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Effect of CXCL12-expressing viral hemorrhagic septicemia virus replicon particles on leukocytes migration and vaccine efficacy in olive flounder (*Paralichthys olivaceus*)

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ABSTRACT

Viral replicon particles are single-cycle viruses defective for function(s) needed for viral replication, which allow them to be recognized as a safer form for the vaccination of animals compared to attenuated live viruses. However, deletion of genes that are critical for the induction of protective immunity can diminish the vaccine potential of viral replicon particles. Therefore, the manipulation of viral replicon particles to produce a molecular adjuvant can be a way to increase immunogenicity of vaccines based on viral replicon particles. Chemokines are a class of chemotactic cytokines that control the migration of diverse cells of vertebrates. CXC chemokine ligand 12 (CXCL12) binds to a receptor CXCR4, and CXCL12-CXCR4 signaling plays an important role in the migration of hematopoietic cells during embryogenesis and the attraction of leukocytes. In the present study, to evaluate the possible use of CXCL12 as a molecular adjuvant for an rVHSV-ΔG vaccine and to know differences between CXCL12a and CXCL12b in the adjuvant ability, we rescued VHSV replicon particles that are expressing olive flounder CXCL12a, CXCL12b, or eGFP (rVHSV-ΔG-CXCL12a, rVHSV-ΔG-CXCL12b, or rVHSV-ΔG-eGFP), and compared the ability to attract olive flounder leukocytes and to induce protection against a VHSV challenge. In the leukocytes migration assay, supernatants collected from cells infected with rVHSV-ΔG-CXCL12a and rVHSV-ΔG-CXCL12b showed significantly higher ability to attract olive flounder leukocytes than the supernatant of cells infected with rVHSV-ΔG-eGFP. Moreover, the significantly higher number of leukocytes were attracted to rVHSV-CXCL12a supernatant compared to rVHSV-CXCL12b supernatant, suggesting that CXCL12a would be more appropriate for the induction of immunity than CXCL12b in olive flounder. In the immunization experiment, olive flounder immunized with rVHSV-ΔG-CXCL12a showed significantly higher survival rate than fish immunized with rVHSV-ΔG-CXCL12b or rVHSV-ΔG-eGFP. In addition, fish immunized with rVHSV-ΔG-CXCL12a showed the highest serum neutralization activity. These results suggest the availability of CXCL12a for a molecular adjuvant of vaccines based on VHSV replicon particles.

1. Introduction

Chemokines are a class of chemotactic cytokines that control the migration of diverse cells of vertebrates [1], and are grouped into four subfamilies (C, CC, CXC, and CX3C) according to the arrangement of cysteine residues in the N-terminal region [2]. Among CXC chemokines, CXC chemokine ligand 12 (CXCL12, also called SDF-1) binds to a receptor CXCR4, and CXCL12-CXCR4 signaling plays an important role in the migration of hematopoietic cells during embryogenesis and the attraction of leukocytes [3–6].

The expression of mammalian CXCL12 gene orthologues has been

reported in several fish species including zebrafish [7], carp [8], catfish [9], large yellow croaker [10], rock bream [11], and orange-spotted grouper [12]. Due to the teleost-specific whole genome duplication [13–15], usually two paralogues, CXCL12a and CXCL12b, are present in fishes, however, differences between 2 paralogues in action or response were reported only from zebrafish and carp [8,16,17].

In fish, the involvement of CXCL12 in cell migration has been mostly investigated in zebrafish. Zebrafish CXCL12a-CXCR4b signaling plays a critical role in the migration of the primordial germ cells [16], the embryonic posterior lateral line primordium [7,18], the endodermal cells during gastrulation [19], and hypothalamic

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gonadotropin-releasing hormone (GnRH) neurons [20]. Furthermore, the neutrophil retention by CXCL12-CXCR4 signaling in hematopoietic tissue was demonstrated in zebrafish [6] and in the little skate (*Leucoraja erinacea*) [21].

Viral hemorrhagic septicemia virus (VHSV) has a negative-stranded RNA genome that contains N, P, M, G, NV and L genes, and has been a principal cause of mass mortalities in cultured fish worldwide [22–25]. As the glycoproteins (G) of rhabdoviruses protruding from the viral envelope can induce neutralizing antibodies which are crucial for the protection of hosts, glycoproteins have been the main target for the development of effective vaccines. To date, various vaccine types including inactivated vaccine, recombinant subunit vaccine, DNA vaccine, and reverse genetically rescued attenuated vaccine have been reported [24,26,27]. Previously, to overcome a safety problem in relation to the replication ability of the NV gene-deleted attenuated VHSV (which might have a possibility to cause a disease in immune-suppressed individuals), we have recently rescued the G gene-deleted VHSV replicon particles (rVHSV-ΔG) as a prophylactic vaccine in olive flounder [28]. Viral replicon particles are single-cycle viruses defective for function(s) needed for viral replication, which allow them to be recognized as a safer form for the vaccination of animals compared to attenuated live viruses. However, deletion of genes that are critical for the induction of protective immunity can diminish the vaccine potential of viral replicon particles. Therefore, the manipulation of viral replicon particles to produce a molecular adjuvant can be a way to increase immunogenicity of vaccines based on viral replicon particles.

