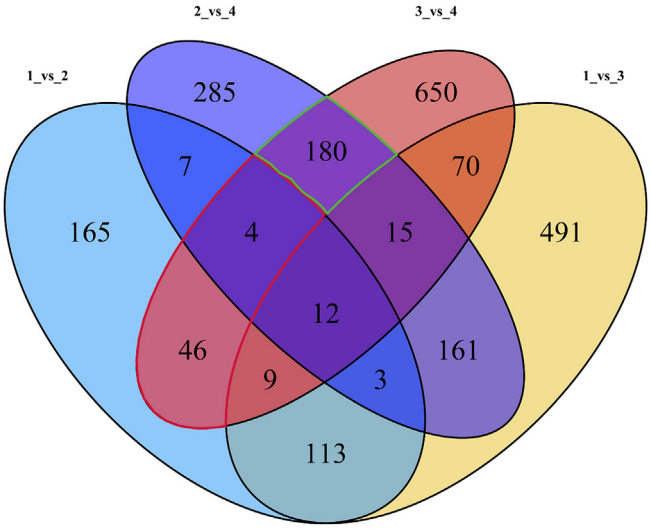

Fig. 3. Venn diagrams showing the number of DEGs in WT PBS compared with WT Vp (1 vs 2), WT PBS compared with *notch1a*$^{-/-}$ PBS (1 vs 3), WT Vp compared with *notch1a*$^{-/-}$ Vp (2 vs 4), and *notch1a*$^{-/-}$ PBS compared with *notch1a*$^{-/-}$ Vp (3 vs 4). The number of DEGs in each comparison are shown in ellipses of different colors. The number of DEGs present in more than one group are shown in overlapping ellipses. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

peptidase inhibitor-related genes *serpinb1l4* and *serpine3* were down-regulated in *notch1a*$^{-/-}$ Vp compared with WT Vp larvae (Fig. 5G). The metallopeptidase related genes *mmp11b* and *mmp14b* were up-regulated in *notch1a*$^{-/-}$ Vp compared with WT PBS or *notch1a*$^{-/-}$ PBS (Fig. 5H). The macrophage-related gene *steap4* and the neutrophil-related gene *ncf1* were up-regulated in *notch1a*$^{-/-}$ Vp compared with WT Vp larvae (Fig. 5I). The PRR-related genes *tlr18* and *nlrx1* were up-regulated in *notch1a*$^{-/-}$ Vp compared with WT Vp larvae, but *itln3* was down-regulated in *notch1a*$^{-/-}$ Vp compared with WT Vp larvae (Fig. 5J). The other cytokine-related genes *dock11*, *prc1b*, *crfb6*, and *irf9* were up-regulated in *notch1a*$^{-/-}$ Vp compared with WT Vp larvae, but *cishb* was down-regulated in *notch1a*$^{-/-}$ Vp compared with WT Vp larvae (Fig. 5K). Furthermore, the gene *prc1b* was up-regulated, whereas the gene *cishb* was down-regulated in *notch1a*$^{-/-}$ PBS compared with WT PBS larvae. In summary, Notch1a plays a role in the activation of the expression of *cfd*, *nfkbiaa*, *ctsll*, *ctsbb*, *ctsc*, *cts12*, *il1b*, *serpinb1l4*, *serpine3*, *itln3*, and *cishb* but inhibits expression of *tnfrsfa*, *c3a.3*, *cfhl5*, *cfb*, *c3a.1*, *cfhl4*, *nkap*, *il13ra1*, *crfb6*, *ccl35.1*, *steap4*, *ncf1*, *tlr18*, *nlrx1*, *dock11*, *prc1b*, *crfb6*, and *irf9* during Vp13 infection.

