Splenic protection network revealed by transcriptome analysis in inactivated vaccine-immunized flounder (*Paralichthys olivaceus*) against *Edwardsiella tarda* infection

Xiaoyan Wu, Jing Xing*, Xiaoqian Tang, Xiuzhen Sheng, Heng Chi and Wenbin Zhan*

Laboratory of Pathology and Immunology of Aquatic Animals, Key Laboratory of Mariculture, MOE, Ocean University of China, Qingdao, China

The protective immune response produced by fish after vaccination is crucial for vaccine effectiveness. Our previous studies have shown inactivated vaccine against *Edwardsiella tarda* can induce immune response in flounder (*Paralichthys olivaceus*). To elucidate the protective immune response at the genetic level, in this study, flounder was immunized with inactivated *E. tarda* for 5 weeks, and then they were challenged with *E. tarda*. The spleen was dissected at 7th day post immunization, 1st and 7th day post challenge, respectively. Transcriptome analysis showed that average of 46 million clean reads were obtained per library, while percentage of clean reads being mapped to reference genome was more than 89% in all cases, which suggested good quality of samples. As for differentially expressed genes (DEGs) identification in inactivated *E. tarda* groups, at 7th day post immunization, 1422 DEGs were identified and significantly enriched in innate immune-related pathways, such as Phagosome, Cell adhesion molecules and NF-kappa B signaling pathway; At 1st post challenge day, 1210 DEGs were identified and enriched to Antigen processing and presentation and Cell adhesion molecules, indicating that the pathogen was rapidly recognized and delivered; At 7th post challenge day, 1929 DEGs were identified, belonged to Toll-like receptor signaling pathway, Antigen processing and presentation, Th1 and Th2 cell differentiation and Th17 cell differentiation. Compared to 7th post immunization day, 73 immune-associated DEGs were identified at 1st post challenge day. Protein-protein interaction networks analysis revealed 11 hub genes (TLR7, TLR3, CXCR4, IFIH1, TLR8 etc), associated with recognition of pathogens and activation of innate immunity; while for 7th post challenge day, 141 immune-associated DEGs were identified. 30 hub genes (IL6, STAT1, HSP90A1, TLR7, IL12β etc) were associated with stimulation of lymphocyte differentiation and activation of cellular immunity. Ten immune-related genes were randomly selected for RT-qPCR validation at each time point. In conclusion, data
revealed protection of flounder against *E. tarda* infection by inactivated vaccine is mediated via immediate recognition of pathogen and subsequently activation of cellular immunity. Results give new aspect for vaccine protection cascades, is good references for vaccine evaluation.

**KEYWORDS**
immune protection, RNA-seq, spleen, *Paralichthys olivaceus*, *Edwardsiella tarda*

**Introduction**

Vaccines induce protective immune responses in fish (1, 2). The immune protection involves the relative percentage survival (RPS), antibody production and T/B lymphocyte response against challenge etc (3–5). RPS has been widely used to assess vaccine protection (6, 7). Besides of the RPS, the production of antibodies is the strategy of vaccines. Antibodies specifically recognize and bind antigens, which promote phagocytosis and achieve clearance of pathogens (8, 9). T/B lymphocytes are essential component of the adaptive immune response for assessing vaccine protection (10, 11). Study in turbot (*Scophthalmus maximus*) immunized with inactivated bivalent vaccine (*IVVah1*) showed high RPS to *Vibrio anguillarum* and *Vibrio harveyi* infection were maintained from week 4 to week 8 post immunization. In addition to RPS, antibody levels showed a trend of increasing and then decreasing, peaking at weeks 2 and 3. After 8 weeks of immunization against the pathogen, antibodies increased within a week. This indicates that at the protein and individual level, turbot immunized with IVVah1 are protected against the pathogen (12). In the study of vaccine-immunized flounder (*Paralichthys olivaceus*) challenged with *Edwardsiella tarda*, antibodies increased significantly at week 3. The percentage of T lymphocytes peaked at day 7. The percentage of IgM+ B lymphocytes showed a trend of increasing and then decreasing, peaking at week 2. This suggested that inactivated vaccine enhanced protective humoral and cellular immune responses in fish after challenge (13). In addition to this, the immune protection of fish vaccines needs to be supported by fine grained networks and comprehensive data. This is urgently needed for the development and application of effective vaccines.

Flounder is valuable marine fish. In recent years, with the rapid development of intensive aquaculture, the incidence of disease outbreaks has been increasing, which seriously affects the culture of flounder (14). *E. tarda* is a gram-negative bacterium with intracellular parasitism (15). It causes high mortality and significant economic losses in flounder through invasion of epithelial cells, production of toxins, and evasion of phagocyte-mediated killing (16, 17). Safe and effective vaccines are essential for the control of bacterial diseases (18, 19), among which inactivated vaccines have become commercialized vaccine species (20). For elucidating the protective immunity mechanism of inactivated vaccines, it is essential to study the response of immune organs in pre-vaccinated flounder after infection (21). Similar to mammals, the spleen of fish is the main peripheral lymphoid organ (22). Here most of the antigens in the blood are captured and engulfed by macrophages. At the same time, it is also the site of aggregation of T and B lymphocytes for antigen presentation and initiation of adaptive immune responses. The spleen is an essential organ for resolving the immune response in fish (23–25).

Transcriptome sequencing analyzes gene expression dynamics at the level of individual transcripts, which can contribute to the resolution of immune-related genetic information and functional molecules (26, 27). RNA-Seq has been applied to analyze immune-related genes and signaling pathways in fish such as flounder, grass carp, large yellow croaker and turbot (28–31). The spleen red blood cells of flounder challenged with *E. tarda* were analyzed by transcriptome. 21 key genes were identified, mainly involved in antigen processing and presentation, pathogen recognition and inflammation (32). The phagosome pathway was activated in the head kidney of turbot inoculated with bivalent inactivated bacteria vaccine *Aeromonas salmonicida* and *Vibrio scophthalmi*. Antigenic peptide transport protein 1 (TAP1), complement fraction 3 (C3) and mannose receptor (MR) were significantly upregulated (33). However, it is often limited to the analysis of tissues such as head kidney, gill and blood at different time points after immunization or infection. This is still inadequate for supporting the molecular mechanisms of immune protection in fish. In addition, genes in organisms are functionally interconnected and together control the activities of the organism (34). For example, kidney from flounder infected with *E. tarda* was sequenced. Immune-related genes were used to construct protein-protein interaction networks (35). The identification of hub genes helps to understand the protective mechanism of the vaccine.

In this study, flounder were immunized with inactivated *E. tarda* vaccine for five weeks and then challenged with *E. tarda*. **Wu et al.** 10.3389/fimmu.2022.1058599
The infection status of flounder was evaluated. Spleens of flounder were sampled for transcriptome analysis at 7th post immunization day, 1st and 7th post challenge day. RNA sequencing (RNA-Seq) was performed using the Illumina Novaseq6000 platform. The results showed that an average of 46 million clean reads were obtained per library, with clean reads accounting for more than 99% of raw reads and Q30 greater than 92% in all cases; while the percentage of clean reads being mapped to the reference genome was more than 89% in all cases. 1422, 1210 and 1929 differentially expressed genes (DEGs) were identified at 7th post immunization, 1st and 7th post challenge day, respectively. Differential genes were annotated into Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional databases. These genes are involved in many immune processes, mainly Toll-like receptor signaling pathways, antigen presentation and presentation, and Th1 and Th2 cell differentiation. At 1st and 7th post challenge day, respectively, 73 and 141 immune-related DEGs were used to construct protein-protein interaction network, predicting 11 and 30 hub genes involved in the immune response. This study provides the basis for further elucidation of the immune protection against bacterial infection in flounder immunized with inactivated vaccine.

Materials and methods

Experiment fish

Healthy flounder (P. olivaceus) (length range: 15-17 cm) were purchased from a farm in Rizhao, Shandong Province, China. The experimental fish were kept in the laboratory basement for two weeks, during which the water temperature was maintained at 21 ± 1°C in aerated seawater. The fish were fed with commercial pellets at 3% of body weight per day, and 1/3 of the seawater in the tanks was replaced. Before the experiment, flounders were randomly selected as free of pathogenic bacteria (36). Fish were anesthetized with tricaine methylate (MS-222, Sigma, USA) before tissue sampling. The treatment of fish in this study was approved by the Institutional Animal Care and Use Committee of the Ocean University of China (permit number: 20150101).