In olive flounder, although Huang et al. [29] reported the expression of CXCL12 in the transcriptome of spleen, no further research has been reported. In the present study, to evaluate the possible use of CXCL12 as a molecular adjuvant for an rVHSV-ΔG vaccine and to know differences between CXCL12a and CXCL12b in the adjuvant ability, we rescued two kinds of VHSV replicon particles that are expressing olive flounder CXCL12a and CXCL12b (rVHSV-ΔG-CXCL12a and rVHSV-ΔG-CXCL12b), and compared the ability to attract olive flounder leucocytes and to induce protection against a VHSV challenge.

2. Materials and methods

2.1. Cells and viruses

Epithelioma papulosum cyprini (EPC) cells and HIRAME natural embryo (HINAE) cells were grown in Leibovitz medium (L-15, Sigma) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% fetal bovine serum (FBS, WelGENE). VHSV KJ2008 that was isolated from diseased olive flounder (*Paralichthys olivaceus*) was propagated in a monolayer of EPC cells at 14 °C in the presence of antibiotics.

2.2. Generation of olive flounder CXCL12-expressing recombinant VHSV replicon particles

The nucleotide sequences of olive flounder CXCL12a (Accession: KU821022.1) and CXCL12b (Accession: KU821021.1) were obtained from the GenBank sequence database in the National Center for Biotechnology Information (NCBI). The ORF of CXCL12a and CXCL12b was PCR amplified from cDNA of olive flounder kidney using each specific primer pair that have NheI (forward) and SalI (reverse) sites (Table 1), and the PCR products were cloned into pGEM-T-easy vector (Promega; pCXCL12a and pCXCL12b). To use as a control, a vector containing the enhanced green fluorescent protein (eGFP) gene ORF was also constructed (peGFP). A previously constructed vector (pVHSV-ΔG) lacking G gene for the generation of single-cycle VHSV replicon particles [28] was used to construct a vector for recombinant VHSV replicon particles that expresses olive flounder CXCL12a, CXCL12b or eGFP. To insert eGFP ORF, the pVHSV-ΔG was digested with NdeI and SalI, and the fragment of eGFP digested with the same enzymes was

ligated between N gene and P gene of pVHSV-ΔG, resulting in pVHSV-ΔG-eGFP. To insert CXCL12a and CXCL12b ORF, the NdeI site behind the N gene was changed to NheI site by site directed mutagenesis kit (SDM, Stratagene). The primers used for SDM are listed in Table 1. Then, the fragment of CXCL12a and CXCL12b digested with NheI and SalI was ligated between N gene and P gene of pVHSV-ΔG (SDM) that was digested with the same enzymes, resulting in pVHSV-ΔG-CXCL12a and pVHSV-ΔG-CXCL12b.

EPC cells expressing T7 RNA polymerase were grown to about 80% confluence and transfected with a mixture of pVHSV-ΔG-CXCL12a (or -CXCL12b or -eGFP) (2 µg) and helper plasmids - pCMV-N (500 ng), pCMV-P (300 ng), pCMV-G (250 ng), and pCMV-L (200 ng) - using FuGENE HD transfection reagent (Promega) according to manufacturer's instructions. After incubation for 24 h at 28 °C, the transfected cells were shifted to 14 °C. When extensive cytopathic effect (CPE) was observed, the cells were submitted to two cycles of freeze-thawing and centrifuged at 1,500 g for 10 min to collect supernatant. After 3 passages in the cells expressing VHSV G protein, the supernatant was collected, filtered using 0.45 µm syringe filters, and used as a viral stock.

The rescue of each viral replicon particles was verified by reverse transcriptase PCR (RT-PCR). Total RNA was extracted from each stock using Trizol reagent (Invitrogen), and treated with DNaseI using Riboclear plus Kit (GeneAll, Korea) according to the manufacturer's instruction. To synthesize first-strand cDNA, 1 µg of total RNA was incubated with 0.5 µl of random primer (0.5 µg/ml, Promega) at 80 °C for 5 min and further incubated at 42 °C for 60 min in reaction mixture containing 2 µl of each 10 mM dNTP mix (Takara), 0.5 µl of M-MLV reverse transcriptase (Promega) and 0.25 µl of RNase inhibitor (Promega) in a final reaction volume of 10 µl. The insertion of olive flounder CXCL12a, CXCL12b, or eGFP gene between N and P genes was confirmed by RT-PCR using primer sets in Table 1. The PCR products were analyzed on a 1% agarose gel containing Nucleic acid stain (Korea labtec) for visualization.