3.3. Gene Ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis of differentially expressed genes

DEGs in WT Vp larvae compared with WT PBS larvae were categorized into 222 GO terms, and the 10 most enriched GO terms were cytokine activity, cytokine receptor binding, immune response, chemokine activity, chemokine receptor binding, cytoskeletal protein binding, immune system processes, extracellular space, receptor binding, and regulation of transcription, DNA-templated (Table 2). Furthermore, 55 KEGG pathways containing DEGs were identified. The 10 most enriched KEGG pathways were tight junction, neurotrophin signaling pathway, biosynthesis of unsaturated fatty acids, malaria, N-glycan, biosynthesis, *Staphylococcus aureus* infection, adrenergic signaling in cardiomyocytes, proteoglycans in cancer, the phototransduction-fly pathway, and lysine degradation (Table 3). DEGs in *notch1a*$^{-/-}$ Vp compared with *notch1a*$^{-/-}$ PBS larvae were categorized into 202 GO terms, and the 10 most enriched GO terms were endopeptidase inhibitor activity, endopeptidase regulator activity, peptidase inhibitor activity, enzyme inhibitor activity, calcium-dependent phospholipid binding, peptidase regulator activity, enzyme regulator activity, biological processes, 4-hydroxyphenylpyruvate dioxygenase activity, and lactoylglutathione lyase activity (Table 4). Furthermore, 77 KEGG pathways containing DEGs were identified. The 10 most enriched KEGG pathways were SNARE interactions in vesicular transport, biosynthesis of unsaturated fatty acids, insulin secretion, measles, cell cycle-yeast, synaptic vesicle cycle, cysteine and methionine metabolism, *S. aureus* infection, meiosis-yeast, and cell cycle (Table 5). Although many immune-related GO terms were enriched in WT Vp compared with WT PBS larvae (Table 2), no immune-related GO terms were enriched in *notch1a*$^{-/-}$ Vp compared with *notch1a*$^{-/-}$ PBS larvae (Table 4). These results demonstrate that Notch signaling is important in the innate immune response. Although more DEGs were identified in *notch1a*$^{-/-}$ Vp compared with *notch1a*$^{-/-}$ PBS larvae, their functions in innate immunity were inconsistent.

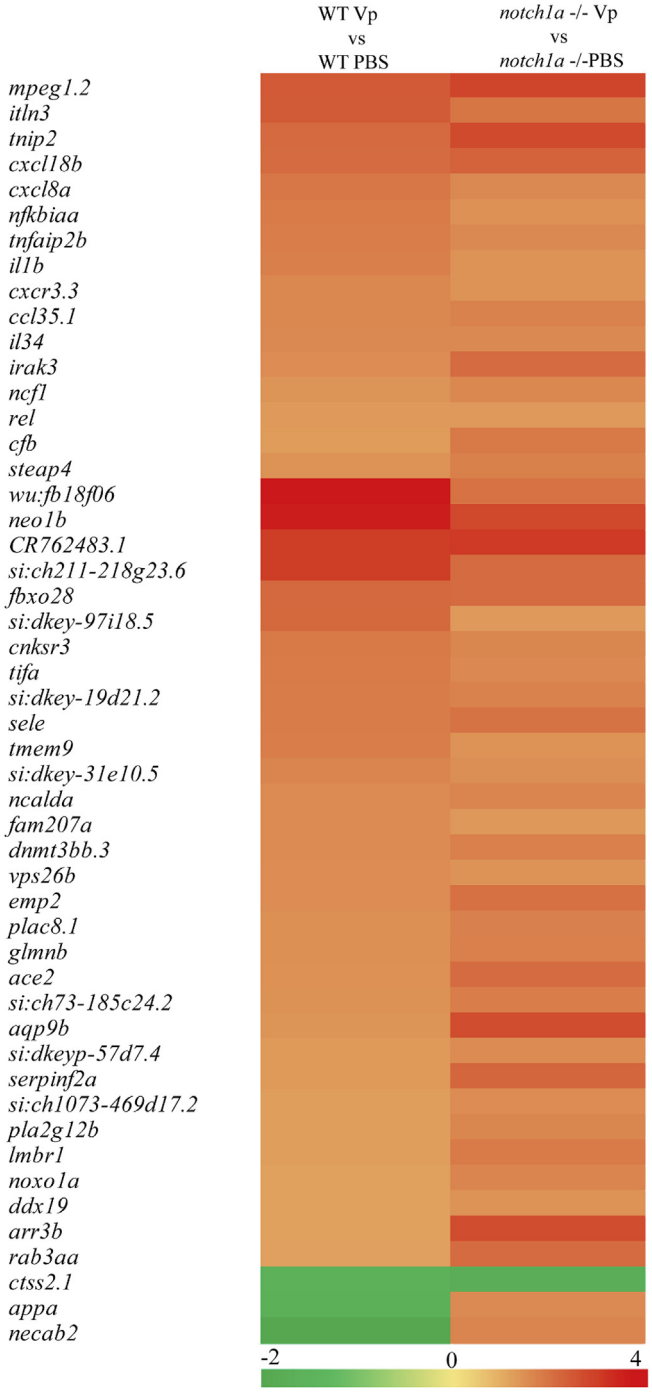

Fig. 4. DEGs in both WT Vp and *notch1a*$^{-/-}$ Vp groups compared with their respective controls. The red color represents the degree of up-regulation and the green color represents the degree of down-regulation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Analysis of differentially expressed genes by RT-qPCR