Inactivated Edwardsiella tarda vaccine

E. tarda HC01090721 strain was isolated from the ascites of diseased flounder by researchers in our laboratory and stored in brain heart infusion (BHI) medium containing 15% glycerol at -80°C (37). The inactivated E. tarda vaccine was prepared by referring to the previous process (38). First, the conserved strains were inoculated on BHI solid medium by continuous scribing and cultured at 28°C for 24 h. Single colonies were inoculated on BHI liquid medium in oscillating incubator at 28°C and 180 rpm for expansion. After the bacteria had grown to the logarithmic growth phase, bacterial precipitates were obtained by centrifugation at 8000 g for 5 min and resuspended in sterilized 0.01 M phosphate-buffered saline (PBS; pH=7.4). The bacterial concentration was adjusted to 10× 10⁹ CFU/mL and inactivated by the adding 0.5% formalin (V/V) and shaking at 4°C for 72 h. The precipitate was collected by centrifugation at 8000 g for 5 min and resuspended with sterile PBS. 200 µL of inactivated bacteria were coated in BHI solid medium and incubated for 24 h at 28°C. The inactivation was proved to be successful if no colonies grew. The bacterial concentration was adjusted to 2.0×10⁹ CFU/mL and stored in a refrigerator at 4°C until use.

Vaccination, challenge and sampling

Healthy flounder were randomly divided into two groups, the PBS group was injected intraperitoneally with 100 µL of PBS with complete Freund’s adjuvant (1:1) and the inactivated vaccine group was injected intraperitoneally with 100 µL of inactivated E. tarda with complete Freund’s adjuvant (1:1). After 5 weeks of immunization, 1.0 × 10⁶ CFU of E. tarda was inoculated for the challenge experiment. At 7th day post immunization and 1st and 7th post challenge, respectively, spleens from three fish in each group were randomly sampled and snap-frozen in liquid nitrogen for RNA extraction. Samples were marked: CS-7 (CS-7-1/CS-7-2/CS-7-3) for fish on the seventh day after immunization with PBS, IPS-7 (IPS-7-1/IPS-7-2/IPS-7-3) for fish on the seventh day after immunization with inactivated vaccine, ACS-1 (ACS-1-1/ACS-1-2/ACS-1-3) for fish on the first day after five weeks of PBS immunization with E. tarda infection, AIPS-1 (AIPS-1-1/AIPS-1-2/AIPS-1-3) for fish on the first day after five weeks of vaccine immunization with E. tarda infection, ACS-7 (ACS-7-1/ACS-7-2/ACS-7-3) for fish on the seventh day after five weeks of PBS immunization with E. tarda infection, AIPS-7 (AIPS-7-1/AIPS-7-2/AIPS-7-3) for fish on the seventh day after five weeks of vaccine immunization with E. tarda infection. To detect the infection status of sampled fish, remaining spleen tissue after challenge was immersed in RNA Later (TaKaRa) for detection of bacterial load and also fully embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek USA) and stored at -80°C for 4 h for immunofluorescence analysis. The specific experimental procedure is shown in Figure 1.

RNA sample preparation, library construction and sequencing

For transcriptome analysis, 18 RNA-seq libraries were constructed using spleens. RNA was extracted from spleen
using the Trizol according to the manufacturer’s instructions for RNA-seq analysis and subsequent RT-qPCR validation of transcriptomic data. RNA samples from spleens were subjected to RNase free agarose gel electrophoresis and Agilent 2100 Bioanalyzer for quality and integrity. The mRNA was enriched by Oligo(dT) beads and fragmented with fragmentation buffer. The mRNA was reverse transcribed into cDNA using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA). cDNA was purified using 1.8X Agencourt AMPure XP Beads, followed by end repair, the addition of base A, and ligation of sequencing adapters. Size selection of ligated fragments was performed by agarose gel electrophoresis and polymerase chain reaction (PCR). Sequencing of 18 cDNA libraries from spleen samples was performed on the Illumina Novaseq6000 platform at Gene Denovo Biotechnology Co. (Guangzhou, China).

Data quality control, sequence alignment analysis and expression statistics

Raw reads of the spleen cDNA libraries contained low quality bases. The reads containing more than 10% unknown nucleotides and containing more than 50% low quality (Q-value ≤ 20) bases and containing adapter are removed according to FASTP (version 0.18.0) (39) to obtain high quality clean reads from the spleen cDNA libraries. The clean reads were aligned to the ribosomal RNA (rRNA) database using the short reads alignment tool bowtie2 (40), and the aligned ribosomal reads in the spleen cDNA libraries were removed. The retained clean reads were used for subsequent transcriptome analysis. The clean reads were mapped to the flounder reference genome (GenBank project accession: PRJNA344006) using HISAT2.2.4 (41). The mapped reads from 18 spleen cDNA libraries were assembled respectively by using StringTie v1.3.1 (42, 43). For each transcribed region, FPKM (fragment per kilobase of transcript per million mapped reads) values were calculated using RSEM (44) to quantify its expression abundance and variation.

Analysis of differentially expressed genes, GO and KEGG functional enrichment

Splenic differentially expressed genes (DEGs) between the PBS and inactivated vaccine groups were analyzed using DESeq2 (45), and the screening criteria for differential genes were $p \leq 0.05$ and expression fold change > 1.5. To further understand the biological functions of the genes, DEGs were mapped to terms in the GO database (http://www.geneontology.org/), and the number of genes per term was calculated. GO terms that were significantly enriched to differential genes compared to background genes were identified. KEGG (http://www.genome.jp/kegg/) is the main database on Pathway. Pathways that were significantly enriched by differential genes compared to background genes were obtained. A hypergeometric test was performed to identify significantly enriched GO terms and KEGG pathways using $p$-value $\leq 0.05$ as the threshold.

Protein-protein interaction network analysis

Venn diagram was used to demonstrate shared and unique DEGs for the three comparison groups after immunization and challenge. String (http://string-db.org) was applied to analyze protein-protein interactions that upregulate immune-related DEGs at 1st and 7th after challenge day, respectively. Cytoscape (V3.7.1) (https://cytoscape.org/) was used to
visualize network file in which genes as nodes and interaction relationships as lines of the network. Studying the protein-protein interaction network helps to identify hub genes.

Quantitative real-time polymerase chain reaction

To analyze the bacterial load in the spleen of *E. tarda* infection, spleen DNA was extracted using the TIANamp Marine Animals DNA Kit (Tiangen, Beijing, China) according to the manufacturer’s instructions. Specific primers (F: TACCTAGAGCTGCTGCA; R: CTCATCTGGCCAGCTCTT) were used for the amplification of *E. tarda* gene fragments. Each sample was taken in triplicate. The bacterial load in the spleen (log_{10} *E. tarda* cells/0.1 g) was quantified according to a previously established standard curve (46).

To verify the reliability of the transcriptome data, immune-related genes were selected for qRT-PCR. RNA was adjusted to the concentration of 1 μg/μL using Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was obtained using HiScript III RT SuperMix for qPCR (Vazyme, Nanjing, China) for RNA reverse transcription. Gene specific primers were designed using Primer Premier 5.0 and the primer sequences are listed in Table 1. β-actin was used as internal control. The mRNA levels of spleens in the PBS group at each time were set to 1. qRT-PCR was performed using the LightCycler® 480 II Real Time System (Roche, Basel, Switzerland). Each reaction system contained 10 μL of 2x Universal SYBR Green Fast qPCR Mix, 2 μL of cDNA template, 0.4 μL each of forward and reverse primers, and 7.2 μL of DEPC water. The reaction procedure was: pre-denaturation at 95°C for 3 min, 40 cycles including annealing at 95°C for 5 s and extension at 60°C for 30 s. Reactions were performed in triplicate. Gene expression levels were analyzed by the 2^{-ΔΔCt} method.

Indirect immunofluorescence assay

To analyze the infection of the spleen by *E. tarda* as described previously (47), in brief, sections obtained using Cryostats (Leica CM1900) were fixed in cold acetone for 15 min. Sections were washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBST) and then sealed with 5% BSA at 37°C for 45 min. Sections were then washed three times with PBST for 5 min each and incubated with rabbit anti-*E. tarda* polyclonal antibody (1:2000) as primary antibody for 1 h. After washing away the unconjugated primary antibody, sections were incubated with Alexa Flour 488-conjugated goat anti-rabbit IgG (1:1000, Thermo Fisher Scientific, Waltham, MA, USA) for 45 min in the dark at 37 °C. After three washes