2.3. Expression of CXCL12a and CXCL12b by rVHSV-ΔG-CXCL12a and rVHSV-ΔG-CXCL12b

The expression of CXCL12a and CXCL12b by the infection of HINAE cells with rVHSV-ΔG-eGFP, rVHSV-ΔG-CXCL12a or rVHSV-ΔG-CXCL12b was analyzed by quantitative real-time PCR (qRT-PCR). Experiments were conducted in triplicate. HINAE cells were cultured in 35 mm dishes (1.0×10^6 cells/dish), and were infected with a multiplicity of infection (MOI) 0.1 of rVHSV-ΔG-eGFP, rVHSV-ΔG-CXCL12a or rVHSV-ΔG-CXCL12b at 14 °C. At 24 h post-infection, total RNA was extracted from cell pellets and cDNA was synthesized according to the above method. qRT-PCR was carried out by a Light Cycler 480 (Roche) using the PCR primer sets shown in Table 1 (β-actin gene was used as an internal control). The PCR reactions in a volume of 20 µl were run using 2 × SYBR Green Premix (Enzynomics, Korea) with 5 µl of 5 fold-diluted cDNA and 5 pM of each primer. Thermal cycling condition was 1 cycle of 15 min at 95 °C (pre-incubation), followed by 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 20 s at 72 °C.

2.4. Transwell migration

To collect CXCL12 containing supernatant, HINAE cells were infected with rVHSV-ΔG-CXCL12a, rVHSV-ΔG-CXCL12b, or rVHSV-ΔG-eGFP at MOI 0.1. After 24 h of infection, each supernatant was collected and used for the transwell migration of leukocytes.

The head kidney of olive flounder (body weight: 300–350 g) that were euthanized with tricaine methanesulfonate (MS222; Sigma) was extracted by ventral incision and transferred to L-15 medium (3 ml) supplemented with heparin (10 units/ml, Sigma), penicillin (100 U/ml, Sigma) and streptomycin (100 U/ml, Sigma). To get leukocytes, the cell suspension obtained by forcing the head kidney through a nylon mesh

Table 1
Summary of primers used in this study.

Name of primer		Sequence (5' to 3')
For cloning and construction of vectors		
pVHSV-ΔG-CXCL12a	F-12a-NheI	<u>GCTAGCATGGATGTCAA</u> ACTGCTGGCAC
	R-12a-Sall	<u>GTCGACTTAGT</u> TTTTCTTGTGTGGCTCTTCTGG
pVHSV-ΔG-CXCL12b	F-12b-NheI	<u>GCTAGCATGGATGTGAA</u> AGTGTGGCTCTC
	R-12b-Sall	<u>GTCGACTCATT</u> AGCCTGCTGTTTTCTCCATC
pVHSV-ΔG-eGFP	F-eGFP-NdeI	<u>CATATGGTGAGCA</u> AGGGCGAGGAGC
	R-eGFP-Sall	<u>GTCGACTTACT</u> TGTACAGCTCGTCCATGCCG
SDM	F-NheI-SDM	TCGCCGTGGAAACCATCATAGCTAGCATCTCAGTGTGGTCTTGG
	R-NheI-SDM	GCAAGACAAACACTGAGAT <u>GCTAGCAT</u> TATGATGGTTCCACGGCGA
For verification of production of recombinant VHSV replicon particles		
From N gene to foreign gene	N-F	CCATGGGGGCGTTGAGGCTCAATG
	12a-R	GTCGACTTAGTTTTTCTTGTGTGGCTCTTCTGG
	12b-R	GTCGACTCATTAGCCTGCTGTTTTCTCCATC
	eGFP-R	ACGCGTTACTTGTACAGCTCGTCCATG
From G gene to NV gene	G-F	ACTGTCTCAAAGAAGTGTGTCAAC
	NV-R	GGATCCTCATGGGGGAGATTCGGAGC
For CXCL12 gene expression by quantitative RT-PCR		
CXCL12a	F	CGGTGTTGGTGTCTGTTCTA
	R	AGACTTCCCTGTGTCTCTTC
CXCL12b	F	GTTTCATCCATACGCCCACT
	R	CATCTTGTGTATAGCGCTCT
β-actin	F	GATCTGGCATCACACCTTCTAC
	R	CATCTTCTCCCTGTTGGCTTTA

Underlined characters represent restriction enzyme sites.

was carefully layered over a 34–47% Percoll density gradient. After centrifugation at 500 g at 4 °C for 30 min, the leukocytes enriched interphase was collected using 1 ml syringe and washed once. Then, the cells were resuspended in culture medium, and an additional Percoll density gradient centrifugation was performed. The interphase cells were isolated and washed 3 times with culture medium (in the last washing step, heparin was not included in the medium). The cell viability was examined with trypan blue exclusion and evaluated to be greater than 98%. Then, 5×10^5 leukocytes (100 μl) were seeded on the upper chamber of transwell migration chamber (Corning cat#3421). The supernatant collected from cells infected with each recombinant VHSV replicon particles was suspended at lower chamber (600 μl). The migration of leukocytes was allowed for 12 h at 20 °C, then, cells migrated to the lower chamber were counted by hemocytometer. The experiment was done with three replicates.