To validate the DEGs identified by transcriptome analysis of larvae 2 hpi, six genes, including *il1b*, *tnfb*, *nfkbiaa*, *il13ra1*, *c3a.3* and *ctslb* were selected for RT-qPCR analysis. Based on that these genes were known to be critical to innate immunity in zebrafish larvae and the transcriptome data revealed that these genes have a significantly different expression pattern in WT Vp group and *notch1a*$^{-/-}$ Vp group, indicating that Notch1a may influent the expression of these genes. The

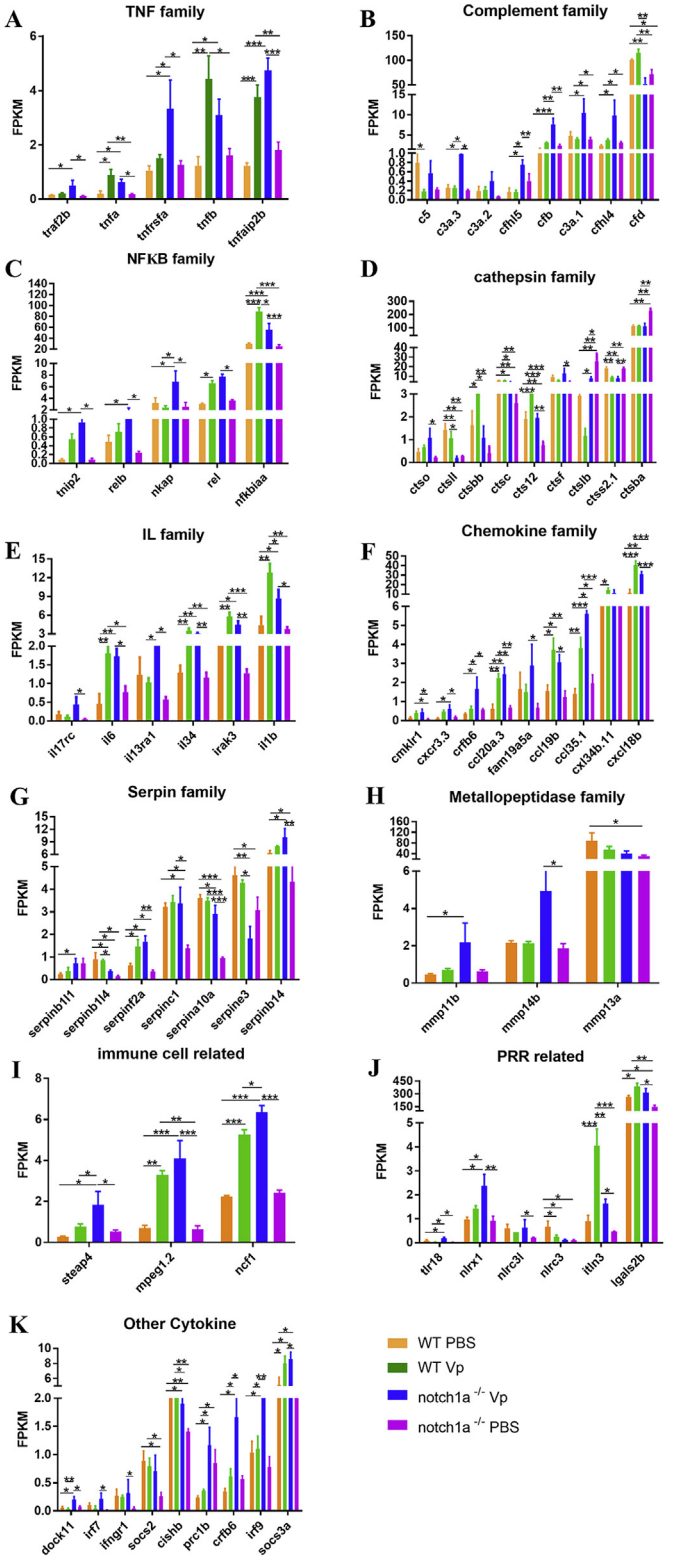

Fig. 5. Fragments per kilobase of exon model per million mapped reads (FPKM) of innate immunity-related genes in WT PBS, WT Vp, *notch1a*$^{-/-}$ PBS, and *notch1a*$^{-/-}$ Vp larvae. (A) Tumor necrosis factor (TNF)-related genes; (B) Complement-related genes; (C) Nuclear factor kappa B (NF-κB)-related genes; (D) Cathepsin-related genes; (E) Interleukin (IL)-related genes; (F) Chemokine-related genes; (G) Serpin peptidase inhibitor-related genes; (H) Metallopeptidase related genes; (I) Innate immunity-related genes; (J) Pattern recognition receptor (PRR)-related genes; (K) Other cytokine-related genes. Each group contained three independent replicates. *p < 0.05, **p < 0.005, ***p < 0.001.