| Primer name | Forward primer (5’-3’) | Reverse primer (5’-3’) | GenBank Accession No. |
|-------------|------------------------|------------------------|-----------------------|
| IL1β        | GAGATGGTGCGGATTCTCTGTG | ATGTTGAGGCTCTGAGGACTG  | XM_020105656.1        |
| CXCL12      | TTTGATTGTCTACCTCTGCA  | TCTTCACCTTGTTGATGGGA  | XM_020084401.1        |
| IL2R        | TACACTTCTCACCTGTCGCA   | CTTCAGGTGAGGTGTTGTTA   | XM_020083502.1        |
| RT1-B       | CGACGAGCTTTGCTTTTCTCC | CGACCTGGCCGATGTTCC    | XM_020102989.1        |
| LPAR4       | TAGCTGTTCCTTCTTCTGCG  | TACCTGATATCTCTCCACAC  | XM_020088202.1        |
| JUN         | TCCCAACAACATGAGATCC   | TCCACTGTTGATGGGAGA    | XM_020109221.1        |
| TLR7        | GCCTCAGCTCCAGGACTGCA  | CTGACACTGACAGGATGAG   | XM_020089659.1        |
| CCL19       | GTAACATCAAGAGATCACA   | GGAACGTGCCGAGGAGA     | XM_020102623.1        |
| IRE3        | CAGTTGAGGTGCTCTGACT   | TGCCCTAGCTGCTGTTGAG   | XM_020107055.1        |
| DHX58       | GGAATCTGAGGACTGACGC   | GGCCTCCAGCTGCTAACATC  | XM_020094280.1        |
| MAPK8       | TTGACCCTCCAAAGAGAAATC | CACACTCCACTACCTAC    | XM_020092592.1        |
| MAPK14      | ATGGACACCCAAACCCCTGTA | ACCAGGGGACAACGCTTTG  | XM_020086273.1        |
| TLR8        | GGTGATTTGGCCTGTGAGT   | CTGCTGTACCCCTGACA    | XM_020089660.1        |
| GADD45β     | ACGTGGTGTCAGGAGGCTGTA | CCGAGGAACAGGAGGTGTA  | XM_020109774.1        |
| PAPPA2B     | CGACCAGCTCCTACTTCTCA | AGGCCGACCGTCAACCTCC   | XM_020083954.1        |
| CCL25       | GGCGGGTGTTAAGAGAGGATG | TGCCACAGACGTCGTTG    | XM_020081297.1        |
| CCL20       | AGGTTGCTGTTGAGTTGTC   | ATGATGGGAGGCCTCTCCA  | XM_020106265.1        |
| HSP90a.1    | CGCTGCTGGGCTCGTCATA  | GTGCTTCCAAACTCTTCTGCT | XM_020091873.1        |
| STAT1       | GCAGCAGGAGGCTCAGTGA   | AAGGGGCCGAGGACACTTG   | XM_020105149.1        |
| IL5Ra       | GTACGAGGTGAGCTGCAAGT  | AAGGATGGGAGGACAGAGA   | XM_020107801.1        |
| CXL8        | AGCTGACGCAATGGAGTCGA  | CCAGACTTAGGTGACCAGG   | XM_020010336.1        |
| MAPK14A     | TAATCATGGTCTGCGTGGAGA | GTGTTATAGGTTGCTGTCG   | XM_020089244.1        |
with PBST, the sections were counterstained with DAPI on the nuclei and incubated for 15 min at room temperature in the dark. After blocking with glycerol, the green positive signal was observed under fluorescent microscope (Olympus DP70, Tokyo, Japan). Rabbit negative serum was used as a negative control (1:1000 diluted in PBS).

Statistical analysis

All experiments were performed three times. Data were presented as mean ± standard deviation (SD). Data analysis was performed by SPSS 20.0 (IBM, Armonk, NY, USA). T-test was used to examine the differences between the PBS and inactivated vaccine groups. Differences were considered statistically significant when *p < 0.05. Graphs were plotted using GraphPad Prism 9 (Inc. San Diego, CA, USA).

Results

The load of *E. tarda* in the spleen of flounder after challenge

The presence of *E. tarda* in spleen samples was characterized quantitatively by qRT-PCR and qualitatively by indirect immunofluorescence (Figure 2). At 1st day post challenge, the bacterial loads in the spleen of PBS and inactivated vaccine groups showed small differences (p > 0.05). At 7th day post challenge, the spleen bacterial loads were significantly higher in the PBS group than in the inactivated vaccine group (p < 0.05). A large amount of specific green fluorescence was present in PBS group, whereas a small amount of green fluorescence was present in the vaccine group.

Transcriptome sequencing and read mapping of flounder

At 7th day post immunization, 1st and 7th day post challenge, respectively, 18 libraries of spleen RNA from PBS and inactivated vaccine group flounders were sequenced. To ensure data quality, raw reads were quality controlled. The six groups produced an average of 43875712, 44863902, 43172840, 55465904, 40599382, and 52051965 clean reads, and the ratio of clean reads to raw reads was above 99%. Among these clean reads, the rates of Q20 and Q30 were above 97% and 92%, respectively, which indicated that the sequencing results were of good quality. Clean reads were mapped to the flounder reference genome at a rate of over 89% in all cases, with an average of 20,542 genes annotated to each library (Table 2). The raw transcriptome sequencing data were submitted to the Sequence Read Archive (SRA) in NCBI. The accession number is PRJNA870695.

Analysis of differentially expressed genes after immunization

The distribution of DEGs between the PBS and inactivated vaccine groups is shown using the volcano plot (Figure 3A). At 7th day post immunization, there were 1020 genes upregulated and 402 genes downregulated (Figure 3D). Compared to the PBS group, interleukin-21 receptor-like (IL21R), interleukin-1 beta-
FIGURE 3
Volcano plots analysis of significantly different genes (DEGs) between samples from the PBS and inactivated vaccine groups at 7th post-immunization day (A) and 1st (B) and 7th (C) post challenge day. The red expression up-regulated and blue (expression down-regulated) points indicate differential expression of genes, and black points are no differences. Statistical plot of DEGs for the PBS and inactivated vaccine groups at three time points (D).

TABLE 2  Summary of transcriptome data from splenic samples.

| Sample   | Raw_Data (bp) | Clean_Data (bp) | Clean reads (%) | Q20 (%) | Q30 (%) | Total_Mapped (%) | Total_Genes |
|----------|---------------|-----------------|-----------------|---------|---------|-----------------|-------------|
| CS-7-1   | 36028808      | 35901210        | 99.65           | 97.22   | 92.57   | 90.23           | 20208       |
| CS-7-2   | 46360062      | 46205862        | 99.67           | 97.54   | 93.21   | 89.11           | 20297       |
| CS-7-3   | 49674892      | 49520064        | 99.69           | 97.72   | 93.62   | 89.18           | 20517       |
| IPS-7-1  | 43635786      | 43487332        | 99.66           | 97.82   | 93.84   | 90.96           | 21438       |
| IPS-7-2  | 44625620      | 44476768        | 99.67           | 97.64   | 93.39   | 90.63           | 20512       |
| IPS-7-3  | 46771046      | 46627606        | 99.69           | 97.73   | 93.65   | 90.47           | 20387       |
| ACS-1-1  | 37501910      | 37371230        | 99.65           | 97.30   | 92.77   | 89.87           | 20420       |
| ACS-1-2  | 45202050      | 45034962        | 99.63           | 97.10   | 92.36   | 90.35           | 20709       |
| ACS-1-3  | 47550540      | 47112328        | 99.11           | 97.47   | 93.08   | 90.40           | 20363       |
| AIPS-1-1 | 68575360      | 68364728        | 99.69           | 97.65   | 93.47   | 90.34           | 21007       |
| AIPS-1-2 | 47851060      | 47700772        | 99.69           | 97.67   | 93.53   | 90.48           | 20541       |
| AIPS-1-3 | 50503214      | 50332212        | 99.66           | 97.39   | 92.84   | 90.35           | 20839       |
| ACS-7-1  | 36282856      | 36157128        | 99.65           | 97.18   | 92.51   | 90.15           | 19969       |
| ACS-7-2  | 40904182      | 40764252        | 99.66           | 97.24   | 92.63   | 90.52           | 20401       |
| ACS-7-3  | 45026286      | 44876766        | 99.67           | 97.31   | 92.72   | 90.52           | 20361       |
| AIPS-7-1 | 47586886      | 47433578        | 99.68           | 97.67   | 93.50   | 90.72           | 20628       |
| AIPS-7-2 | 56620094      | 56488080        | 99.70           | 97.60   | 93.31   | 89.92           | 20335       |
| AIPS-7-3 | 52449960      | 52273510        | 99.68           | 97.66   | 93.49   | 89.85           | 20622       |
like (IL1β), C-C motif chemokine 25-like (CCL25), C-C motif chemokine 20-like (CCL20) and other interleukin and chemokine-related genes were significantly upregulated. Cytoplasmic dynein 1 intermediate chain 1 (DYNC1I1), thrombospondin-4-B-like (THBS4B), thrombospondin-2 isoform X1 (THBS2), tubulin beta chain isoform X2 (TUBB), tubulin beta-4B chain-like (TUBB4B), cathepsin L1-like (CTSS), CD209 antigen-like protein E (CD209E) and other phagosome-related genes were significantly upregulated. Contactin-1 (CNTN1A), claudin-23-like (CLDN23), claudin-3-like (CLDN3), neuroligin-3 (NLGN3), neuroligin-4, X-linked (NLGN4X), contactin-associated protein 1 (CNTNAP1) and other cell adhesion molecules-related genes were significantly upregulated. Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform-like (PIK3CA), phosphatidylinositol 3-kinase regulatory subunit beta-like (PIK3R2) and other phosphatidylinositol 3'-kinase-related genes were signifi-
cantly upregulated. Double-stranded RNA-specific adenosine deaminase (ADAR), cyclic GMP-AMP synthase (MB21D1) and other cytosolic DNA-sensing-related genes were significantly downregulated (Table 3).