2.5. Immunization and challenge

In the immunization experiment, olive flounder fingerlings (average body weight: 3.26 g) confirmed free from pathogens including VHSV were randomly divided into 4 groups, and reared in four 250 L tanks (12 fish/tank; for challenge) and four 50 L tanks (6 fish/tank; for serum isolation) at 20 °C. After 2 weeks of acclimation, fish were intra-muscularly (i.m.) immunized with 5×10^3 PFU/50 μl/fish of rVHSV-ΔG-CXCL12a, rVHSV-ΔG-CXCL12b, or rVHSV-ΔG-eGFP. Fish in the control group were received 50 μl of L-15 alone. At 24 d post-immunization, the water temperature of 250 L tanks was gradually decreased to 14 °C with a refrigerating apparatus over 3 days, then, fish were i.m. challenged with wild-type VHSV KJ2008 at 5×10^4 PFU/50 μl/fish. Mortalities were recorded daily for 18 d post-challenge (at which no more mortality was expected based on the cumulative mortality graph), and the survived fish were euthanized with MS222. The death caused by VHSV was determined by typical external and internal symptoms and RT-PCR analysis. Fish in 50 L tanks were euthanized with MS222 at 24 d post-immunization, then, blood was collected for the analysis of serum neutralization activity.

2.6. Serum neutralization activity

The sera were heat-inactivated at 56 °C for 30 min to inactivate

complement before use in the test. The serially diluted sera were mixed with a fresh serum of an olive flounder (free from VHSV), and incubated with wild-type VHSV KJ2008 in U-shaped 96-well plate at 14 °C for 1 h. Then, 100 μl of each mixture was added to EPC cells monolayer, and observed CPE every day. The titer of each serum was the last dilution at which CPE was not observed.

2.7. Statistical analysis

Statistical significance was analyzed using SPSS for Windows (Chicago, IL, USA). Data were analyzed by using one-way ANOVA followed by Tukey HSD post-hoc test. The Kaplan-Meier method with log-rank test was used to the data on cumulative mortality, and $P < 0.05$ was considered statistically significant.

2.8. Ethics statement

All experiments were conducted in accordance with the ethical guidelines defined by Pukyong National University's Institutional Animal Care and Use Committee (Approval number PKNU-2018-06).

3. Results

3.1. Rescue of recombinant VHSV replicon particles

Recombinant VHSV replicon particles expressing CXCL12a, CXCL12b, and eGFP (rVHSV-ΔG-CXCL12a, rVHSV-ΔG-CXCL12b, and rVHSV-ΔG-eGFP) were successfully rescued through transfection of G gene-expressing EPC cells with the G gene-deleted VHSV genome vectors that harbored olive flounder CXCL12a, CXCL12b, or eGFP gene between N and P genes (Fig. 1A). The exact insertion of each heterologous gene in the genome of each VHSV replicon particles was verified by RT-PCR (Fig. 1B).

The considerably high expression of CXCL12a and CXCL12b in HINAE cells infected with rVHSV-ΔG-CXCL12a and 12b, respectively, was verified by q RT-PCR (Fig. 2).

3.2. Chemotaxis analysis of olive flounder leukocytes

The transwell migration of leukocytes in response to the

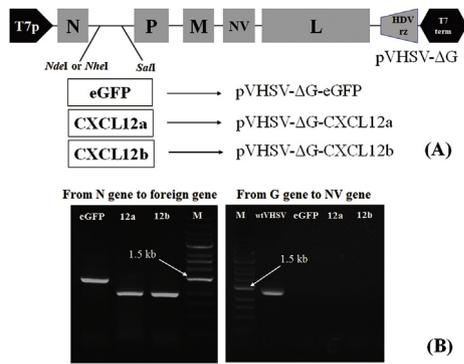


Fig. 1. (A) Construction of vectors for the production of rVHSV-ΔG-eGFP, rVHSV-ΔG-CXCL12a and rVHSV-ΔG-CXCL12b. The ORF of eGFP, CXCL12a, or CXCL12b was inserted between N and P genes of a G gene-deleted VHSV vector (pVHSV-ΔG) by digestion with NdeI (or NheI) and Sall enzymes. (T7p, T7 promoter; HDVr, Hepatitis delta virus ribozyme; T7 term, T7 terminator). (B) Analysis of incorporation of eGFP, CXCL12a, and CXCL12b into the genome of rVHSV-ΔG-eGFP (eGFP), rVHSV-ΔG-CXCL12a (12a) and rVHSV-ΔG-CXCL12b (12b), respectively, by RT-PCR. The deletion of G gene in the VHSV replicon particles was also verified by RT-PCR. (wtVHSV, wild-type VHSV; M, 1 kb DNA ladder).

