Transcriptome Profiling Reveals Th17-Like Immune Responses Induced in Zebrafish Bath-Vaccinated with a Live Attenuated *Vibrio anguillarum*

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**Abstract**

**Background:** A candidate vaccine, live attenuated *Vibrio anguillarum* developed in our laboratory could prevent vibriosis of fish resulted from *V. anguillarum* and *V. alginolyticus*. To elucidate the molecular mechanisms underlying the vaccine protection, we used microarray technology to compare the spleen transcriptomes of bath-vaccinated and unvaccinated zebrafish at 28 days post vaccination.

**Principal Findings:** A total of 2164 genes and transcripts were differentially expressed, accounting for 4.9% of all genes represented on the chip. In addition to iron metabolism related to the innate immunity and the signaling pathways, these differentially expressed genes also involved in the adaptive immunity, mainly including the genes associated with B and T cells activation, proliferation and expansion. Transcription profiles of Th17-related transcription factors, cytokines and cytokine receptors during 35 days post-vaccination implied that Th17 cells be activated in bath-vaccinated zebrafish.

**Conclusion/Significance:** The transcriptome profiling with microarray revealed the Th17-like immune response to bath-vaccination with the live attenuated *V. anguillarum* in zebrafish.

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**Competing Interests:** The authors have declared that no competing interests exist.

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**Introduction**

*Vibrio anguillarum*, a Gram negative, curved rod bacterium, is the causative agent of vibriosis in cultivated fish. The vaccination against *V. anguillarum* is now recognized as a viable strategy for controlling vibriosis. A live attenuated vaccine has been successfully constructed in our laboratory by curing the virulence plasmid pEIB1 which encodes a very efficient iron uptake system mediated Fully constructed in our laboratory by curing the virulence plasmid controlling vibriosis. A live attenuated vaccine has been successively used in the field and is now recognized as a viable strategy for controlling vibriosis. The live attenuated vaccine was developed in our laboratory could prevent vibriosis of fish resulted from *V. anguillarum* and *V. alginolyticus*. To elucidate the molecular mechanisms underlying the vaccine protection, we used microarray technology to compare the spleen transcriptomes of bath-vaccinated and unvaccinated zebrafish at 28 days post vaccination.

**Background:** A candidate vaccine, live attenuated *Vibrio anguillarum* developed in our laboratory could prevent vibriosis of fish resulted from *V. anguillarum* and *V. alginolyticus*. To elucidate the molecular mechanisms underlying the vaccine protection, we used microarray technology to compare the spleen transcriptomes of bath-vaccinated and unvaccinated zebrafish at 28 days post vaccination.

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The ultimate goal of a vaccine is to develop long-lived immunity against a given pathogen depending on long-lived memory cells and effector cells. The memory T-cell compartment consists of both CD4+ and CD8+ T cells which can rapidly acquire effector functions to kill infected cells and/or secret inflammatory cytokines that inhibit replication of the pathogen [3]. Different classes of microbes elicit lineage-specific responses from the T-cell repertoire. While helper Th1 cells produce large quantity of interferon-γ (IFN-γ) to participate in cellular immunity against intracellular pathogen, Th2 cells produce mainly interleukin-4 (IL-4) to mediate the humoral responses to extracellular pathogen. More recently, a subset of CD4+ T cells which were observed to preferentially produce interleukin-17 (IL-17), but not IFN-γ and IL-4, were named Th17 cells. The Th17 cells orchestrate the mucosal defense against pathogen by secreting proinflammatory cytokines IL-17 and IL-22, which stimulate the airway, intestinal and skin epithelia to secrete chemokines and an array of antimicrobial peptides which repel assault from diverse infectious agents [4–6]. Th17 response is likely to emerge as an early response to a number of pathogens not handled well by Th1 or Th2-type immunity, which requires robust tissue inflammation to be cleared. Indeed, through the potent induction of chemokines, Th17 cells attract other subsets of T helper cells to sites of infection at later stages of the inflammatory process [7].
Although Th17 cells have not been identified in teleost, it is thought to be an ancient lineage highly conserved in all vertebrates including the jawless lamprey [8,9]. Zebrafish, for which an enormous amount of information on a genomics scale is available, is a well-studies model for the analysis of host-pathogen interaction during infectious disease [10]. Numerous genes associated with Th17 cells differentiation, function and signaling have been identified in this animal model. Five forms of zebrafish IL-17, the hallmark cytokines of Th17 cells, have been identified [11]. Nuclear RAR-related orphan receptor gamma (RORc), a master regulator of Th17 differentiation, is also conserved in zebrafish [12]. Other cytokines such as IL-21, IL-23 and IL-26 contributing to the Th17 cells differentiation and produced by Th17 cells were discovered in zebrafish too [13,14]. Very recently, the Th17-like immune responses in fish were reported. It was found that Th17 lineage might be related to the severity of side effects in Atlantic salmon vaccinated with oil-adjuvanted vaccine [15] while a Th17-like immune response was demonstrated to be induced in carp infected with a protozoan parasite [16]. However, Th17-like immune response of teleost induced by bath-vaccination with the live attenuated vaccine at 28 days post-vaccination using Zebrafish Agilent gene expression microarray chips was analyzed. Besides notably activated innate immunity, the expression profiling of the genes related to adaptive immunity responses suggested the maturation of B lymphocytes and the activation of Th17 pathway. In order to demonstrate whether Th17-like immune response be involved in the immune protection against Vibrio anguillarum, the differential expression of Th17 pathway-associated genes was investigated in inoculated zebrafish during 35 days post vaccination. The results revealed a Th17-like immune response as well as antibody-mediated immune response mainly contributing to the immunoprotection against Vibrio anguillarum in bath-vaccinated zebrafish.