GO functional analysis showed that DEGs were involved in biological process (BP), molecular function (MF) and cellular component (CC) after immunization (Figure 4). Top 20 GO terms of DEGs enrichment were shown according to p < 0.05, with some GO terms associated with immunization. DEGs were mainly enriched in receptor binding and actin binding of MF; cytoskeleton, extrinsic component of membrane and intermediate filament cytoskeleton of CC; biological adhesion and multimeric organisal process of BP (Figure 5A, Table 4).

DEGs were enriched to six branches in KEGG (Figure 6), and bubble plots were used to demonstrate the top 20 signaling pathways (p < 0.05). At 7th post immunization day, DEGs were significantly enriched to Phagosome, Cell adhesion molecules (CAMs), PI3K-Akt signaling pathway and NF-kappa B signaling pathway (Figure 7A).

**Early transcriptomic responses in the spleen of flounder challenged with *E. tarda***

On the first day after five weeks of immunization with *E. tarda* infection, 1210 DEGs (620 up-regulated genes and 590 down-regulated genes) were identified in the inactivated vaccine group compared with PBS group (Figures 3B, D). Toll-like receptor 3 (TLR3), toll-like receptor 7 (TLR7), toll-like receptor 8 (TLR8), toll/interleukin-1 receptor domain-containing adapter protein isoform X1 (TIRAP) and other toll-like receptor-related genes were significantly upregulated. Functional adhesion molecule B-like (JAM2), cadherin-4-like isoform X1 (CDH4), neural cell adhesion molecule 1-like isoform X1 (NCAM1), contactin-1a-like (CNTN1A) and other cell adhesion molecules-related genes were

| Gene name | ID | Description | Log2 (FoldChange) | PValue |
|-----------|----|-------------|--------------------|--------|
| IL21R | ncbi_109627108 | interleukin-21 receptor-like | 1.213259565 | 4.82E-05 |
| IL1β | ncbi_109641260 | interleukin-1 beta-like | 2.485190275 | 0.001848352 |
| CCL25 | ncbi_109625805 | C-C motif chemokine 25-like | 3.77792616 | 1.42E-05 |
| CCL20 | ncbi_109642248 | C-C motif chemokine 20-like | 2.321928095 | 0.039754988 |
| DYNC1I1 | ncbi_109631575 | cytoplasmic dynein 1 intermediate chain 1 | 2.640289575 | 0.043135722 |
| THBS4B | ncbi_1096264438 | thrombospondin-4-B-like | 3.788246099 | 0.014753712 |
| THBS2 | ncbi_109636524 | thrombospondin-2 isoform X1 | 3.475522895 | 0.00045301 |
| TUBB | ncbi_109633300 | tubulin beta chain isoform X2 | 3.05624296 | 0.031015255 |
| TUBB4B | ncbi_109634278 | tubulin beta-4B chain-like | 1.616223249 | 0.000128822 |
| CTSS | ncbi_109638800 | cathepsin L1-like | 0.716422913 | 0.025319 |
| CD209E | ncbi_109634808 | CD209 antigen-like protein E | 1.250803026 | 0.000128822 |
| CNTN1A | ncbi_109632653 | contactin-1 | 2.83550962 | 0.036055804 |
| CLDN23 | ncbi_109627775 | claudin-23-like | 3.74723393 | 0.012697027 |
| CLDN3 | ncbi_109630440 | claudin-3-like | 3.829564906 | 0.000113599 |
| NLGN3 | ncbi_109625629 | neuroligin-3 | 3.165877762 | 0.006895593 |
| NLGN4X | ncbi_109640043 | neuroligin-4, X-linked | 3.040412468 | 0.004256591 |
| CNTNAP1 | ncbi_109634287 | contactin-associated protein 1 | 2.72631835 | 0.018515565 |
| PIK3CA | ncbi_109642929 | phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform-like | 2.357552005 | 0.028189952 |
| PIK3R2 | ncbi_109623868 | phosphatidylinositol 3-kinase regulatory subunit beta-like | 1.518337769 | 0.019642222 |
| ADAR | ncbi_109633429 | double-stranded RNA-specific adenosine deaminase | -0.68558248 | 0.001415993 |
| MR21D1 | ncbi_109631848 | cyclic GMP-AMP synthase | -0.67861608 | 0.016658127 |
significantly upregulated. Inhibin beta B chain-like (INHBB), C-X-C chemokine receptor type 4-like (CXCR4), interleukin-21 receptor-like (IL21R), bone morphogenetic protein receptor type-1B isoform X1 (BMPR1B) and other Cytokine-cytokine receptor-related genes were significantly upregulated. Rano class II histocompatibility antigen, A beta chain-like (RT1-B), H-2 class II histocompatibility antigen, A-U alpha chain-like (RT1-Ba) and other Antigen processing and presentation-related genes were significantly upregulated. DNA replication ATP-dependent helicase/nuclease DNA2 isoform X1 (DNA2), replication factor C subunit 5 (RFC5), replication factor C subunit 2 (RFC2) and other cell cycle-related genes were significantly downregulated. G2 and S phase-expressed protein 1 isoform X1 (GTSE1), serine/threonine-protein kinase Chk1 (CHEK1), cyclin-G2-like (CCNG2) and other p53-related genes were significantly downregulated (Table 5).

GO functional analysis showed that at 1st day post challenge, DEGs were mainly enriched to motor activity and tubulin binding of MF; cytoskeleton and microtubule cytoskeleton of CC; DNA metabolic process, cell cycle, and cell cycle process of BP (p < 0.05) (Figure 5B, Table 6).

KEGG functional analysis showed that DEGs were significantly enriched to Antigen processing and presentation, Cell adhesion molecules (CAMs), p53 signaling pathway (p < 0.05) (Figure 7B).

**Late transcriptomic responses in the spleen of flounder challenged with E. tarda**

On the seventh day after five weeks of immunization with E. tarda infection, compared with the PBS group, 1106 genes were up-regulated and 823 genes were down-regulated (Figures 3C, D).

Toll-like receptor 7 (TLR7), signal transducer and activator of transcription 1-alpha/beta-like isoform X1 (STAT1), toll-like receptor 5 (TLR5) and other Toll-like receptor-related genes were significantly upregulated. Interleukin-12 receptor subunit beta-2-like (ILL2RB2), interleukin-12 subunit beta-like (IL12B), protein jagged-2-like (JAG2), interleukin-6 (IL6), interleukin-6
TABLE 4 ID, description, class and specific P-value of Top 20 GO terms at 7th post immunization day.

| ID          | Description                                | Class            | P value   |
|-------------|--------------------------------------------|------------------|-----------|
| GO:0005102  | receptor binding                           | Molecular Function | 0.00527  |
| GO:0005882  | intermediate filament                      | Cellular Component | 0.005197 |
| GO:0045111  | intermediate filament cytoskeleton         | Cellular Component | 0.005197 |
| GO:0019898  | extrinsic component of membrane            | Cellular Component | 0.005656 |
| GO:0005856  | cytoskeleton                               | Cellular Component | 0.005944 |
| GO:0002610  | biological adhesion                        | Biological Process | 0.000198 |
| GO:0003779  | actin binding                              | Molecular Function | 0.00324  |
| GO:0005272  | sodium channel activity                    | Molecular Function | 0.003345 |
| GO:0032501  | multicellular organismal process           | Biological Process | 0.000873 |
| GO:004707   | single-multicellular organism process      | Biological Process | 0.001025 |
| GO:0016310  | phosphorylation                            | Biological Process | 0.00132  |
| GO:0001871  | pattern binding                            | Molecular Function | 0.008587 |
| GO:0004312  | fatty acid synthase activity               | Molecular Function | 0.009584 |
| GO:0008905  | blood circulation                          | Biological Process | 0.004093 |
| GO:0008648  | protein phosphorylation                    | Biological Process | 0.004379 |
| GO:0007155  | cell adhesion                              | Biological Process | 0.004788 |
| GO:0090472  | cell-cell adhesion via plasma-membrane adhesion molecules | Biological Process | 0.00598 |
| GO:0007275  | multicellular organism development         | Biological Process | 0.008496 |
| GO:1903522  | regulation of blood circulation            | Biological Process | 0.008771 |
| GO:0051239  | regulation of multicellular organismal process | Biological Process | 0.011304 |

receptor subunit alpha-like (IL6R), heat shock protein HSP 90-alpha (HSP90A.1) and other T cell differentiation-related genes were significantly upregulated. Fibroblast growth factor 1 (FGF1), lysophosphatidic acid receptor 3 (LPAR3), lysophosphatidic acid receptor 4 (LPAR4), laminin subunit beta-1 (LAMB1), integrin beta-4 isoform XI (ITGB4) and other PI3K-Akt signalling pathway-related genes were significantly upregulated. Claudin-3-like (CLDN3), E-selectin
SELE, L-selectin-like (SELL) and other Cell adhesion molecules-related genes were significantly upregulated. Mitogen-activated protein kinase 14A-like isoform X2 (MAPK14A), mitogen-activated protein kinase 8-like isoform X1 (MAPK8) and other MAPK-related genes were significantly downregulated (Table 7).