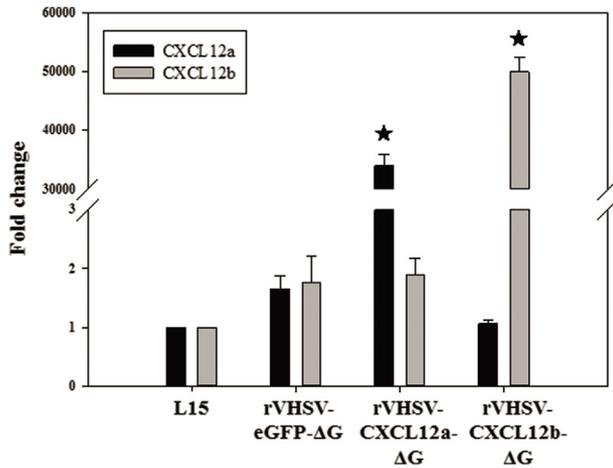


Fig. 2. Quantitative real-time PCR (qRT-PCR) analysis of CXCL12a and CXCL12b expression. HINAe cells were infected with rVHSV-ΔG-eGFP, rVHSV-ΔG-CXCL12a or rVHSV-ΔG-CXCL12b at MOI 0.1. After 24 h of infection, total RNA was extracted from cell pellets and synthesized cDNA for qRT-PCR. The asterisk on the bar represents significantly different at $P < 0.05$.

supernatants derived from cells infected with each type of recombinant VHSV replicon particles was significantly higher than to the control supernatant. Significantly higher number of leukocytes migrated in response to the supernatant from cells infected with rVHSV-ΔG-CXCL12a or rVHSV-ΔG-CXCL12b than from cells infected with rVHSV-ΔG-eGFP. Among all experimental groups, supernatant from rVHSV-ΔG-CXCL12a infected cells attracted the highest number of leukocytes (Fig. 3).

3.3. Immunization

Olive flounder fingerlings immunized with rVHSV-ΔG-CXCL12a showed the lowest cumulative mortalities when challenged with wild-type VHSV (Fig. 4). However, the cumulative mortalities of fish immunized with rVHSV-ΔG-CXCL12b were similar to those of fish immunized with rVHSV-ΔG-eGFP (Fig. 4).

Fish immunized with each VHSV replicon particles showed significantly higher serum neutralization activity than fish immunized with L15 alone (Fig. 5). Furthermore, fish immunized with rVHSV-ΔG-

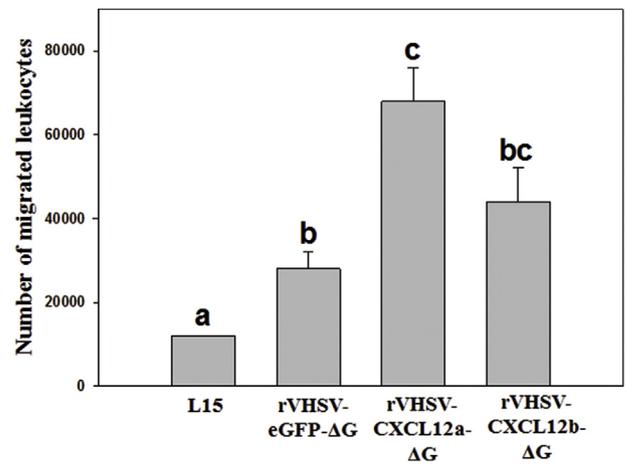


Fig. 3. The effect of supernatant of EPC cells infected with rVHSV-ΔG-eGFP, rVHSV-ΔG-CXCL12a or rVHSV-ΔG-CXCL12b on the chemotaxis of olive flounder leukocytes. Different letters on the bars represent significantly different at $P < 0.05$.

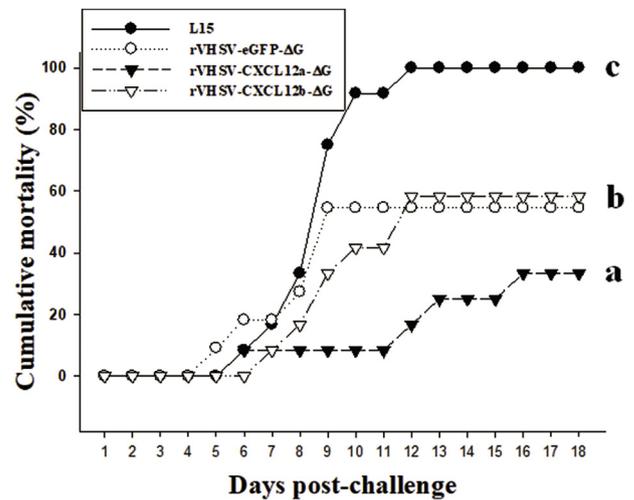


Fig. 4. Cumulative mortality of olive flounder immunized with rVHSV-ΔG-CXCL12a, rVHSV-ΔG-CXCL12b, or rVHSV-ΔG-eGFP. Fish in the control group were received 50 μl of L-15 alone. After 4 weeks post-immunization, fish were intramuscularly challenged with wild-type VHSV KJ2008. Mortalities were recorded daily for 18 d post-challenge. Different letters represent significantly different at $P < 0.05$.

