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Poly (I:C) and imiquimod induced immune responses and their effects on the survival of olive flounder (*Paralichthys olivaceus*) from viral haemorrhagic septicaemia

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ABSTRACT

The stimulation of immune genes by polyinosinic:polycytidylic acid (poly (I:C)) and imiquimod in olive flounder (*Paralichthys olivaceus*) and their role in control of viral haemorrhagic septicaemia virus (VHSV) infection were examined. Poly (I:C) (100 μg/fish) treated olive flounder had very low mortality (5%) post VHSV infection, while the imiquimod treated group had 65% and 85% mortality at a dose of 100 μg/fish and 50 μg/fish, respectively. Though the imiquimod treated group had high mortality, it was lower than the untreated group, which had 90% mortality. *In vivo* experiments were conducted to determine effect of the two ligands on immune modulation in the head kidney of olive flounder. Poly (I:C) activated the immune genes (TLR-3, TLR-7, MDA-5, LGP-2, IRF-3, IRF-7, IL-1β type I IFN and Mx) very early, within 1 d post stimulation, faster and stronger than imiquimod. Though Mx levels were enhanced by imiquimod, the host was still susceptible to VHSV. The poly (I:C) treated group had a high immune response at the time of infection and 1 dpi, though it decreased at later stages. The imiquimod treated group and the unstimulated group had a higher immune response to VHSV compared to the poly (I:C) treated group. The nucleoprotein copies of VHSV were very low in the poly (I:C) treated group but interestingly, were high in both untreated and imiquimod treated fish. Thus, host survival from a viral infection does not only depend on the quantity of immune response but also the time of response. Although imiquimod enhanced immune gene expression in olive flounder, a delayed response could be the reason for high mortality to VHS compared with poly (I:C), which induced the immune system effectively and efficiently to protect the host.

1. Introduction

Olive flounder (*Paralichthys olivaceus*) is a highly valued commercially important aquaculture species in East Asian countries, including Korea and Japan [1]; since 2001, viral haemorrhagic septicaemia virus (VHSV) has resulted in large scale economic losses in the culture industry [2–5]. Viral haemorrhagic septicaemia is one of the most severe viral diseases of teleosts in Europe, North America and East Asia. Worldwide, VHSV infects more than 48 different fish species [6]. VHSV is an enveloped negative-sense single-strand RNA (ssRNA) virus of genus *Novirhabdovirus*, Rhabdoviridae and the genotype of the virus found in the Korean peninsula has been classified as genotype IVa based on the glycoprotein sequence [3,6,7].

The innate immune system is the first line of host defence; it is activated immediately upon pathogen entry and is effective against a broad range of pathogens with no need for pathogen-specific immunity [8]. Pattern recognition receptors (PRRs) initiate an innate immune response by selectively sensing the pathogen-associated molecular patterns (PAMPs) of the invading pathogen and play a pivotal role in the host immune response [9]. Therefore, the stimulation of PRRs, toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) using synthetic ligands, such as polyinosinic:polycytidylic acid (poly (I:C)) or imiquimod, has proven to be very effective against malignant and viral diseases in mammals [10–13]. Similarly, such ligands have been highly efficient in the control of viral diseases in fish [14–22]. Although poly (I:C) has been shown to promote a high level of host survival in VHS, no significant information is available about other ligands such as imiquimod. Imiquimod and other synthetic compounds that imitate viral ssRNA are able to stimulate TLR-7, cytokines and interferon (IFN) stimulated genes (ISGs) in various fishes [23–26]. A comparative study of these two types of ligands may provide additional information on immune-

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stimulation in the olive flounder and survival after VHSV infection. Moreover, the immune gene kinetics in response to these ligands and their role in imparting host defence against viral infection need to be studied.

Our earlier studies showed that olive flounder at 15 °C was highly susceptible to VHSV despite expressing a high level of ISGs, whereas at 20 °C no mortality and a quicker immune response was observed [27]. Furthermore, the hosts were found to be deficient in the expression of crucial immune genes (such as TLR-7, caspase-3) at 15 °C, which could lead to susceptibility [27,28]. We challenged olive flounders with VHSV after treatment with the ligands. The immune gene kinetics in response to the ligands were studied to determine their effectiveness in elevating innate immunity. We extended the experiments by conducting an *in vivo* experiment to understand the host immune response in pre-stimulated olive flounders against the virus.

2. Materials and methods

2.1. Culture of viral haemorrhagic septicaemia virus and ligand preparation

VHSV was propagated in fathead minnow (FHM) cells cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) with 2% foetal bovine serum (FBS) (Gibco, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco, USA). The final viral titre was $10^{8.8}$ $TCID_{50}$/ml and it was stored in aliquots at −80 °C.