GO functional analysis showed that DEGs were mainly enriched to NADH dehydrogenase activity, G-protein coupled nucleotide receptor activity and iron ion binding of MF, glycerol-3-phosphate metabolic process and endothelial cell differentiation of BP (Figure 5C, Table 8).

KEGG functional analysis showed that DEGs were significantly enriched to Th1 and Th2 cell differentiation, Th17 cell differentiation, IL-17 signaling pathway, Antigen processing and presentation, Toll-like receptor signaling pathway and RIG-I-like receptor signaling pathway, which are closely related to immunity (Figure 7C).

Compared with immunization, the protection of vaccine on flounder after challenge

To explore the protection of vaccine after challenge compared to immunization. Venn diagram was used to show the common and specific profiles of upregulated DEGs at three time points. A specific 512 DEGs were upregulated at 1st day post challenge compared to 7th day post immunization (Figure 8B). Immune-related 73 DEGs were used to construct protein-protein interaction networks (Table S1). Their expression levels at three time points are represented by heat map (Figure 8D). 57 DEGs showed interactive network relationships. 11 hub genes (TLR7, TLR3, CXCR4, TLR8 etc) were identified in the network according to the number of node connections, most of which belong to the Toll-like receptor signaling pathway (Figure 9, Table 9). A specific 1042 DEGs were upregulated at 7th day post challenge compared to 7th day post immunization (Figure 8C). Immune-related 141 DEGs were used to construct protein-protein interaction networks (Table S2). Their expression levels at three time points are also represented by heat map (Figure 8E). 127 DEGs showed interactive network relationships. 30 hub genes (IL6, STAT1, HSP90A1, TLR7, IL12B etc) were identified in the network according to the number of node connections (Table 10). In addition, a total of 9 DEGs were upregulated at the three time points. The immune-related DEGs were IL21R, Lpar4 (Figure 8A).

qRT-PCR validation of transcriptomic immune-related genes

The expression of ten immune-related genes at each time point was randomly examined using qRT-PCR. At 7th day post immunization, the expression of IL1b, CXCL12, IL21R, RT1-B and LPAR4 were upregulated, while the expression of TLR7, CCL19, IRF5 and DHX58 were down-regulated (Figure 11A). At 1st day post challenge, the expressions of TLR7, IL21R, MAPK8, CXCL14, LPAR4 and TLR8 were up-regulated, while the expressions of GADD45B, PPAP2B, CCL25 and CCL20 expressions were down-regulated (Figure 11B). At 7th day post challenge, the expressions of IRF5, HSP90α1, IL21R, TLR7, STAT1 and LPAR4 were up-regulated, while the expressions of IL5Rα, CXCL8, MAPK14A and JUN were down-regulated (Figure 11C). The expression levels of immune-related genes were basically consistent with the transcriptome results at the three time points, indicating that the sequencing results were accurate and reliable.
### Table 5: Summary of immune-related genes in the inactivated vaccine group compared to the PBS group at 1st post challenge day.

| Genes name | ID                | Description                                                                 | Log2 (FoldChange) | PValue |
|------------|-------------------|------------------------------------------------------------------------------|-------------------|--------|
| TLR3       | ncbi_109641908    | toll-like receptor 3                                                          | 1.16598316        | 3.13E-06 |
| TLR7       | ncbi_109631070    | toll-like receptor 7                                                          | 1.439806437       | 3.30E-08 |
| TIRAP      | ncbi_109634523    | toll/interleukin-1 receptor domain-containing adapter protein isoform X1     | 0.794222571       | 0.01509113 |
| JAM2       | ncbi_109631164    | junctional adhesion molecule B-like                                           | 6.936441641       | 3.41E-05 |
| CDH4       | ncbi_109628880    | cadherin-4-like isoform X1                                                    | 1.854149134       | 0.014162315 |
| NCAM1      | ncbi_109632006    | neural cell adhesion molecule 1-like isoform X1                             | 1.784131965       | 0.000125865 |
| CNTN1A     | ncbi_109624594    | contactin-1a-like                                                            | 1.438370003       | 0.027450591 |
| INHBB      | ncbi_109637318    | inhibin beta B chain-like                                                     | 7.69161905        | 0.00451912 |
| CXCR4      | ncbi_109647982    | C-X-C chemokine receptor type 4-like                                         | 3.93412064        | 0.000209948 |
| IL21R      | ncbi_109627108    | interleukin-21 receptor-like                                                  | 1.57052058        | 0.004386107 |
| BMPRIB     | ncbi_109638193    | bone morphogenetic protein receptor type-1B isoform X1                       | 1.304854582       | 0.010382372 |
| RT1-B      | ncbi_109633037    | rano class II histocompatibility antigen, A beta chain-like                  | 1.699037395       | 0.001496312 |
| RT1-BA     | ncbi_109634417    | H-2 class II histocompatibility antigen, A-U alpha chain-like                | 1.42949101        | 0.003743397 |
| DNA2       | ncbi_109636867    | DNA replication ATP-dependent helicase/nuclease DNA2 isoform X1              | -0.735787403      | 0.012755301 |
| RFC5       | ncbi_109637136    | replication factor C subunit 5                                               | -0.629558444      | 0.005990157 |
| RFC2       | ncbi_109641693    | replication factor C subunit 2                                               | -0.910283724      | 1.77E-06 |
| GTSE1      | ncbi_109624619    | G2 and S phase-expressed protein 1 isoform X1                               | -1.002573944      | 0.004375909 |
| CHEK1      | ncbi_109628292    | serine/threonine-protein kinase Chk1                                         | -0.625982809      | 0.026969525 |
| CCNG2      | ncbi_109646257    | cyclin-G2-like                                                               | -0.680328178      | 0.000142464 |

### Table 6: ID, description, class and specific P-value of Top 20 GO terms at 1st post challenge day.

| ID          | Description                      | Class               | P value |
|-------------|----------------------------------|---------------------|---------|
| GO:0006259  | DNA metabolic process            | Biological Process  | 0       |
| GO:0003774  | motor activity                   | Molecular Function  | 0       |
| GO:0005875  | microtubule associated complex   | Cellular Component  | 0.00002 |
| GO:0007049  | cell cycle                       | Biological Process  | 0.00001 |
| GO:0015631  | tubulin binding                  | Molecular Function  | 0.00007 |
| GO:0044430  | cytoskeletal part                 | Cellular Component  | 0.00032 |
| GO:0022402  | cell cycle process               | Biological Process  | 0.00006 |
| GO:0015630  | microtubule cytoskeleton         | Cellular Component  | 0.00065 |
| GO:0007017  | microtubule-based process        | Biological Process  | 0.00012 |
| GO:0005856  | cytoskeleton                     | Cellular Component  | 0.00024 |
| GO:0000280  | nuclear division                 | Biological Process  | 0.00075 |
| GO:0048285  | organelle fission                | Biological Process  | 0.00089 |
| GO:0034502  | protein localization to chromosome | Biological Process | 0.00138 |
| GO:0051276  | chromosome organization          | Biological Process  | 0.000427 |
| GO:0006887  | nitrogen compound metabolic process | Biological Process | 0.000582 |
| GO:0006725  | cellular aromatic compound metabolic process | Biological Process | 0.000622 |
| GO:0015669  | gas transport                    | Biological Process  | 0.000707 |
| GO:0008996  | organelle organization           | Biological Process  | 0.00093 |
| GO:0009987  | cellular process                 | Biological Process  | 0.001137 |
| GO:1901380  | organic cyclic compound metabolic process | Biological Process | 0.001235 |
TABLE 7  Summary of immune-related genes in the inactivated vaccine group compared to the PBS group at 7th post challenge day.