Table 2
Top10 GO terms enrichment in WT Vp compared with WT PBS groups.

ID	Description	DEGs	Bankground number	p_uncorrected	p_bonferroni	Type
GO:0005125	cytokine activity	7	39	2.33E-07	0.0002	molecular_function
GO:0005126	cytokine receptor binding	7	49	1.18E-06	0.0011	molecular_function
GO:0006955	immune response	8	86	5.62E-06	0.0055	biological_process
GO:0008009	chemokine activity	4	21	7.92E-05	0.0784	molecular_function
GO:0042379	chemokine receptor binding	4	21	7.92E-05	0.0784	molecular_function
GO:0008092	cytoskeletal protein binding	9	176	0.0001	0.1730	molecular_function
GO:0002376	immune system process	8	145	0.0002	0.2360	biological_process
GO:0005615	extracellular space	7	110	0.0002	0.2420	cellular_component
GO:0005102	receptor binding	10	229	0.0002	0.2760	molecular_function
GO:0006355	regulation of transcription, DNA-templated	24	984	0.0005	0.4950	biological_process

Table 3
Top10 KEGG pathways enrichment in WT Vp compared with WT PBS groups.

Term	ID	Sample number	Background number	P-Value	Corrected P-Value
Tight junction	ko04530	3	33	0.0062	0.3521
Neurotrophin signaling pathway	ko04722	2	31	0.0471	0.3971
Biosynthesis of unsaturated fatty acids	ko01040	1	5	0.0618	0.3971
Malaria	ko05144	1	6	0.0718	0.397
N-Glycan biosynthesis	ko00510	1	10	0.1105	0.397
Staphylococcus aureus infection	ko05150	1	10	0.1105	0.397
Adrenergic signaling in cardiomyocytes	ko04261	2	52	0.1112	0.397
Proteoglycans in cancer	ko05205	2	54	0.1181	0.397
Phototransduction - fly	ko04745	1	11	0.1199	0.397
Lysine degradation	ko00310	1	11	0.1199	0.397

Table 4
Top10 GO terms enrichment in *notch1a* $^{-/-}$ Vp compared with *notch1a* $^{-/-}$ PBS groups.

ID	Description	DEGs	Bankground number	p_uncorrected	p_bonferroni	Type
GO:0004866	endopeptidase inhibitor activity	9	55	4.19E-05	0.0826	molecular_function
GO:0061135	endopeptidase regulator activity	9	55	4.19E-05	0.0826	molecular_function
GO:0030414	peptidase inhibitor activity	9	61	9.70E-05	0.191	molecular_function
GO:0004857	enzyme inhibitor activity	10	75	9.70E-05	0.191	molecular_function
GO:0005544	calcium-dependent phospholipid binding	4	9	9.85E-05	0.194	molecular_function
GO:0061134	peptidase regulator activity	9	63	0.0001	0.247	molecular_function
GO:0030234	enzyme regulator activity	17	209	0.0002	0.534	molecular_function
GO:0008150	biological_process	266	7155	0.0004	0.908	biological_process
GO:0003868	4-hydroxyphenylpyruvate dioxygenase activity	2	2	0.0009	1	molecular_function
GO:0004462	lactoylglutathione lyase activity	2	2	0.0009	1	molecular_function

Table 5
Top10 KEGG pathways enrichment in *notch1a* $^{-/-}$ Vp compared with *notch1a* $^{-/-}$ PBS groups.

Term	ID	Sample number	Background number	P-Value	Corrected P-Value
SNARE interactions in vesicular transport	ko04130	3	10	0.0086	0.7607
Biosynthesis of unsaturated fatty acids	ko01040	2	5	0.0215	0.7607
Insulin secretion	ko04911	4	34	0.0388	0.7607
Measles	ko05162	3	22	0.0514	0.7607
Cell cycle - yeast	ko04111	2	9	0.0517	0.7607
Synaptic vesicle cycle	ko04721	3	23	0.0567	0.7607
Cysteine and methionine metabolism	ko00270	2	10	0.0606	0.7607
Staphylococcus aureus infection	ko05150	2	10	0.0606	0.7607
Meiosis - yeast	ko04113	2	10	0.0606	0.7607
Cell cycle	ko04110	3	25	0.0680	0.7607

RT-qPCR results of all examined genes matched the expression patterns of the transcriptome sequencing data (Fig. 6).