CXCL12a showed significantly higher neutralization activity than fish immunized with rVHSV-ΔG-eGFP. However, there were no significance in neutralization activity between fish immunized with rVHSV-ΔG-CXCL12b and rVHSV-ΔG-eGFP (Fig. 5).

4. Discussion

Although the present CXCL12 expressing-VHSV replicon particles (rVHSV-ΔG-CXCL12a & rVHSV-ΔG-CXCL12b) are unable to produce infective viral particles because of lacking G gene in the genome, they still can transcribe their genes in the infected cells. The transcription level of each gene in rhabdoviruses is gradually decreased according to the order of genes from 3' to 5' [30,31]. Therefore, in this study, to maximally express CXCL12, the ORF of CXCL12 gene was inserted between N and P gene, and a considerably high amount of CXCL12 transcript in the cells infected with rVHSV-ΔG-CXCL12a or rVHSV-ΔG-CXCL12b was verified.

The critical role of CXCL12-CXCR4 signaling in leukocytes trafficking has also been demonstrated in fish [6,21]. In the present study,

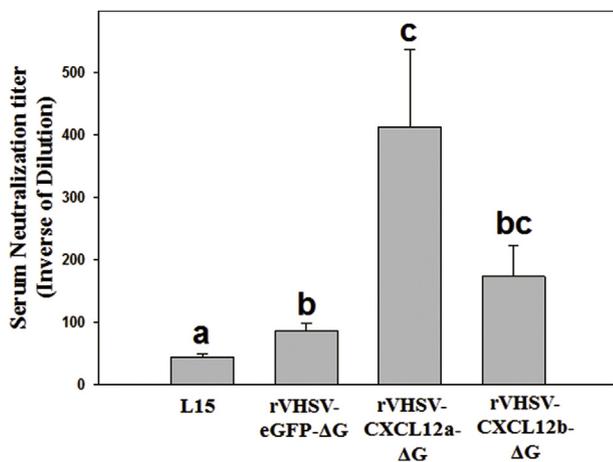


Fig. 5. Serum neutralization activity. The sera of olive flounder immunized with rVHSV-ΔG-CXCL12a, rVHSV-ΔG-CXCL12b, or rVHSV-ΔG-eGFP were collected at 24 d post-immunization and used for neutralization test. Fish in control group were injected L15 alone. Values are mean \pm standard error. Different letters on the bars represent significantly different at $P < 0.05$.

we generated olive flounder CXCL12a-, CXCL12b-, and eGFP-expressing recombinant VHSV replicon particles, and demonstrated the significantly higher ability of the supernatant collected from cells infected with rVHSV-ΔG-CXCL12a & b to attract olive flounder leukocytes than the supernatant of cells infected with rVHSV-ΔG-eGFP. This result suggests that the CXCL12 of olive flounder has the ability to attract leukocytes. However, the supernatant from rVHSV-ΔG-eGFP also attracted significantly higher number of leukocytes than control supernatant, suggesting that the present recombinant VHSV replicon particles can stimulate the secretion of chemotactic factors of cells to make leukocytes migrate to the infected regions. Therefore, the present results suggest that the ability of leukocytes attraction in rVHSV-ΔG-CXCL12a & -CXCL12b was further strengthened by the addition of chemokine-expressing ability. Moreover, the significantly higher attraction of leukocytes by rVHSV-CXCL12a supernatant compared to rVHSV-CXCL12b supernatant suggests that CXCL12a would be more appropriate for the induction of immunity than CXCL12b in olive flounder.

The up-regulation of CXCL12 gene expression by polyinosinic: polycytidylic acid (poly I:C), lipopolysaccharide (LPS), viruses, or bacteria has been reported in several fish species [10–12], which suggests that CXCL12 may be involved in inflammatory responses and may have an adjuvant ability. Thus, in this study, we immunized olive flounder with rVHSV replicon particles expressing olive flounder CXCL12a or CXCL12b, and the results showed higher survival rates of fish immunized with rVHSV-ΔG-CXCL12a than fish immunized with rVHSV-ΔG-CXCL12b or rVHSV-ΔG-eGFP when challenged with wild-type VHSV. This result suggests that the protective potential of VHSV replicon particles against VHSV infection in olive flounder can be enhanced by supplemental expression of CXCL12a.

Although much less is known in fish about the effect of CXCL12 on lymphocyte activities, the critical role of CXCL12 in the trafficking of T and B lymphocyte has been well demonstrated in mammals [32–34]. In the present study, fish immunized with rVHSV-ΔG-CXCL12a showed the highest serum neutralization activity suggesting that CXCL12a has the ability to enhance the adaptive humoral immune response in fish.