| Gene name | ID Description | Log2 (FoldChange) | PValue |
|-----------|----------------|-------------------|--------|
| TLR7      | ncbi_109631070 toll-like receptor 7 | 1.260077653 | 0.007232326 |
| STAT1     | ncbi_109640914 signal transducer and activator of transcription 1-alpha/beta-like isoform X1 | 1.103908424 | 1.16E-09 |
| TLR5      | ncbi_109643067 toll-like receptor 5 | 1.009899047 | 0.01759484 |
| IL12RB2   | ncbi_109625390 interleukin-12 receptor subunit beta-2-like | 2.231325546 | 0.018502643 |
| IL12B     | ncbi_109636980 interleukin-12 subunit beta-like | 2.127755547 | 0.03061254 |
| JAG2      | ncbi_109645635 protein jagged-2-like | 0.753996675 | 0.004852413 |
| IL6       | ncbi_109631714 interleukin-6 | 9.120669887 | 0.01019172 |
| IL6R      | ncbi_109633010 interleukin-6 receptor subunit alpha-like | 0.701641389 | 0.030149799 |
| HSP90A.1  | ncbi_109632540 heat shock protein HSP 90-alpha | 1.50077805 | 6.78E-08 |
| FGF1      | ncbi_109635763 fibroblast growth factor 1 | 1.919829651 | 0.046466637 |
| LPAR3     | ncbi_109625857 lysosphosphatic acid receptor 3 | 1.460125389 | 9.64E-05 |
| LPAR4     | ncbi_109630081 lysosphosphatic acid receptor 4 | 1.259106188 | 9.13E-05 |
| LAMB1     | ncbi_109642034 laminin subunit beta-1 | 1.245061497 | 9.77E-07 |
| ITGB4     | ncbi_109627076 integrin beta-4 isoform X1 | 0.976370254 | 0.031286644 |
| CLDN3     | ncbi_109630427 claudin-3-like | 2.611434712 | 0.042749421 |
| SELE      | ncbi_109625902 E-selectin | 0.845011148 | 0.048004897 |
| SELL      | ncbi_109625903 L-selectin-like | 0.823795888 | 0.010752208 |
| MAPK14A   | ncbi_109630822 mitogen-activated protein kinase 14A-like isoform X2 | -0.602102745 | 0.011441485 |
| MAPK8     | ncbi_109633007 mitogen-activated protein kinase 8-like isoform X1 | -0.633336555 | 0.018499353 |

TABLE 8  ID, description, class and specific P-value of Top 20 GO terms at 7th post challenge day.

| ID Description | Class | P value |
|----------------|-------|---------|
| GO:0003954     | NADH dehydrogenase activity | Molecular Function | 0.000882 |
| GO:0016655     | oxidoreductase activity, acting on NAD(P)H, quinone or similar compound as acceptor | Molecular Function | 0.000882 |
| GO:0005136     | NADH dehydrogenase (quinone) activity | Molecular Function | 0.000882 |
| GO:0001680     | G-protein coupled nucleotide receptor activity | Molecular Function | 0.001157 |
| GO:0016705     | oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen | Molecular Function | 0.001347 |
| GO:0005506     | iron ion binding | Molecular Function | 0.003659 |
| GO:0016651     | oxidoreductase activity, acting on NAD(P)H | Molecular Function | 0.004839 |
| GO:0016297     | acyl-[acyl-carrier-protein] hydrolase activity | Molecular Function | 0.006364 |
| GO:0016491     | oxidoreductase activity | Molecular Function | 0.008463 |
| GO:0003700     | transcription factor activity, sequence-specific DNA binding | Molecular Function | 0.009143 |
| GO:0006072     | glycerol-3-phosphate metabolic process | Biological Process | 0.01019172 |
| GO:00070997    | neuron death | Biological Process | 0.01019172 |
| GO:0045446     | endothelial cell differentiation | Biological Process | 0.01019172 |
| GO:0006739     | NADP metabolic process | Biological Process | 0.01019172 |
| GO:0035588     | G-protein coupled purinergic receptor signaling pathway | Biological Process | 0.005481 |
| GO:0035587     | purinergic receptor signaling pathway | Biological Process | 0.006307 |
| GO:0008154     | mitogen, cytokine or hormone receptor activity | Biological Process | 0.008987 |
| GO:0032535     | regulation of cellular component size | Biological Process | 0.009897 |
response in fish after vaccination and the status of the pathogen after infection. The spleen is the secondary immune organ with an important role in hematopoiesis and immunity. Here, flounder spleens were collected for RNA-Seq at 7th day post immunization, 1st and 7th day post *E. tarda* challenge, respectively. DEGs were functionally annotated to explore immune response.

Vaccines trigger the body to produce the immune response in order to provide protection in case of pathogenic challenge. When vaccination is administered, the immune system makes a complex series of responses (3, 5, 49). First, many cytokines and chemokines are induced, causing an inflammatory response (50, 51). On the seventh day of marbled sleeper goby vaccination with inactivated iridovirus and rhabdovirus bivalent vaccine, pro-

**FIGURE 8**

Venn diagram of upregulated genes between the PBS and inactivated vaccine groups at the three time points (A). Venn diagram of genes upregulated between the seventh day after immunization and the first day after challenge (B) and heat map of 73 immune-related genes specifically upregulated on the first day after challenge between the PBS and inactivated vaccine groups at the three time points (D). Venn diagram of genes upregulated between day 7 after immunization and day 7 after challenge (C) and heat map of 141 immune-associated genes specifically upregulated on day 7 after challenge in the PBS and inactivated vaccine groups at three time points (E). The color shades in the heat map represent the gene expression levels. Closer to red indicates greater expression and closer to blue less expression.
Inflammatory cytokines such as IL-1β, IFN-γ, and IL-2R are upregulated to activate the innate immune response and, more importantly, to trigger a specific immune response (52). In study of IL-1β, IL-8, TNF-α and G-CSF as adjuvants for OmpV of E. tarda subunit vaccine, IL-1β and IL-8 were reported to significantly enhance serum antibodies and sIg+B lymphocytes, and the expression of genes (CD4-1, CD4-2, MHCIIa and IgM) related to cellular and humoral immunity (53, 54). Similarly, in this study, inflammatory and chemotaxis-related genes such as IL21R, IL1b, CCL25, and CCL20 were significantly upregulated at day 7 after immunization, activating cell recruitment and laying the foundation for triggering adaptive immunity.

Phagocytosis is the process of host defense against pathogens. In innate immunity, macrophages, dendritic cells and neutrophils take up antigens through endocytosis. The antigen is then digested by lysosomes and the antigen signal is presented to the specific immune system (55). In adaptive immunity, B lymphocytes have been demonstrated to have phagocytic effect in fish. A previous study showed that B lymphocytes of dental flounder could phagocytose inactivated Lactococcus lactis (L. lactis). Transcriptome sequencing analysis of B lymphocytes after L. lactis stimulation showed that many DEGs were enriched to the phagocytic pathway. Further studies revealed the key role of Fc receptor (FcR) in regulating phagocytosis and bactericidal activity of B lymphocytes (56, 57). After 2 and 4 weeks of turbot (Scophthalmus maximus) inoculation with the bivalent inactivated bacteria vaccine Aeromonas salmonicida and Vibrio scophthalmi, Phage-associated genes such as Calreticulin (CALR), Antigen peptide transporter 1 (TAP1), and Integrin beta-3 (αVβ3) C-type mannose receptor 2 (MRC2) were upregulated in the kidney (53). Integrin beta-5-like (ITGB5), cytoplasmic dynein 1 intermediate chain 1 (DYNCLI1), and thrombospondin-4-B-like (THBS4B) of the Phagosome pathway were also significantly enriched in this study, indicating that the early immune response underwent antigen processing. Cell adhesion molecules are involved in recognition between cells by means of ligand and receptor binding. In addition, in the immune response, it transmits signals for the antigen delivery process (58). In muscles around the injection site of flounder (Paralichthys olivaceus) vaccinated with VAA DNA vaccine, cell adhesion molecules enhance the local immune response by mediating the recruitment of immune cells to the site of inflammation (59).

In addition, the PI3K-Akt signaling pathway is important node for signaling. It promotes cell proliferation, differentiation and anti-apoptosis after receiving extracellular signals (60). PIK3CD and PIK3R2 were identified as hub genes involved in the immune response in flounder gill infected with E. tarda. It was found that the expression of PIK3CD decreased continuously, while the expression of PIK3R2 increased and then decreased.