Next, the relative expression of these six genes was analyzed 2, 4, 8, 12, and 24 hpi in WT PBS, WT Vp, *notch1a*$^{-/-}$ PBS, and *notch1a*$^{-/-}$ Vp larvae (Fig. 7). In WT Vp larvae, the genes *il1b* and *nfkbiaa* were significantly up-regulated at 2, 4, and 8 hpi, and mRNA levels peaked 4 hpi. The gene *tnfb* was up-regulated at 2 and 4 hpi. The gene *ctslb* was significantly down-regulated at 2 and 4 hpi, but its expression was not significantly different at other time points. The gene *il13ra1* was significantly up-regulated at 4, 8, 12, and 24 hpi but not 2 hpi. The gene *c3a.3* was up-regulated at all time points. In *notch1a*$^{-/-}$ PBS larvae, there was no difference in expression of *il1b*, *tnfb*, *nfkbiaa*, *il13ra1*, or *c3a.3*, but the gene *ctslb* was significantly up-regulated. In *notch1a*$^{-/-}$ Vp larvae, the genes *il1b*, *tnfb*, and *nfkbiaa* were up-regulated 2, 4, and 8 hpi, the gene *ctslb* was up-regulated 2, 8, 12, and 24 hpi, and the genes *il13ra1* and *c3a.3* were up-regulated at all time points. However, expression of the genes *il1b*, *tnfb*, and *nfkbiaa* was lower, whereas expression of the genes *il13ra1* and *c3a.3* was higher in *notch1a*$^{-/-}$ Vp