Although VHSV vaccines based on viral replicon particles are effective for the induction of protective immune responses, they are classified as gene modified organisms (GMOs). While the practical use of DNA vaccines that have been showing highly effective against fish rhabdoviral diseases has been limited since the fishes immunized with DNA vaccines are regarded as GMOs in many countries including Korea. Therefore, more endeavors to address environmental safety issues

related to VHSV replicon particles and DNA vaccines are needed to be permitted as vaccines for aquaculture farms.

In conclusion, we demonstrated the potential of CXCL12a as a molecular adjuvant of vaccines based on VHSV replicon particles. The interaction between CXCL12 and adaptive immune factors should be further investigated to know the mechanism of the present enhancement of protection.

Acknowledgements

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References

- [1] J.G. Cyster, Chemokines and cell migration in secondary lymphoid organs, *Science* 286 (1999) 2098–2102.
- [2] A. Zlotnik, O. Yoshie, Chemokines: a new classification system and their role in immunity, *Immunity* 12 (2000) 121–127.
- [3] C.C. Bleul, R.C. Fuhlbrigge, J.M. Casasnovas, A. Aiuti, T.A. Springer, A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1), *J. Exp. Med.* 184 (1996) 1101–1109.
- [4] Y.R. Zou, A.H. Kottmann, M. Kuroda, I. Taniuchi, D.R. Littman, Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development, *Nature* 393 (1998) 595–599.
- [5] T. Ara, Y. Nakamura, T. Egawa, T. Sugiyama, K. Abe, T. Kishimoto, Y. Matsui, T. Nagasawa, Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1), *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 5319–5323.
- [6] K.B. Walters, J.M. Green, J.C. Surfus, S.K. Yoo, A. Huttenlocher, Live imaging of neutrophil motility in a zebrafish model of WHIM syndrome, *Blood* 116 (2010) 2803–2811.
- [7] N.B. David, D. Sapède, L. Saint-Etienne, C. Thisse, B. Thisse, C. Dambly-Chaudière, F.M. Rosa, A. Ghysen, Molecular basis of cell migration in the fish lateral line: role of the chemokine receptor CXCR4 and of its ligand, SDF1, *Proc. Natl. Acad. Sci. U. S. A.* 99 (2002) 16297–16302.
- [8] M.O. Huising, T. van der Meulen, G. Flik, B.M. Verburg-van Kemenade, Three novel carp CXC chemokines are expressed early in ontogeny and at nonimmune sites, *Eur. J. Biochem.* 271 (2004) 4094–4106.
- [9] P. Baoprasertkul, C. He, E. Peatman, S. Zhang, P. Li, Z. Liu, Constitutive expression of three novel catfish CXC chemokines: homeostatic chemokines in teleost fish, *Mol. Immunol.* 42 (2005) 1355–1366.
- [10] X. Wan, X. Chen, Molecular cloning and expression analysis of a CXC chemokine gene from large yellow croaker *Pseudosciaena crocea*, *Vet. Immunol. Immunopathol.* 127 (2009) 156–161.
- [11] W.S. Thulasitha, N. Umasuthan, I. Whang, B.S. Lim, H.B. Jung, J.K. Noh, J. Lee, A CXC chemokine gene, CXCL12, from rock bream, *Oplegnathus fasciatus*: molecular characterization and transcriptional profile, *Fish Shellfish Immunol.* 45 (2015) 560–566.
- [12] C.S. Wu, T.Y. Wang, C.F. Liu, H.P. Lin, Y.M. Chen, T.Y. Chen, Molecular cloning and characterization of orange-spotted grouper (*Epinephelus coioides*) CXC chemokine ligand 12, *Fish Shellfish Immunol.* 47 (2015) 996–1005.
- [13] A. Amores, A. Force, Y.L. Yan, L. Joly, C. Amemiya, A. Fritz, R.K. Ho, J. Langeland, V. Prince, Y.L. Wang, M. Westerfield, M. Ekker, J.H. Postlethwait, Zebrafish hox clusters and vertebrate genome evolution, *Science* 282 (1998) 1711–1714.
- [14] O. Jaillon, J.M. Aury, F. Brunet, J.L. Petit, N. Stange-Thomann, E. Maucci, et al., Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype, *Nature* 431 (2004) 946–957.
- [15] M. Kasahara, K. Naruse, S. Sasaki, Y. Nakatani, W. Qu, B. Ahsan, et al., The medaka draft genome and insights into vertebrate genome evolution, *Nature* 447 (2007) 714–719.
- [16] M. Doitsidou, M. Reichman-Fried, J. Stebler, M. Kopranner, J. Dorries, D. Meyer, C.V. Esguerra, T. Leung, E. Raz, Guidance of primordial germ cell migration by the chemokine SDF-1, *Cell* 111 (2002) 647–659.
- [17] H. Knaut, C. Werz, R. Geisler, C. Nusslein-Volhard, A zebrafish homologue of the chemokine receptor Cxcr4 is a germ-cell guidance receptor, *Nature* 421 (2003) 279–282.
- [18] Q. Li, K. Shirabe, J.Y. Kuwada, Chemokine signaling regulates sensory cell migration in zebrafish, *Dev. Biol.* 269 (2004) 123–136.
- [19] T. Mizoguchi, H. Verkade, J.K. Heath, A. Kuroiwa, Y. Kikuchi, Sdf1/Cxcr4 signaling controls the dorsal migration of endodermal cells during zebrafish gastrulation, *Development* 135 (2008) 2521–2529.
- [20] O. Palevitch, E. Abraham, N. Borodovsky, G. Levkowitz, Y. Zohar, Y. Gothilf, Cxcl12a-Cxcr4b signaling is important for proper development of the forebrain GnRH system in zebrafish, *Gen. Comp. Endocrinol.* 165 (2010) 262–268.
- [21] T.A. Hersh, A.L. Dimond, B.A. Ruth, N.V. Lupica, J.C. Bruce, J.M. Kelley, B.L. King, B.V. Luton, A role for the CXCR4-CXCL12 axis in the little skate, *Leucoraja erinacea*, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 315 (2018) R218–R229.
- [22] H. Schutze, E. Mundt, T.C. Mettenleiter, Complete genomic sequence of viral hemorrhagic septicemia virus, a fish rhabdovirus, *Virus Gene.* 19 (1999) 59–65.