**TABLE 9** Description and degree of connectivity of the 11 immune-associated upregulated hub genes on the first day after challenge.

| Hub Gene | ID          | Description                  | Degree |
|----------|-------------|------------------------------|--------|
| TLR7     | ncbi_109631070 | toll-like receptor 7         | 12     |
| TLR3     | ncbi_109641908 | toll-like receptor 3         | 10     |
| IFIH1    | ncbi_109631067 | interferon-induced helicase C domain-containing protein 1 | 8      |
| MAPK8    | ncbi_109633007 | mitogen-activated protein kinase 8-like isoform X1 | 8      |
| TLR8     | ncbi_109631071 | toll-like receptor 8         | 7      |
| CXCR4    | ncbi_109647982 | C-X-C chemokine receptor type 4-like | 6      |
| GRIN2B   | ncbi_109627266 | glutamate receptor ionotropic, NMDA 2B-like isoform X1 | 6      |
| TRIM25   | ncbi_109627517 | E3 ubiquitin/SG515 ligase TRIM25-like | 6      |
| DHX58    | ncbi_109634039 | probable ATP-dependent RNA helicase DHX58 | 5      |
| NCAM1    | ncbi_109632006 | neural cell adhesion molecule 1-like isoform X1 | 5      |
| NRXN1A   | ncbi_109627041 | neurexin-1a-like isoform X1  | 5      |
**FIGURE 10**
Protein interaction network analysis of immune-related upregulated genes on day 7 after challenge.

**TABLE 10** Description and degree of connectivity of the 30 immune-associated upregulated hub genes on day 7 after challenge.

| Hub Gene | ID          | Description                                      | Degree |
|----------|-------------|--------------------------------------------------|--------|
| IL6      | ncbi_109631714 | interleukin-6                                    | 57     |
| EGFR     | ncbi_109646379  | epidermal growth factor receptor                 | 46     |
| STAT1    | ncbi_109640914  | signal transducer and activator of transcription 1-alpha/beta-like isoform X1 | 38     |
| FN1      | ncbi_109624813  | fibronectin-like                                 | 36     |
| NFkBIA   | ncbi_109632273  | NF-kappa-B inhibitor alpha-like                  | 33     |
| HSP90A.1 | ncbi_109632540  | heat shock protein HSP 90-alpha                 | 29     |
| HRAS     | ncbi_109641467  | GTPase HRas-like                                | 27     |
| PTK2     | ncbi_109638831  | focal adhesion kinase 1 isoform X2              | 26     |
| MAPK14   | ncbi_109632714  | mitogen-activated protein kinase 14-like        | 24     |
| TLR7     | ncbi_109631070  | toll-like receptor 7                             | 23     |
| CASP1    | ncbi_109630250  | caspase-1-like isoform X1                       | 22     |
| SOCS3    | ncbi_109633948  | suppressor of cytokine signaling 3              | 21     |
| MAPK11   | ncbi_109632714  | mitogen-activated protein kinase 14-like        | 18     |
| FAS      | ncbi_109631170  | tumor necrosis factor receptor superfamily member 6-like isoform X1 | 17     |
| IRE3     | ncbi_109642328  | interferon regulatory factor 3-like            | 17     |
| SOCS1    | ncbi_109645254  | suppressor of cytokine signaling 1             | 17     |
| HSPA5    | ncbi_109626157  | 78 kDa glucose-regulated protein               | 16     |
| IFIH1    | ncbi_109631067  | interferon-induced helicase C domain-containing protein 1 | 16     |
| CALR     | ncbi_109645752  | calreticulin-like                               | 15     |
| EZR      | ncbi_109647436  | ezrin-like                                      | 15     |
| ITGA2    | ncbi_109636955  | integrin alpha-2                                | 15     |
| FCGR3    | ncbi_109630247  | low affinity immunoglobulin gamma Fc region receptor III-like | 14     |
| TNFSF10  | ncbi_109645712  | tumor necrosis factor ligand superfamily member 10-like | 14     |
| SELP     | ncbi_109648028  | P-selectin-like                                 | 14     |
| HMOX     | ncbi_109627701  | heme oxygenase-like                             | 13     |
| HSC71    | ncbi_109628432  | heat shock cognate 71 kDa protein              | 13     |
| HSPB1    | ncbi_109628310  | heat shock protein beta-1                      | 13     |
| IL12B    | ncbi_109636980  | interleukin-12 subunit beta-like               | 13     |
| MAPK9    | ncbi_109626552  | mitogen-activated protein kinase 9-like        | 13     |
| SELL     | ncbi_109625903  | L-selectin-like                                 | 13     |
during infection. Combined with its transduction of antigenic signals, it affects the specific immune response, which is inhibited by *E. tarda* (61). In the present study, genes related to CAM (CNTN1A, CLDN23, CLDN3, NLGN3, NLGN4X) and PI3K family (PIK3R2, PIK3CA) were significantly upregulated after immunization.

At 1st day post challenge, pattern recognition receptors (PRRs), such as Toll-like receptors, elicit the body’s immune response by binding to pathogen-associated molecular patterns (PAMPs) (62). Eleven TLR family members were identified in flounder (63). TLR3, TLR7, TLR8 and TIRAP were significantly upregulated in this study. In mammals, TLR3, TLR7 and TLR8, located in intracellular vesicles, are critical receptors for the recognition of viral nucleic acids in the antiviral response (64). TLR7 was upregulated in both head kidney and spleen within 48 h after tongue sole infection with *Pseudomonas fluorescens*, and knockdown of TLR7 resulted in significantly higher bacterial load in tissues than in control (65). In the intestine of black rockfish *Sebastes schlegelii* infected with *E. tarda*, TLR3 showed upregulation at 2h, 6h, 12h and 24h (66). The TLR family of bony fish has a more complex immune response to pathogens such as viruses and bacteria than that of mammals. In the protective response of vaccines, antigen delivery is the initiating step of adaptive immunity. Antigen signals are presented to T cells, which can specifically bind and kill target cells or release cytokines to stimulate B cell proliferation and differentiation (67). Among them, MHC molecules expressed on antigen-presenting cells such as dendritic cells and B cells are the markers of presentation (68). MHC class I presents antigenic fragments to CD8+ T cells for killing of target cells by releasing cytoxic particles (perforin and granzyme) (69, 70). MHC class II presents antigenic fragments to CD4+ T cells to achieve expanded and increased immune effector functions through synthesis and release of cytokines (71). The expression of MHC Iα, MHC Iβ, CD4-1 and CD8α was significantly upregulated in immunized tissues when flounder was immunized with inactivated *E. tarda* vaccine. In addition, these genes were also significantly elevated in the spleen and head kidney after five weeks of immunization with *E. tarda* infection. This indicates that immunization activates both cellular and humoral immune responses (13, 47). Vaccinated Arctic Charr showed significant expression of TLR7 after infection with *Aeromonas salmonicida*, activating B cells and DC to produce IFN-α and triggering Th1 and CD8+ T cell responses to demonstrate the effect of vaccination (72). Antigen processing and presentation-related genes (TAP1, TAP2, ABCB9 and PSME2) were identified in flounder spleen erythrocytes infected with *E. tarda* for 24h (32). Full-length transcriptome sequencing was performed on several tissues (liver, kidney, intestine, skin, gill) involved in immune and metabolic processes in black rockfish (*Sebastes schlegelii*). Four immune-related genes annotated as H-2 class II histocompatibility antigens were mined (73). In the present study, RT1-B and RT1-Ba were identified. In addition, Cytokine-cytokine receptor interaction as well as adhesion molecules (CAMs) remained functional after *E. tarda* infection. Toll-like receptors form the initial barrier against pathogens by specifically recognizing pathogens. In addition, TLR signaling induces DCs to produce IL-1β, IL-6, IL-12, and chemokine receptors that regulate antigen-specific Th1 and Th2 cell differentiation, linking innate and adaptive immunity (74–77). Marbled sleeper goby vaccinated with inactivated iridovirus and rhabdovirus bivalent vaccine showed consistent upregulation of MHC I, CD8, TCR, MHC II, CD4 and IgM expression after 2 days of *Oxyeleotris marmoratus rhabdovirus* challenge, indicating rapid induction of cellular and humoral immunity (52). At 7th day post challenge, TLR5, TLR7, il6, il1b, and IL12B were still highly expressed, suggesting that TLR may play a role in both early and late stages of infection. In mammals, stimulated by different antigens, CD4+ T lymphocytes differentiate into different cell subtypes (Th1, Th2, Th17 and Treg cells). This mechanism has also been demonstrated in teleost fish (78). Flounder were immunized with the NADP-dependent isocitrate dehydrogenase (IDH) subunit vaccine of *E.

![Image](image-url)
The expression of Th1 and Th2 immune-related genes (IL-1β, TNF-α, IL-8, IL-6, NKEF, IFN-γ) was significantly increased. After infection with *E. tarda*, the bacterial load in the tissues was significantly reduced and the RPS reached 73.3%, which provided good protection against edwardsiellosis (46). In the present study, Th1 and Th2 cell differentiation, Th17 cell differentiation were significantly enriched, indicating that activation of cellular immunity plays an important role in vaccine protection against bacterial infection.