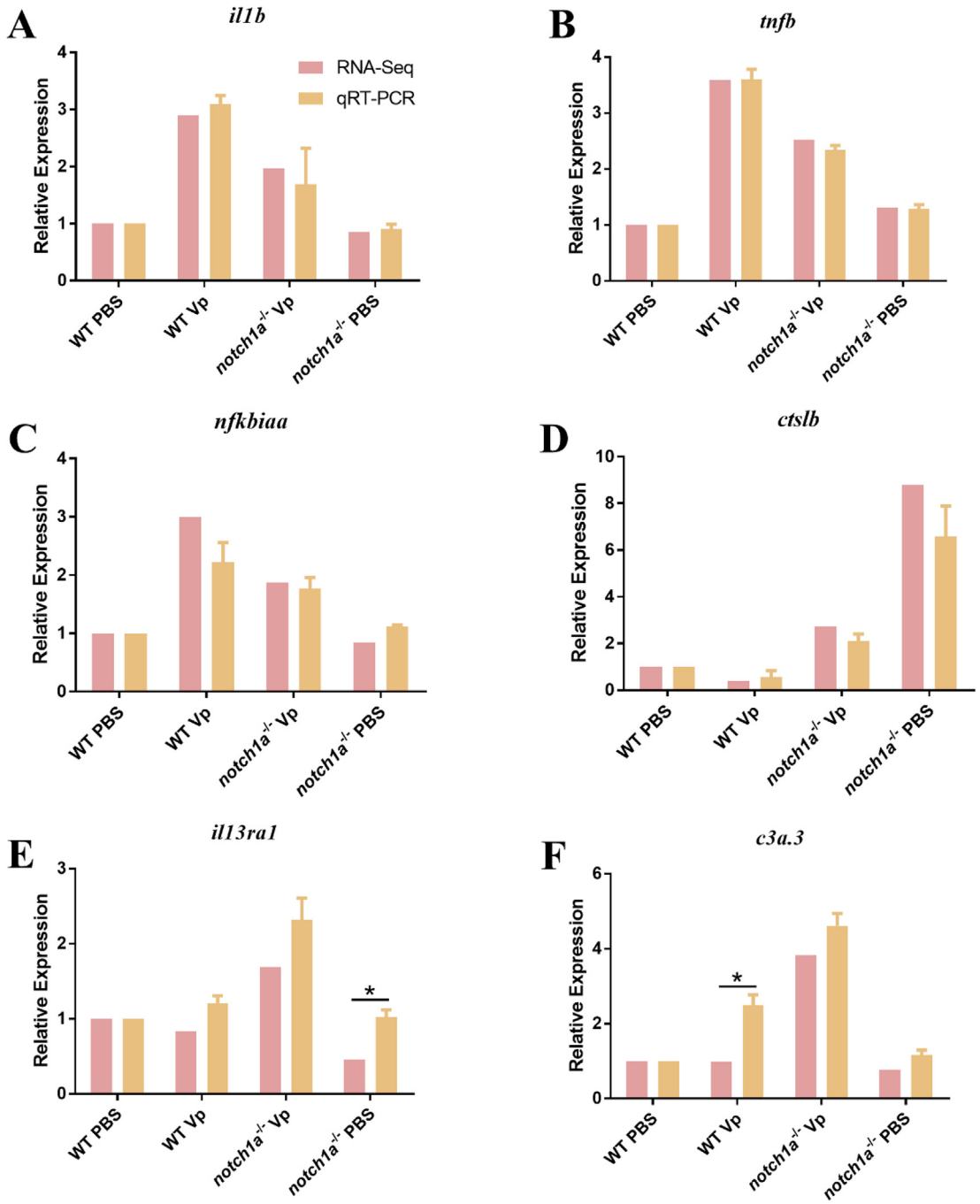

Fig. 6. Validation of transcriptome data by quantitative reverse transcription-polymerase chain reaction (RT-qPCR). Relative expression refers to the ratio of gene expression of WT Vp, *notch1a*$^{-/-}$ Vp, and *notch1a*$^{-/-}$ PBS with WT PBS groups. The following six genes were analyzed: (A) *il1b*, (B) *tnfb*, (C) *nfkbiaa*, (D) *ctslb*, (E) *il13ra1*, and (F) *c3a.3*. RT-qPCR results were normalized to the zebrafish *ef1a* gene. *p < 0.05.

larvae than in WT Vp larvae. Although the gene *ctslb* was up-regulated in *notch1a*$^{-/-}$ Vp 2, 8, 12, and 24 hpi, its expression level was lower than in *notch1a*$^{-/-}$ PBS larvae. These results indicate that the genes *il1b*, *tnfb*, *nfkbiaa*, *ctslb*, *il13ra1*, and *c3a.3* indeed play a role in the innate immune response to Vp13, and Notch1a promotes the expression of *il1b*, *tnfb*, and *nfkbiaa* while inhibiting the expression of *ctslb*, *il13ra1*, and *c3a.3*.

4. Discussion

The innate immune response is activated at 2 hpi in *V. parahaemolyticus*-infected WT and *notch1a*$^{-/-}$ larvae. In the present study, 359 DEGs (246 down-regulated genes and 113 up-regulated genes) were observed in infected WT larvae compared with control WT larvae, and 986 DEGs (82 down-regulated genes and 904 up-regulated genes) were observed in infected *notch1a*$^{-/-}$ larvae compared with control *notch1a*$^{-/-}$ larvae. More genes were up-regulated in infected *notch1a*$^{-/-}$ larvae, indicating that Notch1a plays an important role in inhibiting innate immune responses during infection. Additionally, although more genes were significantly up-regulated, the LD_{50} of *V. parahaemolyticus* was lower in *notch1a*$^{-/-}$ larvae, meaning that the *notch1a*$^{-/-}$ survival rate was lower than that of WT larvae. We speculated that *notch1a*$^{-/-}$ larvae were more sensitive to infection based on the facts that more genes were differentially expressed in infected *notch1a*$^{-/-}$ larvae and that most