- [23] K.E. Einer-Jensen, P. Ahrens, R. Forsberg, N. Lorenzen, Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus, *J. Virol.* 85 (2004) 1167–1179.
- [24] H.F. Skall, N.J. Olesen, S. Møllergaard, Viral hemorrhagic septicemia virus in marine fish and its implications for fish farming - a review, *J. Fish Dis.* 28 (2005) 509–529.
- [25] N. Tordo, A. Benmansour, C. Calisher, R.G. Dietzgen, R.-X. Fang, A.O. Jackson, G. Kurath, S. Nadin-Davis, R.B. Tesh, P.J. Walker, Family rhabdoviridae, in: C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger, L.A. Ball (Eds.), *Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses*, Elsevier Academic Press, San Diego, California, 2005, pp. 623–644.
- [26] E. Gomez-Casado, A. Estepa, J.M. Coll, A comparative review on European-farmed finfish RNA viruses and their vaccines, *Vaccine* 29 (2011) 2657–2671.
- [27] M.S. Kim, D.S. Kim, K.H. Kim, Oral immunization of olive flounder (*Paralichthys olivaceus*) with recombinant live viral hemorrhagic septicemia virus (VHSV) induces protection against VHSV infection, *Fish Shellfish Immunol.* 31 (2011) 212–216.
- [28] M.S. Kim, J.S. Park, K.H. Kim, Generation of G gene-deleted viral hemorrhagic septicemia virus (VHSV) and evaluation of its vaccine potential in olive flounder (*Paralichthys olivaceus*), *Fish Shellfish Immunol.* 45 (2015) 666–671.
- [29] L. Huang, G. Li, Z. Mo, P. Xiao, J. Li, J. Huang, De Novo assembly of the Japanese flounder (*Paralichthys olivaceus*) spleen transcriptome to identify putative genes involved in immunity, *PLoS One* 10 (2015) e0117642.
- [30] J.N. Barr, S.P. Whelan, G.W. Wertz, Transcriptional control of the RNA-dependent RNA polymerase of vesicular stomatitis virus, *Biochim. Biophys. Acta* 1577 (2002) 337–353.
- [31] S.P. Whelan, J.N. Barr, G.W. Wertz, Transcription and replication of nonsegmented negative-strand RNA viruses, *Curr. Top. Microbiol. Immunol.* 283 (2004) 61–119.
- [32] C.C. Bleul, M. Farzan, H. Choe, C. Parolin, I. Clark-Lewis, J. Sodroski, T.A. Springer, The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry, *Nature* 382 (1996) 829–833.
- [33] T. Nanki, K. Hayashida, H.S. El-Gabalawy, S. Suson, K. Shi, H.J. Girschick, S. Yavuz, P.E. Lipsky, Stromal cell-derived factor-1-CXC chemokine receptor 4 interactions play a central role in CD4+ T cell accumulation in rheumatoid arthritis synovium, *J. Immunol.* 165 (2000) 6590–6598.
- [34] T. Nakayama, K. Hieshima, D. Izawa, Y. Tatsumi, A. Kanamaru, O. Yoshie, Cutting edge: profile of chemokine receptor expression on human plasma cells accounts for their efficient recruitment to target tissues, *J. Immunol.* 170 (2003) 1136–1140.