| Term       | GO ID       | Description                                      | Gene No. | %*   | p value  |
|------------|-------------|--------------------------------------------------|----------|------|----------|
| BP_2       | GO:0044237  | Cellular metabolic process                        | 292      | 20.3 | 0.0000   |
| BP_2       | GO:0009058  | Biosynthetic process                              | 140      | 9.7  | 0.0015   |
| BP_2       | GO:0006807  | Nitrogen compound metabolic process               | 138      | 9.6  | 0.0035   |
| BP_2       | GO:0048522  | Positive regulation of cellular process           | 25       | 1.7  | 0.0114   |
| BP_2       | GO:0048518  | Positive regulation of biological process         | 26       | 1.8  | 0.0139   |
| BP_2       | GO:0055085  | Transmembrane transport                           | 44       | 3.1  | 0.0141   |
| BP_2       | GO:0009893  | Positive regulation of metabolic process          | 13       | 0.9  | 0.0148   |
| BP_2       | GO:0009056  | Catabolic process                                 | 46       | 3.2  | 0.0198   |
| BP_2       | GO:0006996  | Organelle organization                            | 39       | 2.7  | 0.0328   |
| BP_2       | GO:0043170  | Macromolecule metabolic process                   | 237      | 16.5 | 0.0390   |
| BP_2       | GO:0042440  | Pigment metabolic process                         | 5        | 0.3  | 0.0520   |
| BP_2       | GO:0044238  | Primary metabolic process                         | 293      | 20.4 | 0.0524   |
| BP_2       | GO:0043933  | Macromolecular complex subunit organization       | 18       | 1.2  | 0.0724   |
| BP_2       | GO:0048523  | Negative regulation of cellular process           | 21       | 1.5  | 0.0859   |
| BP_2       | GO:0006950  | Response to stress                                | 36       | 2.5  | 0.0889   |
| BP_2       | GO:0007163  | Establishment or maintenance of cell polarity     | 4        | 0.3  | 0.0980   |
| MF_2       | GO:0000166  | Nucleotide binding                                | 166      | 11.6 | 0.0016   |
| MF_2       | GO:0016563  | Transcription activator activity                  | 11       | 0.8  | 0.0046   |
| MF_2       | GO:0003676  | Nucleic acid binding                              | 198      | 13.8 | 0.0089   |
| MF_2       | GO:0008135  | Translation factor activity                       | 14       | 1.0  | 0.0160   |
| MF_2       | GO:0001882  | Nucleoside binding                                | 104      | 7.2  | 0.0330   |
| MF_2       | GO:0048037  | Cofactor binding                                  | 24       | 1.7  | 0.0390   |
| MF_2       | GO:0016874  | Ligase activity                                   | 26       | 1.8  | 0.0560   |
| MF_2       | GO:0008289  | Lipid binding                                     | 21       | 1.5  | 0.0580   |
| CC_2       | GO:0044424  | Intracellular part                                | 323      | 22.5 | 0.0015   |
| CC_2       | GO:0005622  | Intracellular                                     | 395      | 27.55| 0.0016   |
| CC_2       | GO:0031967  | Organelle envelope                                | 25       | 1.75 | 0.0182   |
| CC_2       | GO:0043227  | Membrane-bounded organelle                        | 223      | 15.55| 0.0429   |
| CC_2       | GO:0043233  | Organelle lumen                                   | 25       | 1.75 | 0.0525   |
| CC_2       | GO:0043229  | Intracellular organelle                           | 257      | 17.9 | 0.0686   |
| CC_2       | GO:0044422  | Organelle part                                    | 87       | 6.0  | 0.0816   |
| CC_2       | GO:0044446  | Intracellular organelle part                      | 87       | 6.0  | 0.0816   |

*the percentage of genes in the specific subcategory from each of the three GO ontologies.

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Materials and Methods

Ethics Statement

The animal work presented here was approved by the Animal Care Committee, East China University of Science and Technology (approval ID: 2006(272)).