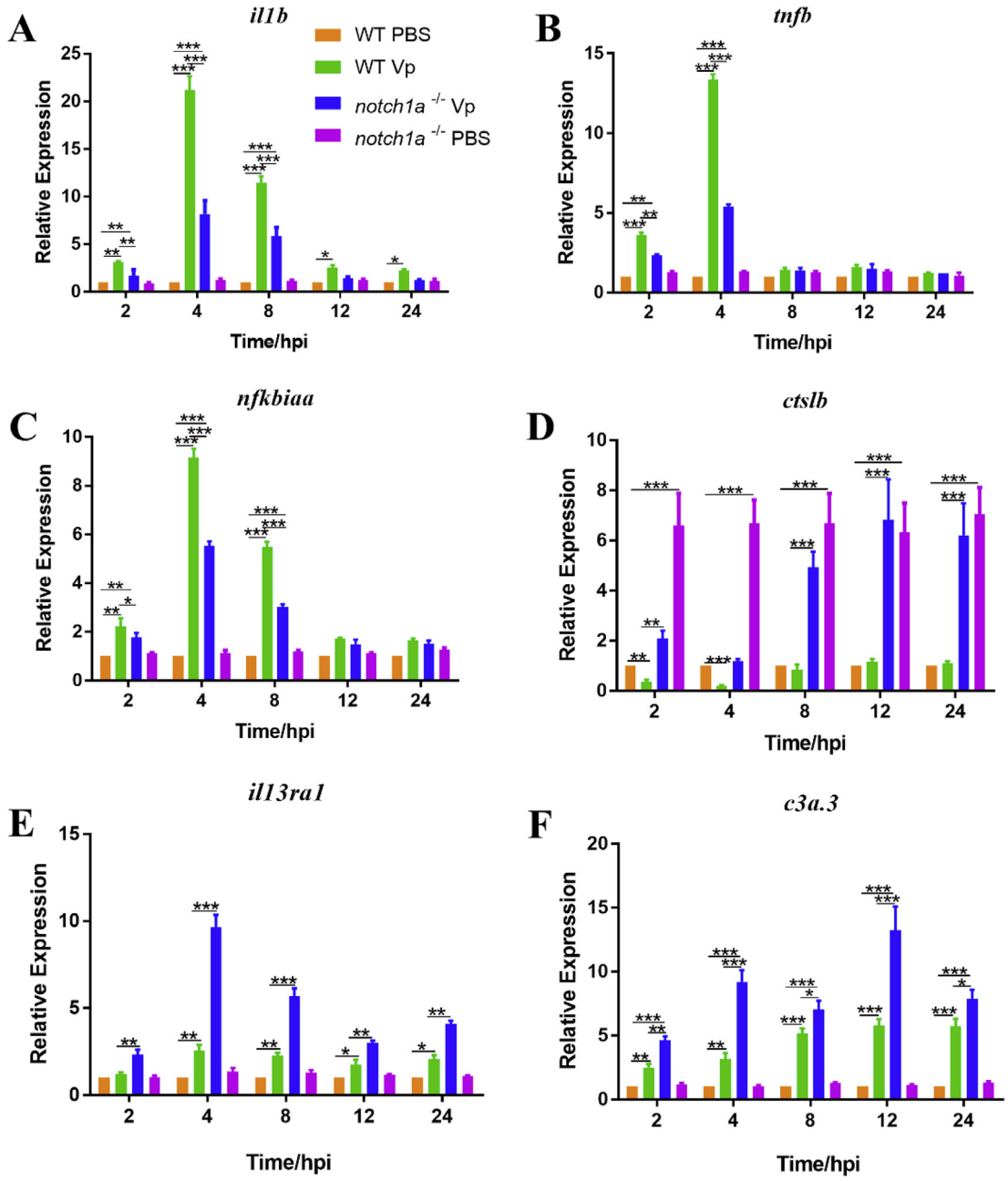

Fig. 7. RT-qPCR analysis of genes expression of WT PBS, WT Vp, *notch1a*$^{-/-}$ PBS and *notch1a*$^{-/-}$ Vp groups at 2, 4, 8, 12 and 24 h post infection (hpi). (A) *il1b*, (B) *tnfb*, (C) *nfkbiaa*, (D) *ctslb*, (E) *il13ra1*, and (F) *c3a.3*. RT-qPCR results were normalized to the zebrafish *ef1a* gene. *p < 0.05, **p < 0.005, ***p < 0.001.

DEGs were up-regulated. As a result, the balance between the response to infection and the avoidance of unregulated inflammation was disturbed [28]. Alternatively, *notch1a*$^{-/-}$ larvae may be weaker than WT larvae, as *notch1a*$^{-/-}$ larvae died before 15 dpf, and the activation of more genes in larvae with weak immune systems may lead to a lower tolerance to infection and immune- or pathogen-inflicted damage [29]. The lack of resistance and tolerance to infection in *notch1a*$^{-/-}$ larvae suggests that the functions of innate immune cells, such as pathogen recognition, replication, and migration of neutrophils, monocytes, and natural killer (NK)-like cells, were impaired, leading to tissue damage during infection [29,30].