Imiquimod (Enzo Life sciences, USA) and Poly (I:C) (Sigma-Aldrich, Israel) were suspended or dissolved in phosphate buffered saline (PBS) at 1 μg/μl final concentration. The toxicity of imiquimod was analysed by injecting different doses (200 μg/fish, 100 μg/fish and 50 μg/fish) of imiquimod into nine olive flounders (16 ± 3 g) in each group (at 15 ± 0.5 °C). The internal organs were observed by sacrificing three individuals at each sampling time of 7, 14 and 21 days post injection. Though no dose caused mortality, 200 μg/fish caused liver congestion, while 50 and 100 μg/fish were found to be safer (data not shown). Therefore, 50 μg/fish and 100 μg/fish doses of imiquimod were used for the experiment. In our experiment, a dose of 100 μg poly (I:C)/fish was used, since that dose has been confirmed to be safe and efficient in immune modulation in olive flounder [29].

2.2. Mortality of pre-ligand treated/VHSV infected olive flounder (challenge 1)

The olive flounder (16 ± 3 g) were transported from our culture centre to a temperature controlled indoor facility, which is equipped with seawater filtration and UV-sterilization units. The experimental animals were acclimatized to 15 ± 0.5 °C for a period of 7 days and were maintained with regular feeding and aeration. Approximately 50–60% water was siphoned out daily, and an equal volume of fresh seawater (15 ± 0.5 °C) was added. The olive flounder were divided into five groups, each containing 20 individuals. Individuals from each group were injected (intraperitoneally - ip) with 100 μl of either 50 μg or 100 μg imiquimod, or 100 μg poly (I:C) or 100 μl of PBS as a control. Two days post stimulation (dps), one of the two control groups (positive control) and the pre-stimulated groups were infected (ip) with 100 μl of VHSV (10^7 $TCID_{50}$/fish). Another control group (negative control) was injected (ip) with 100 μl of culture medium, DMEM (with 2% FBS). The fish groups were observed for 21 days at 15 ± 0.5 °C. The dead fish were immediately removed. All moribund and dead fish had dark body colour and severe dropsy due to clear ascites.

2.3. Immune gene kinetics in olive flounder injected with imiquimod or poly (I:C) stimulation

Time course analysis of gene expression from 6 h to 4 d after ligand stimulation was conducted to know immune responses of olive flounder against imiquimod or poly (I:C), then VHSV infection time point for further experiment was selected. Another batch of olive flounder (16 ± 3 g) were used for this experiment. Three batches of olive flounder, each containing 15 individuals were maintained at 15 ± 0.5 °C as explained above. Individual fish from each group was injected (ip) with 100 μl of either 100 μg imiquimod, 100 μg poly (I:C) or PBS and maintained at 15 ± 0.5 °C. At 6 and 12 h post stimulation (hps) and 1, 2 and 4 days post stimulation (dps), olive flounder (n = 3) from each group (including the control) were sacrificed, and their head kidneys were aseptically collected, flash frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted using RNAiso Plus (Takara, Japan) as described in the manufacturer's instructions. The quality of the RNA was assessed by agarose gel (2.0%) electrophoresis, quantified with a NanoDrop™ 1000 spectrophotometer (Thermo Scientific) and stored at −80 °C in aliquots for further use.

2.4. Immune gene kinetics of pre-ligand treated/VHSV infected olive flounder (challenge 2)

Two days post treatment, both the ligands stimulated high immune response in olive flounder and hence all the groups except one PBS treated group were challenged with VHSV. Olive flounder (22 ± 2 g) were brought to our temperature-regulated fish rearing room from the culturing facility and acclimatized to 15 ± 0.5 °C for one week. Four groups of twenty individuals were stocked in each tank and maintained as described above. First two groups were injected (ip) with imiquimod (100 μg/fish) or poly (I:C) (100 μg/fish), while remaining two groups were injected with 100 μl of PBS. Two days post treatment, all the groups except one PBS treated group were challenged with 100 μl of VHSV (10^7 $TCID_{50}$/fish), and respective group was named group-I (imiquimod treated), group-P (poly (I:C) treated) and group-V (VHSV alone). Last group of PBS injected fish was given a mock injection of MEM (with 2% FBS) and named group-C (Control). Sampling was performed before VHSV challenge (0 h) and 1, 3 and 5 days post VHSV challenge. Another set of the four groups were maintained to determine the mortality rate, as explained in section 2.2.

2.5. Reverse transcription and real-time PCR gene expression

Total RNA was treated with Recombinant DNase I (Takara Bio Inc, Japan) as prescribed by the manufacturer to obtain total RNA free of genomic DNA. The efficacy of the DNase treatment was analysed by real-time PCR using the DNase-treated RNA as a template. The integrity of the RNA was checked by gel electrophoresis. RNA quantification was performed using a NanoDrop™ 1000 spectrophotometer. Genomic DNA free total RNA was reverse transcribed using ReverTra Ace qPCR RT kit (Toyobo, Japan). Total RNA (1 μg) was incubated at 65 °C for 5 min and cooled on ice for 15 min. The mixture consisted of 2 μl of reaction buffer, 0.5 μl of reverse transcriptase, and 0.5 μl of primer mix, and the total volume was brought to 10 μl by adding nuclease-free water that was included in the kit. The mixture was incubated at 37 °C for 30 min before inactivating the reverse transcription reaction at 80 °C for 2 min. The resultant cDNA was diluted tenfold using nuclease-free water and stored at −20 °C. One μl of cDNA was used as template for real-time PCR using AccuPower 2x Greenstar qPCR Master mix (Bioneer, Korea). Each reaction included 10 μl of master mix, 1 μl forward and reverse primers, 1 μl of cDNA template and nuclease free water (supplied with the kit) to make up to 20 μl of reaction volume. The PCR programme began with a 10-min initial denaturation at 94 °C, followed by 35 cycles of 20 s denaturation (94 °C), annealing (temperature is given in Table 1) and scanning. The efficiency of amplification was monitored by melting curve analysis after completion of 35 cycles of amplification. Each sample was run in duplicate. Primers were designed and standardized as previously described [30]. Details of the primer used in this study are given in Table 1. Absolute quantification of VHSV was performed by real-time PCR as explained earlier [30]. Fold change in host immune gene expression was quantified relative to β-actin using the equation