According to previous experiments, the immune response was initially activated at 7th day post immunization (36, 79). The response of the organism to the pathogen is strong and rapid after infection, with 24 h being important time point. 73 immune-related genes were activated on day 1 after *E. tarda* challenge compared to day 7 after immunization. 11 hub gene were identified, in which TLR family members were more enriched. TLR7 acts in organelles such as endoplasmic reticulum and lysosomes, which recognize viral single-stranded RNA and induce IFN-α, cytokine and chemokine production (80). In addition, the Toll-IL-1 receptor domain interacts with the junctional protein MyD88 in the antimicrobial immune response, activating the downstream NF-κB signaling pathway and producing pro-inflammatory cytokines. The bacterial load in the tissues of tongue sole (*Cynoglossus semilaevis*) infected with *Pseudomonas fluorescences* TSS was significantly enhanced after knockdown of CaTLR7. The results suggest that CaTLR7 has a positive role in the clearance of bacterial pathogens (65).

In mammals, TLR3 specifically recognizes viral double-stranded RNA (dsRNA) and triggers toll interleukin-1 receptor domain (TIR) through the myeloid differentiation factor 88 (MyD88) non-dependent pathway, activating downstream type I interferon gene expression and the NF-κB signaling pathway to induce an antiviral response in the organism (81). High expression of TLR3 was detected in the spleen and head kidney of channel catfish infected with virulent * Edwardsiella ictalurid* (82). TLR3 showed upregulation in the intestine of black rockfish *Sebastes schlegeli* for 24 h after *Edwardsiella tarda* infection (66). In the present study, TLR3 expression was upregulated on the first day after infection, suggesting that TLR3 in fish also plays a role in resistance to bacterial infection. TLR8, which is highly homologous to TLR7, functions in the lysosome to recognize bacterial or viral single-stranded RNA (83). TLR8 expression was detected in mucosal tissues (skin, gill and intestine) of turbot after infection with *Vibrio anguillarum* and *Streptococcus iniae* (84). Based on their central position in the interaction network, TLRs may play the important function in the anti-infection response of the fish spleen.

141 immune-related genes were activated on day 7 after *E. tarda* challenge compared to day 7 after immunization. Inflammatory cytokines (IL6, IL12B, IL6R, IL12Rβ2, IL17D), transcription factors (STAT1, IRF3) showed the strong interaction according to the functional classification. IL-12, which is involved in Th1 differentiation, is produced by DCs. IL12B (p40) and IL-12A (p35) together encode IL12, which acts by inducing the production of IFN-γ. IL-12Rβ1 and IL-12Rβ2 form the IL-12R complex, which is mainly expressed by activated T cells and natural killer cell. IL-12 and IL-12R binding activates the JAK2/STAT4 pathway to increase IFN-γ production as well as induce shift of T cells to Th1 phenotype (85). Th1 cells secrete IFN-γ, TNF-β, IL-2, etc. to mediate cellular immunity, which effectively defends against infection by intracellular pathogens (86, 87). A previous study showed that T-beta is a transcription factor involved in the immune response of Th1 cells. The expression was significantly increased after *E. tarda* infection, which laterally corroborates the important role of Th1 cells in responding to pathogenic infections. Moreover, IFN-γ and IL-2 were able to upregulate T-beta expression and contribute to the differentiation of Th0 cells to Th1 cell type (88). On day 14 of immunization with inactivated iridovirus and rhabdovirus bivalent vaccine and on day 7 of *Oxyeleotris marmoratus* iridovirus infection, the expression of inflammatory cytokines such as IL-12 and IL-1β was upregulated in the spleen of marbled sleepy goby, realizing the inflammatory of the innate immune response and leading to the development of adaptive immunity (52). In the present study, IL-6 was the hub gene of the protective immune network at 7th post challenge day. Previous studies have shown that significant upregulation of IL-6 was also detected after immunization of flounder with rIDH vaccine (46). IL-6 is pro-inflammatory cytokine produced mainly by macrophages and Th2 cells. IL-6 forms a complex with IL-6R, which binds to the membrane protein gp130, which activates intracellular signal transduction to function. It can promote the proliferation activation of T cells and the expression of IL-2 receptor on the surface of T cells, which further assists the proliferation and differentiation of B cells and the production of antibodies to participate in the humoral immune response (89, 90). A previous study showed that cyclosporine A (CsA) inhibited T lymphocyte expression by blocking activation of the transcription factor NFAT, and then inhibited B lymphocyte expression and antibody production. This suggests that T lymphocytes have an important regulatory role on B lymphocytes in the immune response (91, 92). In response to cytokines (IL-6, IFNγ) and growth factors (epidermal growth factor), STAT1 forms dimers that are transported to the nucleus to regulate apoptosis and the cell cycle (93). After *P. Olivaceus* was infected with *E. tarda* for 8h and 48h, STAT1 was identified as hub gene in blood, gill, and kidney expression profiling (35, 61, 94). In the present study, IL21R was significantly upregulated at all three time points, corroborating its protective role in immunity and infection. IL21R is cytokine receptor for IL21. It is expressed on activated NK cells and belongs to the type I cytokine
receptor. The receptor binds to IL21, leading to the activation of several downstream molecules (JAK1, JAK3, STAT1 and STAT3), while inducing the proliferation and differentiation of T cells, B cells and natural killer (NK) cells (95, 96).

The spleen, as an important lymphoid organ, is the main site of the immune response. Previous studies have found strong immune response in the spleen after flounder immunization or infection (13, 47, 97, 98). In the study of flounder immunized with inactivated E. tarda vaccine, the uptake of antigen and antigen presentation-related immune genes (MHC Iα, MHC IIα, CD4-1 and CD8α etc) were significantly elevated in the spleen. The immunohistochemical results of the spleen showed that CD4+ and CD8+ T lymphocyte were distributed around the melanocyte macrophage center (MMC). This fully demonstrates the important role of the spleen in capturing antigens, aggregating macrophages and lymphocyte populations, and presenting antigens to lymphocytes to activate the adaptive immunity (47, 97). Also in the study of immunized flounder infected with E. tarda, the immune response in the spleen varied significantly at the tissue, T/B lymphocyte and genetic levels and was the main tissue for monitoring the protective effect (13, 98). Keeping in line with previous studies, in this work, the spleen was sampled for transcriptome sequencing analysis after immunization with inactivated vaccine and infection with E. tarda. In terms of immune and protective responses, the strong immune response (cytokines, T- and B-cell related factors, etc.) in the spleen was also confirmed. In addition, in the immune system of fish, there are temporal differences in the production of cytokines, T/B lymphocytes, and antibodies, which are closely related to immune protection (12, 13). The overall immune response requires a multitemporal analysis, which is important for a comprehensive and detailed elucidation of vaccine immunization mechanism (99, 100). For example, the humoral immune response and the production of antibodies require a longer response time to be effective. IgM was significantly upregulated in marbled sleepy goby immunized with inactivated iridovirus and rhabdovirus bivalent vaccine for two weeks, indicating that the humoral immune response was activated (52). In the vaccine evaluation, antibodies were produced in fish during the immunization phase of two weeks to four weeks (12, 101, 102). The study of multiple time points of immune response is also worth doing to fully reveal the mechanism of fish vaccines.

Conclusions

This study investigated the mechanism of immune protection in flounder inoculated with inactivated E. tarda vaccine. Transcriptome sequencing analysis of the flounder spleen was performed on the seventh day after immunization and on the first and seventh day after five weeks of immunization with E. tarda infection. 1422, 1210 and 1929 DEGs were identified, respectively, which were significantly enriched in immune-related pathways such as Toll-like receptor signaling pathway and Th1 and Th2 cell differentiation. In addition, immune-related hub genes were identified after E. tarda infection compared to after immunization, in which TLR family members (TLR3, TLR5, TLR7, TLR8), pro-inflammatory cytokines and their receptors (IL6, IL6R, IL12B, IL12RB2, IL17D) were the main regulators that exerted immune protection. These genes are closely associated with the recognition and presentation of pathogens and the activation of cellular immunity. This study analyzed the transcriptional profiles of flounder spleen after immunization and infection, providing basis for further elucidation of the immune protection mechanisms in flounder immunized with inactivated E. tarda vaccine.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Ocean University of China (permit number: 20150101).

Author contributions

XW and JX contributed to the conception and design of this experiment, performed most of the experiments and statistical analysis, drafted and revised the manuscript. XT, HC, and XS participated in the design of the study, helped analyzed experiments and data. JX and WZ design the study, provided reagents, instruments and experiment space. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the National Natural Science Foundation of China (32173005; 31730101; 31672684; 31672685), Shandong Provincial Natural Science Foundation (ZR2020KC025), the National Key Research and Development Program of China (2018YFD0900503).
Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1058599/full#supplementary-material

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