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Short communication

Profiling immune response in zebrafish intestine, skin, spleen and kidney bath-vaccinated with a live attenuated *Vibrio anguillarum* vaccine

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1. Introduction

A live attenuated *Vibrio anguillarum* vaccine MVAV6203 for preventing and curing vibriosis of cultivated fish was successfully constructed in our laboratory [1]. The vaccine was proved to induce both innate and adaptive immune response via bath-vaccination. For instance, a highly specific antibody level was detected in the peripheral blood of zebrafish [2]. Significant changes of transcripts of both iron metabolism related to innate immunity and B and T cells activation, proliferation and expansion involved in adaptive immunity have been found [3]. Recently, a Th17-like immune response was also found in mucosal organs of zebrafish [4] and mucosal immune response such as antigen uptake and inflammation was observed in the intestine of zebrafish [5]. The aim of this work was to investigate the immune response induced by the live attenuated vaccine from the initial site of antigen-contact including intestine and skin to immune organs such as kidney and spleen spatiotemporally using a gene expression profiling.

2. Materials and methods

2.1. Zebrafish maintenance

Six-month-old zebrafish *Danio rerio* weighing 0.2 ± 0.05 g were obtained from the animal center at the East China University of Science and Technology (Shanghai, China) and acclimatized in our laboratory for two weeks before experimental manipulation. Fish were reared in running dechlorinated and aerated water at 24 ± 2 °C on a 12 h/12 h light/dark rhythm and fed with commercial blood worm twice daily. Prior to each experiment, fish were randomly sampled to examine specific-pathogen infection from their kidney, spleen, intestine and skin. Briefly, samples were collected and homogenized in sterile phosphate-buffered saline (PBS) using tissue grinders. The homogenates were serially diluted and plated onto Luria–Bertani (LB) plates at 30 °C for 12 h. Colonies were amplified and the genomic DNA was extracted from the cultures using TIANamp Bacteria DNA Kit (Tiangen, China). 16S DNA gene was amplified using 16S bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The products were randomly sequenced to ensure whether fish were infected by the specific-pathogens such as *V. anguillarum*, *Edwardsiella tarda*, *Aeromonas hydrophila* and *Mycobacterium* spp. All the zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (http://ZFIN.org). The vaccination experiment was approved by the animal center at the East China University of Science and Technology (Shanghai, China). In the experiment, zebrafish were immersed in 100 ng ml^{-1} tricaine methanesulphonate (MS-222, Sigma, USA) for anesthetization and in 300 ng ml^{-1} MS-222 at least 10 min for euthanasia.

2.2. Vaccination and sampling

The live attenuated *V. anguillarum* strain MVAV6203 was constructed in our previous work. Strains were grown in LB mediums supplemented with 2% NaCl (LB20) at 30 °C for 16 h. Cells were

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harvested by centrifugation at 5000 rpm for 5 min and washed twice with sterile physiological sea water (PSW, NaCl, 20 g l^{-1}; KCl, 0.7 g l^{-1}; $MgCl_2 \cdot 6H_2O$, 4.8 g l^{-1}; $NaHCO_3$, 0.11 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 3.5 g l^{-1}; and $CaCl_2 \cdot 2H_2O$, 1.6 g l^{-1}; pH 7.2). Then, cells were resuspended and diluted to 10^8 colony forming units (CFU) ml^{-1} with PSW. Sixty acclimated healthy zebrafish were bath-vaccinated in the aerated cell-resuspended PSW for 10 min and transferred to clean water for 5 min and then returned to the recovery tanks. Equal fish were mock-vaccinated in PSW as a control. At 3, 7, 14, 21 and 28 d post vaccination (p.v.), ten zebrafish from each group were anesthetized and intestine, skin, spleen and kidney were pooled aseptically. Samples were stored at −80 °C until RNA extraction. The experiment was performed in triplicate.

2.3. Total RNA isolation, cDNA synthesis and RT-qPCR

Total RNA was extracted from samples by Trizol (Invitrogen, USA) according to the manufacturer's instructions. RNA samples were digested with RNase-free DNase I (Promega, USA) to eliminate genomic DNA contaminant. Afterwards, 1 μg of total RNA was amplified in cDNA synthesis reaction by using PrimeScript® RT reagent kit (TaKaRa, Japan). Negative controls lacking reverse transcriptase or RNA were included for each group. Finally, 20 μl cDNA reaction mixtures were diluted with 80 μl water and stored at −20 °C for RT-qPCR. The primers for each gene were listed in Table 1. Each primer pair was designed and tested for specificity. Primer efficiency was determined by performing serial dilutions of reference cDNA. RT-qPCR assay was conducted with ABI 7500 Real-Time Detection System (Applied Biosystems, USA). Reaction mixtures (20 μl) contained 10 μl SYBR® Select Master Mix, 1 μl of determined cDNA samples, 10 μmol l^{-1} each of forward and reverse primers. The standard cycling included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/elongation at 60 °C for 60 s. The relative expression of each immune-related gene was determined by comparative threshold cycle method ($2^{-\Delta\Delta Ct}$ method) with β-actin as reference gene. The data was analyzed using Cluster 3.0 software.