Fish, Vaccination and Sampling

Six-month-old zebrafish weighing about 0.3 g were obtained from the animal center in our laboratory (Shanghai, China). They were reared in running dechlorinated and aerated water at 24°C on a 12:12 h light/dark rhythm and fed with commercial feed for aquatic animal twice per day. After 6 days of acclimation, 420 zebrafish were randomly divided into three vaccinated groups and three control groups. *V. anguillarum* MVAV6203 was cultured in high-salt Luria (LB) medium at 30°C for 16 h. The cells were harvested by centrifugation and rinsed twice in 2% saline. The desired number of cells was adjusted to 1×10^8 CFU/ml with 2% saline. Six groups of 70 zebrafish were immersed in the aerated cell-resuspended saline or 2% saline for 10 min at 24°C. At 1, 7, 14, 21, 28 and 35 days post-vaccination (dpv), 10 zebrafish from each group were euthanized and sacrificed to isolate the spleen tissue. For euthanasia, they were immersed in 300 ng ml^{-1} tricaine methanesulphonate (MS-222, Sigma, USA) for at least 10 min. At 28 days post-vaccination additional pool of spleen tissue of 10 zebrafish from each group was harvested for microarray hybridization.

RNA Preparation, Microarray Hybridization and Data Analysis

Total RNA was isolated from spleen samples by Trizol (Invitrogen, USA) according to the manufacturer’s instructions. The RNA samples were digested with DNase (Promega, USA) to eliminate genomic DNA contaminant. The quality of RNA samples was assessed using NanoDrop ND-1000 spectrophotometer (Labtech, USA). The integrity of RNA was assessed using standard denaturing agarose gel electrophoresis. From each sample, 1 μg of total RNA was amplified and transcribed into fluorescent cDNA with the manufacturer’s Agilent’s Quick Amp Labeling protocol (version 5.7, Agilent Technologies). The labeled cDNAs were hybridized onto the Whole Zebrafish Genome Oligo Microarray (4×44 K, Agilent Technologies). After having washed the slides, the arrays were scanned by the Agilent Scanner G2505B. Agilent Feature Extraction software (version 10.5.1.1) was used to analyze acquired array images. The software determines feature intensities (including background subtraction), rejects outliers and calculates statistical confidences. Median normalization and subsequent data processing were performed.

| Table 2. Top 20 of Statistically significant KEGG pathways in response to vaccination. |
|----------------------------------------|--------|---------------|-------|
| Category                               | Gene No. | p value | FDR     |
| Ribosome                               | 20      | 0.0000     | 0.0000 |
| Metabolic pathways                     | 84      | 0.0000     | 0.0000 |
| MAPK signaling pathway                 | 31      | 0.0000     | 0.0000 |
| Spliceosome                            | 18      | 0.0000     | 0.0000 |
| Protein processing in endoplasmic reticulum | 21  | 0.0000     | 0.0000 |
| RNA transport                          | 18      | 0.0000     | 0.0003 |
| Insulin signaling pathway              | 18      | 0.0000     | 0.0006 |
| Hedgehog signaling pathway             | 10      | 0.0001     | 0.0016 |
| Phenylalanine metabolism               | 5       | 0.0002     | 0.0026 |
| Dorsal-ventral axis formation          | 6       | 0.0004     | 0.0054 |
| Wnt signaling pathway                  | 17      | 0.0004     | 0.0054 |
| Pyrimidine metabolism                  | 12      | 0.0004     | 0.0054 |
| Regulation of actin cytoskeleton       | 20      | 0.0010     | 0.0109 |
| Melanogenesis                          | 13      | 0.0010     | 0.0109 |
| Protein export                         | 5       | 0.0013     | 0.0134 |
| Cytokine-cytokine receptor interaction | 14      | 0.0018     | 0.0159 |
| Endocytosis                            | 20      | 0.0018     | 0.0159 |
| Tight junction                         | 14      | 0.0019     | 0.0159 |
| Purine metabolism                      | 15      | 0.0021     | 0.0167 |
| RIG-I-like receptor signaling pathway  | 7       | 0.0044     | 0.0336 |

Figure 1. Validation of relative expression between microarray data and RT-qPCR results at 28 days post vaccination. iNOS2a: inducible nitric oxide synthase 2, Itga3b: integrin alpha 3b, STAT5: Signal Transducer and Activator of Transcription 5, IL7R: interleukin 7 receptor, IL22: interleukine 22, Bcl6ab: B-cell CLL/lymphoma 6a, Cdc42l: cell division cycle 42 like, Mycb: myelocytomatosis oncogene b, ABCB8: ATP-binding cassette sub-family B member 8, Cts1a: cathepsin L 1 a.