Notch1a regulates the TLR signaling pathway by regulating TLRs, NF-κB, and downstream cytokines. Previous studies have shown that the Notch signaling pathway can influence the function of macrophages [6–8]. Comparison of the transcriptome data of the WT Vp and *notch1a*$^{-/-}$ Vp groups revealed that the genes *tlr18*, *ccl35.1*, *il13ra1*, *nkap*, *crfb6*, *irf9*, and *tnfrsfa* were significantly up-regulated, whereas the genes *nfkbiaa* and *il1b* were significantly down-regulated in the *notch1a*$^{-/-}$ Vp group. The gene *tlr18* is a TLR1 family member and a substitute for mammalian TLR6 or TLR10 [31–33]. The gene *nkap* encodes an NF-κB-activating protein, and the gene *nfkbiaa* encodes an NF-κB inhibitor. These results are consistent with a previous study, which showed that the TLR signaling pathway is negatively regulated by the Notch signaling pathway [7]. We speculated that the function of Notch1a was to limit the expression of the cytokines *ccl35.1*, *il13ra1*, *crfb6*, *irf9*, and *tnfrsfa* in WT larvae infected with Vp13, but the mechanism of regulation was unclear. However, the expression of *il1b* was down-regulated in *notch1a*$^{-/-}$ larvae. Therefore, we speculate that other mechanisms regulate *il1b* expression. It is known that lectins mediate pattern recognition by functioning as phagocytosis receptors, soluble opsonins, and agglutinins. Intelectins are up-regulated in mammals and fishes after bacterial infection or lipopolysaccharide stimulation [34].

Galectins are synthesized by immune cells and can be released upon pathogen invasion [35]. In our results, the NLR family-related genes *nlrx1*, *nlrc3l*, and *nlrc3*, the intelectin family gene *itln3*, and the galectin family gene *lgals2b* were differentially expressed in *notch1a*$^{-/-}$ PBS and *notch1a*$^{-/-}$ Vp groups. Therefore, we speculate that Notch1a may also regulate NLR and lectin to mediate the function of PRRs.

The Notch signaling pathway also mediates cathepsin and serpin family members in the innate immune response. Cathepsins are a family of lysosomal proteases synthesized as inactive proenzymes that block the active site of enzymes. Previous studies have shown that cathepsins play a role in the fish immune system [36–40]. The serpins are a superfamily of serine protease inhibitors that control proteolytic activity and play critical roles in biological processes including blood coagulation, immune regulation, inflammation, and fibrinolysis [41–43]. In our results, nine cathepsin family genes and seven serpin family genes were differentially expressed in WT Vp, *notch1a*$^{-/-}$ Vp, or *notch1a*$^{-/-}$ PBS groups compared with the WT PBS group, indicating that the two families indeed play critical roles in innate immune responses to *V. parahaemolyticus* infection. In addition, the genes *ctsll*, *ctsbb*, *ctsc*, *serpinb1l4*, and *serpine3* were significantly down-regulated and the gene *ctslb* was significantly up-regulated in the *notch1a*$^{-/-}$ Vp group compared with the WT Vp group, demonstrating that Notch1a regulates the innate immune response by promoting *ctsll*, *ctsbb*, *ctsc*, *serpinb1l4*, and *serpine3* expression and inhibiting *ctslb* expression during infection.

The Notch signaling pathway can influence the function of immune-related pathways. In this study, we analyzed enriched GO terms and KEGG pathways in WT Vp and *notch1a*$^{-/-}$ Vp groups. In WT Vp groups, the most enriched GO terms and KEGG pathways were related to cytokine and chemokine pathways. However, no immune-related pathways were enriched in *notch1a*$^{-/-}$ Vp groups, although physiological processes such as enzyme activity, synthesis, transport, secretion and metabolism were enriched. During infection, the production and secretion of cytokines by immune cells in response to stimuli is an important process [44]. Chemokines, which can regulate the migration of leukocytes, are crucial to host defense, immunosurveillance, and immune cell recruitment [45]. In our results, mutation of *notch1a* led to changes in physiological process-related pathways, whereas no immune-related pathways were enriched, indicating that Notch1a is important for host maintenance of normal physiological processes and mediates the immune system, including cytokine and chemokine pathways, to defend the host during the initial stages of infection.

5. Conclusion

In summary, this is the first report of the comprehensive functions of Notch1a in the innate immune response. Previous study demonstrated that the Notch signaling pathway is an inhibitor of most immune-related pathways. However, our findings indicate that Notch1a may play various roles related to the innate immune response by regulating PRR signaling pathways, innate immune cells, cathepsin and serpin family members. Further studies are required to completely understand the interaction between the Notch signaling pathway and other innate immunity-related pathways.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.06.058.

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