3. Results and discussion

3.1. Antigen recognition and uptake

The expression of genes related to antigen recognition and uptake in the intestine, skin, spleen and kidney was firstly determined. In our previous work, significant up-regulation of TLR5 was observed in mucosal tissues than the other Toll-like receptors [5]. Herein, the expression of TLR5 reached the peak in the skin as well as in the spleen and kidney at 3 d p.v. while no significant change was observed in the intestine (Fig. 1). It was reported that the basic principle of innate control of adaptive immunity is based on establishing an association between the antigens recognition by lymphocytes and the pathogen associated molecular patterns recognition (PAMP) by PRRs [6]. Interestingly, immune cells involved in antigens recognition such as macrophages and B cells mainly populate in the anterior kidney and spleen in teleosts [6]. It was reported that the expression of TLR5 was significantly increased with 5 and 10-fold in the spleen and kidney of common carp after *A. hydrophila* infection [7]. Therefore, the live attenuated vaccine was recognized both in antigen-contact tissues and in immune organs after it entered into the zebrafish. However, the immune response of recognition probably lasted for a short time in antigen-contact tissues since TLR5 was elevated notably within 12 h in the intestine after the vaccination [5]. Generally, the expression of TLRs represents the capacity of antigen recognition at the initial stage of infection or vaccination. Herein, we also proposed it as a parameter for vaccine efficacy. The significant up-regulation of TLR5 in the skin at 21 d p.v. and in the kidney at 28 dp.v. suggested that the live vaccine enhanced the immune system's capacity of discrimination against pathogens. Besides, the up-regulation of TLR5 in antigen-contact tissues might be due to the migration of cells expressing TLR5 from immune organs.

Mononuclear phagocyte system (MPS), an important innate immune component, has been characterized in teleosts [8]. In this study, the expression of a MPS marker, b7r, was determined to monitor the process of antigen uptake in antigen-contact tissues

Table 1
Primers used for RT-qPCR analysis.

Primer	Forward primer (5′-3′)	Reverse primer (5′-3′)
β-actin	ATGGATGAGGAAATCGCTGCC	CTCCCTGATGTCTGGGTCGTC
TLR5	GAAACATTCACCCTGGCACA	CTACAACCAGCACCACCAGAATG
b7r	TCAGCAACAGTCATCAACAG	TTAGCACCGTCATTCTCG
IL-1β	TGGACTTCGCAGCACAAAATG	GTTCACTTCACGCTCTTGGATG
IL-8	GTCGCTGCATTGAAACAGAA	CTTAACCCATGGAGCAGAGG
MHC I	GGAGTTCACCTTGCTTATGC	CCCTCTGACCCATTCTTGT
MHC II	TGACTCAACTGTCCGTGATA	CCATTAGCCATCTCCATAGTG
CD4	GTGGTCTTCATCTTGCTTGT	AATCCCTTTGGCTGTTTGTT
CD8	AAGAGCATAGCACCGTAG	GACTTCCGTCTGCTTTGCG
IL-12	GAAACTCAACTGACCTCAACTG	CTTTATCTGGCTTGACAATGTCTC
T-bet	TAGTAGCCACAGCGTCAG	CTTCAGAACACCTCCACAT
IL-4/13A	CCTGAAGTGTGAGCATGATTA	AGCGTGACTCCTGATCTC
GATA3	CAGCATCCTGAGACACATC	CCACATCTTCCGCAAGTT
IL-22	CATCGAGGAACAACGGTGTACA	CACGAGCACAGCAAAGCAAT
ROR-γ	CGTGATGCGGTGAAGTTTGG	CCTGGGACTGCTGGTGCTT
TGF-β	TGGGCTGGCGGTGGAT	CCTCTGGGTTCAGCGTGTT
Foxp3	GCAACCAGCCTTTTCCACAAGC	GACTATATGGATGCTTCCCAGTA
IgM	GTGTTTGTGACTTGGCTTG	CACCCGTCCACTCTGAATT
IgZ1	CACCCAGCATTCTACAGCAAAC	GTCGGTACAAGAACCAAACTCAG
IgZ2	CAGAATGGAGCAAGCCTGAC	TAACTGTGCCCTCTTGGTGTATT

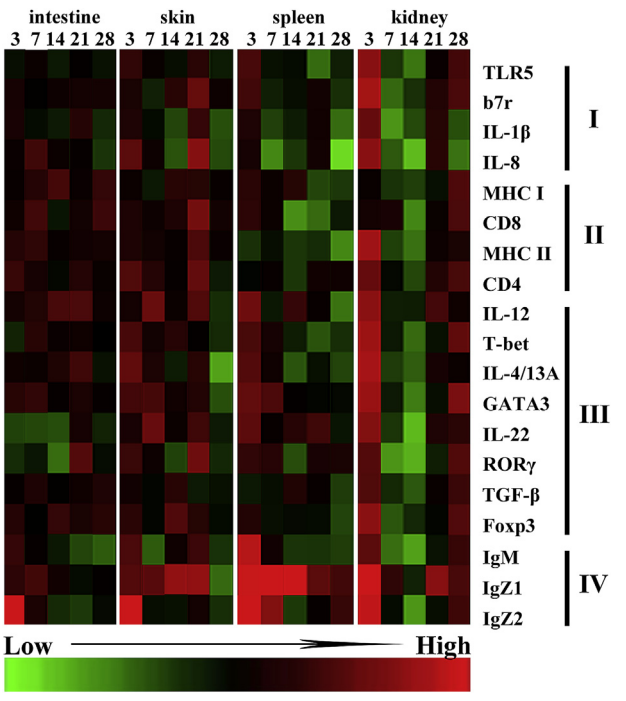

Fig. 1. Expressions of immune response-related genes in the intestine, skin, spleen and kidney of zebrafish after *V. anguillarum* MVAV6203 bath-vaccination. I: antigen recognition and uptake related genes; II: antigen processing and presentation related genes; III, T cell proliferation and differentiation related genes; IV, antibodies related genes. Zebrafish were bath-vaccinated with 10^8 CFU ml^{-1} *V. anguillarum* MVAV6203 or mock-vaccinated in PSW as a control. Intestine, skin, spleen and kidney of ten zebrafish from vaccination groups and control groups were sampled at 3, 7, 14, 21 and 28 days post vaccination.

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