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using the GeneSpring GX v11.0 software package (Agilent Technologies). After median normalization of the raw data, genes that at least 4 out of 8 samples have flags in Present (“All Targets Value”) were chosen for further data analysis. Differentially expressed genes with statistical significance were identified through Volcano Plot filtering. To identify differentially expressed genes with statistical significance Fold Change $\geq 1.5$ ($p \leq 0.05$) was used as a cutoff. Pathway analysis and GO enrichment analysis were applied to determine the function of differentially expressed genes in different biological pathways or GO terms. GO analysis was performed with DAVID software tools for Functional Classification and Functional Annotation Clustering (http://david.abcc.ncifcrf.gov/home.jsp) and KEGG pathway database with default parameters. All data set can be downloaded from Gene Expression Omnibus public data base at www.ncbi.nlm.nih.gov/geo/ with the GEO accession number GSE39914 (Table S1).

**Real-time Quantitative PCR Analysis**

Real-time quantitative PCR was performed using the ABI Prism 7500 Detection System (Applied Biosystems, USA) with SYBR Green (Roche, USA) as the fluorescent detection dye according to the manufacturer’s protocol. Total RNA was isolated from spleen samples by Trizol (Invitrogen) according to the manufacturer’s instructions. The RNA samples were digested with DNase (Promega) to eliminate genomic DNA contaminant. First strand cDNA was synthesized by PrimeScript RT reagent kit (TaKaRa, Dalian, China) with oligo d(T) primer and random 6 mers using DNase digested total RNA as template. Primers were designed using Primer Express 3 software (Applied Biosystems) (Table S2). The thermal cycling profile consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s an appropriate annealing/extension temperature at 60°C for 60 s. An additional temperature ramping step was utilized to produce melting-curves of the reaction from 60°C to 95°C. For each gene the triplicate fluorescence intensities of the control and treatment groups were measured. The relative expression of each gene was determined by comparative threshold cycle method ($2^{-\Delta\Delta C_{t}}$ method) with house-keeping gene $\beta$-actin as reference gene. For each primer a standard curve was generated by analyzing serial dilutions of cDNA to optimize the designed

| Accession Number | Gene Name | $p$ value | Fold | Description |
|------------------|-----------|----------|------|-------------|
| XM_002662721     | LTB4R     | 0.0057   | 9.19 | Leukotriene B4 receptor 1-like |
| NM_001104937     | iNOS2a    | 0.0310   | 7.50 | Nitric oxide synthase 2a, inducible |
| XR_084418.1      | MRC1      | 0.0004   | 6.33 | Mannose receptor C type 1-like (LOC100329625) |
| NM_201503        | MMP13a    | 0.0481   | 6.12 | Matrix metalloproteinase 13a |
| XM_683237        | Prf1      | 0.0088   | 5.69 | Novel protein similar to mouse and rat perforin 1 (Pore forming protein) (Prf1) Fragment |
| NM_205554        | atp60cb   | 0.0109   | 2.33 | ATPase, H+ transporting, lysosomal, V0 subunit c, b |
| NM_173255        | atp60ca   | 0.0248   | 2.27 | ATPase, H+ transporting, lysosomal, V0 subunit c, a |
| NM_213123        | MMP9      | 0.0192   | 2.05 | Matrix metalloproteinase 9 |
| NM_199865        | Cdc42l    | 0.0206   | 1.66 | Cell division cycle 42, like |
| NM_205554        | atp60cb   | 0.0078   | 1.62 | ATPase, H+ transporting, lysosomal, V0 subunit c, b |
| NM_001017734     | Steap4    | 0.0156   | 3.10 | STEAP family member 4 |
| NM_001128234     | SLC30a10  | 0.0365   | 2.75 | Solute carrier family 30 (zinc transporter), member 10 |
| BC092881         | ISCU      | 0.0303   | 2.45 | Iron-sulfur cluster scaffold homolog |
| NM_001103139     | Hmox2a    | 0.0445   | 2.32 | Heme oxygenase (decycling) 2 |
| NM_001076602     | SLC25a38a | 0.0370   | 2.27 | Solute carrier family 25, member 38 |
| NM_001040370     | SLC11a2   | 0.0007   | 2.22 | Solute carrier family 11 (proton-coupled divalent metal iron transporters), member 2 |
| NM_200486        | SLC25a39  | 0.0350   | 2.02 | Solute carrier family 25, member 39 |
| NM_201192        | PCBP2     | 0.0212   | 1.91 | Poly(rC) binding protein 2 |
| XM_00341755      | IREB2     | 0.0009   | 1.64 | Iron-responsive element binding protein 2 |
| NM_001017544     | ABCB8     | 0.0059   | $-3.09$ | ATP-binding cassette, sub-family B (MDR/TAP), member 8 |
| NM_199659        | cul1a     | 0.0387   | $-4.77$ | culin 1a |
| NM_001076601     | Bl4na     | 0.0460   | $-6.08$ | Biliverdinreductase A |
| NM_001076662     | SFXN4     | 0.0030   | $-8.34$ | Sideroflexin 4 |
| NM_001045438     | TMEM14c   | 0.0039   | $-10.68$ | Transmembrane protein 14C |
| NM_213021        | Glx5      | 0.0204   | $-11.97$ | Glutaredoxin 5 homolog (S. cerevisiae) |
| BC107969         | TFR2      | 0.0051   | $-12.93$ | Transferrin receptor 2 |
| NM_201306        | LRPAP1    | 0.0141   | $-13.79$ | Low density lipoprotein receptor-related protein associated protein 1 |

Table 3. List of differentially expressed genes related to innate immunity.
primer. Student’s t-test was used to determine whether the detected expression differences were statistically significant ($p<0.05$).

**Results and Discussion**

**Global Changes in Gene Expression upon Vaccination**

A total of 2164 genes and transcripts were differentially expressed using Fold Change $\geq 1.5$ ($p\leq0.05$) as a cutoff, accounting for 4.9% of all genes represented on the chip (Table S3). Using the web-based database for annotation, visualization and integrated discovery (DAVID), the gene ontology (GO) analysis of the genes was performed with default settings and the Ensembl Gene IDs was used as input during the process. DAVID had functional annotation for 1436 genes, making up 66.3% of all differently expressed genes. In the “molecular function” and “biological process” categories, “nucleic acid binding” and “cellular metabolic process” were the most abundant GO terms, making up 13.8% and 20.3% of each subcategory, respectively. GO analysis indicated that live attenuated *V. anguillarum* up- and down-regulated genes were involved in cellular metabolic process, response to stress, transcription activator activity (Table 1).

The 2164 genes were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) to identify the biological pathways that were activated in the zebrafish in response to the vaccination. A total of 1766 genes of zebrafish transcriptome were mapped to KEGG and 46 statistically remarkable categories ($p<0.05$) were identified (Table S4), 20 of which were listed in Table 2. Several signaling pathways, including MAPK, insulin, hedgehog, Wnt and RIG-I-like receptor signaling pathways, are significantly modulated.

To validate the differentially expressed genes identified by microarray, 10 differentially expressed genes such as inducible nitric oxide synthase 2 (iNOS2a), itga3b, STAT5, IL7R, IL22, Bcl6ab, Cdc42l, Mycb, ABCB8, and Ctsl1a were selected for real-time qPCR analysis. The expressions of all examined genes matched the microarray data (Figure 1).

Many genes associated with the innate immunity were modulated in vaccinated zebrafish with the live attenuated *V. anguillarum* (Table 3). A receptor of inflammatory mediator, Leukotriene B4 receptor, was over-expressed significantly (fold = 9.19) while a scavenger receptor named mannose receptor C type 1 was up-regulated (fold = 6.33). Metalloproteins (MMPs) regulate the cell matrix composition and are evaluated as markers

**Figure 2. Iron uptake and metabolism were enhanced in the bath-vaccinated zebrafish.** Genes related to putative iron metabolism of intracellular iron and competition for extracellular iron were differentially modulated after vaccination with the live attenuated *V. anguillarum*. Red: up-regulated, Green: down-regulated, Black: not found to be modulated.

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of inflammation [17]. Of them, MMP-9 is important for leukocyte migration and inflammation owing to its ability to degrade basement membranes and components of the extra cellular matrix [18]. MMP-13 has a key role in the MMP activation cascade and also contributes to wound repair [19]. The expressions of the two metalloproteins, MMP9 and MMP13, were up-regulated for 6.12 fold and 2.05 fold, respectively.

Additionally, a number of genes related to iron metabolism were differentially expressed in the spleen of zebrafish at 28 days post bath-vaccination. The nature resistance associated macro-

Table 4. List of differentially expressed genes related to adaptive immunity.

| Accession number | Gene name | p value | Fold | Description |
|------------------|-----------|---------|------|-------------|
| **Cytokines and cytokine receptors** | | | | |
| NM_001043334 | EpoR | 0.0025 | 9.24 | Erythropoietin receptor, involved with erythroid and lymphoid differentiation |
| NM_001113595 | CCL20 | 0.0119 | 3.27 | Chemokine (C-C motif) ligand 20 |
| BC083364 | XCR1a | 0.0043 | 3.16 | Chemokine (C motif) receptor 1 |
| NM_001037683 | TGFBR1a | 0.0367 | 3.12 | Transforming growth factor, beta receptor 1a |
| BC098597 | IL1β | 0.0395 | 2.62 | Interleukin 1, beta |
| NM_194386 | TGFβ3 | 0.0326 | 2.52 | Transforming growth factor, beta 3 |
| NM_001083868 | crfb4 | 0.0098 | 2.35 | Cytokine receptor family member b4 |
| NM_001113507 | IL7R | 0.0109 | 2.24 | Interleukin 7 receptor |
| BC163192 | IL22 | 0.0228 | 2.08 | Interleukin 22 |
| NM_207640 | ifnph1 | 0.0467 | 1.94 | Interferon phi 1 (IFNΦ), mRNA |
| NM_205762 | traf4a | 0.0449 | 1.58 | TNF receptor-associated factor 4a |
| NM_001113625 | trap1 | 0.0165 | 1.55 | TNF receptor-associated protein 1 |
| **Antigen present, T-cell and B cell activation** | | | | |
| AF246168 | TCRAV | 0.0061 | 5.96 | Isolate G5209 T-cell receptor alpha variable region mRNA, partial cds. |
| NM_001190309 | itga3b | 0.0181 | 4.44 | Integrin, alpha 3b |
| NM_212634 | alcamb | 0.0073 | 3.38 | Activated leukocyte cell adhesion molecule b |
| XM_002666544 | Nfx1 | 0.0003 | 3.32 | Nuclear transcription factor, X-box binding-like 1 (Named Xbp1 in human) |
| XM_001923447 | CD28 | 0.0236 | 3.23 | T-cell-specific surface glycoprotein CD28-like (LOC100151365) |
| NM_194387 | STAT5.1 | 0.0112 | 3.20 | Signal transducer and activator of transcription 5.1 |
| DNBN9368 | itgb7 | 0.0016 | 3.13 | Integrin, beta 7 |
| NM_001040369 | tnfrsf9a | 0.0465 | 3.13 | TNF receptor superfamily, member 9a |
| AY841759 | Prdm1a | 0.0248 | 3.07 | PR domain containing 1a, with ZNF domain (homolog to Blimp of human) |
| NM_001082997 | CD40 | 0.0037 | 2.57 | CD40 molecule, TNF receptor superfamily member 5 |
| NM_198878 | rbppa | 0.0033 | 2.47 | Recombination signal binding protein for immunoglobulin kappa J region a |
| NM_200909 | creb1a | 0.0042 | 2.31 | cAMP responsive element binding protein 1a |
| NM_001007167 | MHC II | 0.0483 | 1.88 | Major histocompatibility complex class II (MHC II) DAB gene |
| NM_203366 | STAT6 | 0.0162 | 1.87 | Signal transducer and activator of transcription 6, interleukin-4 induced |
| NM_001100074 | Bcl6ab | 0.0287 | 1.86 | B-cell CLL/lymphoma 6a, genome duplicate b (similar to Bcl-6 of human) |
| NM_001110403 | hsap8 | 0.0149 | 1.81 | Heat shock protein 8 |
| NM_001080622 | CD276 | 0.0421 | 1.75 | CD276 molecule |
| NM_214716 | hsap4a | 0.0492 | 1.70 | Heat shock protein 4a |
| NM_200172 | Mycb | 0.0217 | −1.88 | Myelocytomatosis oncogene b |
| NM_131198 | Ctnlb1 | 0.0120 | −2.05 | Cathepsin L b, hatching gland gene 1 |
| NM_001045076 | hsap14 | 0.0310 | −2.07 | Heat shock protein 14 |
| NM_001089476 | hsf5 | 0.0168 | −2.06 | Heat shock transcription factor family member 5 |
| BC0090693 | FN2YBb | 0.0455 | −2.38 | Nuclear transcription factor Y, beta b |
| BC063995 | mhc1ze | 0.0477 | −3.25 | Major histocompatibility complex class I ZE gene |
| NM_212584 | Ctn1a | 0.0435 | −3.80 | Cathepsin L 1a |
| NM_200075 | hsbp1 | 0.0307 | −4.90 | HSPA (heat shock 70 kDa) binding protein, cytoplasmic cochaperone 1 |
| NM_213522 | igbp1 | 0.0051 | −4.95 | Immunoglobulin (CD79A) binding protein 1 |
| XM_001343036 | Ciita | 0.0413 | −5.61 | Class II, major histocompatibility complex, transactivator |
| NM_198210 | hsp90b1 | 0.0277 | −8.64 | Heat shock protein 90, beta (grp94), member 1 |

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phage protein 2 (Nramp2, also known as SLC11a2) is a transporter associated with export of iron from phagosomes [20]. Its up-regulated expression (fold = 2.22) might increase the iron uptake from transferrin and low-molecular-weight iron complexes. In vitro uptake of hemopexin-heme complex mediated by CD91 could be inhibited by LRPAP1 (low density lipoprotein receptor-related protein associated protein 1) [21]. The expression of LRPAP1 was significantly suppressed (fold = 13.79). The expression of IREB2 (iron responsive element binding protein 2) which plays a central role in iron metabolism was slightly increased (fold = 1.64). IREB2 can sense cytosolic iron levels and posttranscriptionally regulate iron metabolism genes including transferrin receptor 1 (TIR1) and ferritin H and L subunits, by binding to iron-responsive elements (IREs) within target transcripts. Its up-regulated expression implied the more iron uptake. Although the expression of transferrin receptor 2 (TfR2) which might play a role in cellular iron uptake through binding and internalizing a carrier protein transferrin (Tf) decreased considerably, its substantially lower affinity to Tf was identified and its expression was independent on cellular status of iron in contrast to Tf1 [22]. Its decreased expression might be related to other function. Additionally, NO is a critical regulator of cellular iron homeostasis via activation of IRP (iron regulatory protein) binding to IRE (iron responsive element). The expression of iNOS2a, which could produce important antimicrobial effectors namely nitric oxide (NO) [23], was highly up-regulated (fold = 7.50). As a ferritin iron chaperone, poly(rC) binding protein (PCBP) could deliver iron to ferritin. Its up-regulated expression implied that excess iron was stored as the form of ferritin. Taken together, the genes expression changes indicated that iron uptake was increased after bath-vaccination (Figure 2).

Meanwhile, the expressions of numerous genes relevant to adaptive immunity responses were regulated, including cytokines, receptors and other proteins involved in the antigen presentation and the T-cell and B cell activation (Table 4). Expression of some immunologically significant genes increased, including TCRVR, CD28, CREB, MHCII, CD276 (B7) and CD40, which could provide the activating signal and co-stimulation signal to naive T cell. From these evidences, helper T lymphocytes were activated by bath-vaccination of the live attenuated V. anguillarum. Besides, The expressions of four transcription factors Bcl6, Prdm1a, Nfxl1, and STAT6 which were found to play important roles in B cells activation and differentiation and another transcription factor RBPJ (recombination signal binding protein for immunoglobulin kappa J region) which was involved in cell fate determination of margin zone B cell [24] were all elevated. Especially, Bcl6 is a master factor of B cell differentiation. These results suggest that the differentiation and maturation of B lymphocytes were induced. In our laboratory, unambiguous specific antibody response was detected in serum of vaccinated zebrafish during 28 days post vaccination [2]. Significantly enhanced specific antibody and changes in gene expression related to B cell differentiation at 28 days post bath-vaccination of live attenuated V. anguillarum confirmed responses of humoral immunity.

Cytokines, Membrane Receptors and Transcription Factors Associated with Th17 Cell

As the functions of those genes related to adaptive immunity were further explored, we found that several genes associated with Th17 differentiation and amplification were significantly modulated. In contrast to Th1 and Th2 cells which depend on their respective effector cytokines (IFN-γ and IL-4) for differentiation, Th17 differentiation is initiated by the combined action of IL-6, TGF-β [25,26] and IL-1β [27]. The genes encoding IL-1β, TGF-β and TGF-β receptor in vaccinated zebrafish were up-regulated. Furthermore, the mannose receptor (MRC1) was overexpressed by 6.33 fold (p = 4.4 × 10^-4). Although its immunological role is still unknown, a possible clue is via a role in inducing Th17 response during infection [28].

The expression of gene encoding IL22, one of cytokines secreted by Th17 effector, was elevated by 2.08 fold. Furthermore, Cua et al recently indicated that IL-23 might play an important role in the terminal differentiation of Th17, potentially through its effect on re-expression of IL7R on Th17 cells [29]. Activated Th17 cells...
and other effector T cells showed surface expression of interleukin 7 receptor (IL7R). IL7R is essential in survival and development of cells differentiating to T cells as well as in mature T cells [30]. In addition, IL7R signaling combined with activation of STAT5 appears to play an essential, although not sufficient, role for development of memory CD4+ T cell [31,32]. In this work, significant up-regulations of IL23R, IL7R and STAT5 expressions were found in 28 days post bath-vaccinated zebrafish.

IL-17 strongly recruits and activates neutrophils [33] and stimulates the release of a variety of mediators of inflammation.
including matrix metalloproteinases such as MMP9 [34] and MMP13 [35] which facilitate neutrophil infiltration. CCL20, a small cytokine belonging to the CC chemokine family, is strongly and specifically chemotactic for Th17 cells by its CCR6 chemokine receptor and is secreted by Th17 cells [36]. In this work, the gene expressions of all three mediators CCL20, MMP9, and MMP13 were up-regulated. Accordingly, it could be believed that the Th17-like immune response was activated in bath-vaccinated zebrafish with the live attenuated \textit{V. anguillarum} (Figure 3).

Temporal Expression Profiling of Th17-related Genes

Microarray analysis showed the signs of adaptive T cell immunity. Several up-regulated cytokines (TGF-β, IL-1β, IL-22), surface receptors (TGF-βR, IL-23R and IL-7R), and transcription factor STAT3 illustrated Th17 differentiation and expansion. To confirm the activation of Th17, a set of Th17-related markers were included in a temporal expression analysis during 35 days post bath-vaccination with the live attenuated \textit{V. anguillarum}. Of the 16 genes measured, the expressions of 14 genes were significantly changed in zebrafish after vaccination as compared to control group \((p<0.05)\). Among them, the expressions of 6 genes were significantly increased at 14 dpv \((p<0.05)\), including TGF-β,
IL-6, IL-23p19, IL-17A/F2, IL-17A/F3 and IL-17D (Figure 4, A, B, C, F). While TGF-β and IL-6 expressions were slightly, but significantly, elevated, IL-23p19 expression increased with a high peak (4-fold) at 14 dpv. The expressions of the cytokine receptors of TGF-β and IL-23 were up-regulated at 14 dpv. All three cytokines TGF-β, IL-6 and IL-23p19 play important roles in the initiation and maintenance of Th17 differentiation. IL-23 expands and stabilizes Th17 cells to produce their effector cytokines [42]. Particularly, the expression of IL-17A/F2 was highly up-regulated to the peak of over 7-fold at 14 dpv with a gently up-regulated expression of its transcription factor RORc. The expressions of two other transcription factors, STAT3 and STAT5, gradually increased after 7 dpv and reached a peak at 28 dpv (Figure 4, E). Two other cytokines IL-21 and IL-22, mainly secreted by Th17 effector, were elevated with a peak at 21 dpv and 28 dpv, respectively (Figure 4, D). Taken together, these results suggest that Th17 effector cell be activated between 14 dpv to 21dpv. The significantly up-regulated expression of the IL7R and integrin β7 at 28 days and 35 days post vaccination (Figure 4, G, H) imply the exist of immunological memory and mucosal-related immunity.

The expression profiles supported the Th17-like immune response to the live attenuated V. anguillarum. In mammals, studies of infectious disease highlight the critical role of Th17 response in host defense against extracellular pathogens, particularly Gram-negative bacteria that colonize mucosal surfaces. Mice infected by Klebsiella pneumonia [43,44], Bordetella pertussis [45], or Streptococcus pneumonia, for example, mount a Th17 response and disruption of IL-17 signaling increase susceptibility in these model. The activation of Th17 lineage by pertussis toxin and pneumococcal antigen is necessary to confer full protection against subsequent infection [46]. Similar to K. pneumonia and B. pertussis, V. anguillarum is a Gram-negative, extracellular pathogenic bacterium. Following these reality and results, we assume that Th17 lineage provide protection against further infection in the bath-vaccinated zebrafish, although more evidences need to be established.

Signaling Pathway and Adaptive Immunity

Several signaling pathway, such as Wnt and hedgehog (Hh) were significantly modulated at 28 days after bath-vaccination. During embryonic development complex but delicate interactions of these pathways are crucial for stem cell maintenance, body patterning, cell fate determination and organogenesis. Recent studies indicated that the signaling pathway was also associated with lymphocytes development, activation and differentiation. Among them, Wnt-signaling plays a prominent role in the immune system for regulating effector T-cell development, regulatory T-cell activation and dendritic-cell maturation [47]. The expressions of most genes involved in canonical and non-canonical Wnt-signaling pathways were affected in 28 days post vaccination of the zebrafish with the live attenuated V. anguillarum (Figure 5). Two reports demonstrated the critical requirements of the Wnt-β-catenin pathway for Th2 differentiation [48,49]. Although the TCF-1/β-catenin complex positively regulates Th2 initiation and further differentiation, TCF-1 represses alternative Th1 and Th17 fates in activated CD4+ T cells [50]. Given the timing for microarray hybridization is at 28 dpv, it is suggested that at this time point Th17-mediated immune response decrease and Th2-mediated B cell maturation are induced. Emerging trends also highlight the capacity of Th17 cells to bridge the gap between innate and adaptive immunity and attract other subset of T helper cell to sites of infection at later stages of the inflammatory process [51]. This deduction is consistent with our results that peak of Th17-mediated immune response appears at 14 dpv and specific antibody response at 28 dpv. Furthermore, Wnt/β-catenin signaling activates NFAT, NF-kB and Bcl6 and up-regulates CD4 expression. NFAT may intensify Wnt/β-catenin signaling by activating NF-kB and Bcl6 for protection of B cells [52]. Hedgehog signaling modulates mature T cell functions through the regulation of cell cycle progression [53–55]. Our observation indicated that adaptive immunity induced by the live attenuated V. anguillarum are mainly mediated by Th17 cells and B cells.

Conclusions

The analysis of the transcriptome and gene expression in the live attenuated V. anguillarum-vaccinated zebrafish revealed the changes of genes involved in both innate and adaptive immunity. Adaptive immunity associated with Th17-like immune response and antibody response can account for the high level RPS of bath-vaccinated zebrafish. Activation of Th17 pathway implies that bath-vaccination of the live attenuated V. anguillarum evokes mucosa immune response which plays pivotal role in orchestrating the mucosal barrier against pathogen. These findings provided valuable leads for further investigation into the mucosal immune response induced by immersion route. The modification of immunity factors and anti-infection at the mucosal site of entry may be a focus in the next work.

Supporting Information

Table S1 The matrix table deposited at GEO. (XLS)

Table S2 Primers used for real-time quantitative PCR analysis. (XLS)

Table S3 Complete list of differentially expressed genes. (XLS)

Table S4 Complete list of KEGG pathways of differentially expressed genes. (XLS)

Author Contributions

Conceived and designed the experiments: HW QL QW YZ. Performed the experiments: HZ CF. Analyzed the data: HZ CF. Contributed reagents/materials/analysis tools: HZ CF MY. Wrote the paper: HZ HW YZ.

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