Table 1
Details of primers used for real-time PCR.

Target gene	GenBank Acc. No	Sense Primer	Antisense Primer	Ta[a]	PCR Efficiency	Reference
β-actin	HQ386788	cctcttccagccttcattc	tggttcctccagatagcac	56	2.0980	[30]
VHSV N	JF792424	atctggaggcaaagtgcaag	ccatgaggttgtcgttgttg	62	2.0221	[30]
TLR-3	AB109394	aacgcctggttcatcaagtg	cgaatgtcgaagtgcaagag	58	1.9199	[30]
TLR-7	HQ845984	cctgggaaatctggaagaac	tttgagggaggagaaactgc	62	2.0291	[30]
MDA-5	HQ401014	acgagcgacctcttgatttg	agcgtcaccacgaagttttg	60	2.0795	[27]
LGP-2	HM070372	gatgatgcagatgatccaagactaca	ctcgctgctctaaaatcaccacat	60	1.9977	[27]
IRF-3	GU017418	acaccatgaaccagagcaac	tgtccaaaagtgtccctgtg	62	2.0354	[30]
IRF-7	GU017419	tctgatctgtcggcactttc	ccgaacacggagttaatgag	58	1.8257	[30]
Type I IFN	AB511962	caggtgtcaaatgcatcagc	tggaatcctcctcaaacagg	62	2.0254	[30]
Mx	AB110446	tcactggatttcccaacctc	tgtcactcaaactgctgctg	62	2.0376	[30]
IL-1β	AB070835	aaagaagcatcaccactgtct	ctactcaacaacgccaccttt	56	1.9473	[30]
Caspase-3	JQ394697	acatcatgacacgggtgaac	tccttcgtcagcattgacac	58	1.9146	[28]

[a] Ta: Annealing temperature.

described by Ref. [31]:

$$\text{Relative expression} = E_t^{\,(\text{control Ct-infection Ct})} / E_c^{\,(\text{control Ct-infection Ct})}$$

where E_t is the PCR efficiency of the target gene primer and E_c is that of reference gene (β-actin) primer; Ct is the Cycle threshold.

2.6. Statistical analysis

Statistical analysis was carried out in SAS 9.3. The significance of the differences in gene expression at different sampling time was evaluated by one-way analysis of variance (ANOVA) with Tukey's Studentized Range (HSD) Test ($\alpha = 0.05$). An unpaired *t*-test was used to ascertain the significance of the differences in gene expression. Two-way ANOVA with Tukey's Studentized Range (HSD) Test ($\alpha = 0.05$) was used to determine the significance of the differences in gene expression in challenge 2. A value of $p < 0.05$ was considered to be significant. The mean expression values and the standard error at each time point were determined.

3. Results

3.1. Mortality of pre-ligand treated/VHSV infected olive flounder

Olive flounder with no immune stimulation had a 90% cumulative percentage mortality (CPM) when infected with VHSV, whereas poly (I:C) pre-treated olive flounder had only a 5% CPM. Unexpectedly, imiquimod pre-treated olive flounder had high mortality, at 85% in the 50 μg/fish group and 65% in the 100 μg/fish group (Fig. 1A). However, the mortality of the infected fish was delayed in the imiquimod treated group compared to the untreated group. A similar observation was made in the second challenge trial conducted and depicted in Fig. 1B. Imiquimod treatment delayed mortality of the host though cumulative mortality was 75% compared to 95% in untreated group. Mortality was recorded between 3 and 16 days post infection (dpi), and all the dead fish showed clinical signs of VHS. The control group was healthy, and no fish died.

3.2. Immune gene kinetics by imiquimod or poly (I:C) stimulation

The immune response of olive flounder to poly (I:C) and imiquimod is detailed in Table 2 and graphically presented in Fig. 2. In response to poly (I:C) stimulation, TLR-3 expression was significantly higher than the imiquimod treated group at 6 hps and peaked at 12 hps (30-fold), whereas imiquimod caused no considerable changes and remained similar to control level. Both the ligands stimulated TLR-7 to a maximum expression level at 1–2 dps. Poly (I:C) induced Melanoma Differentiation-Associated-5 (MDA-5) and Laboratory of Genetics and Physiology-2 (LGP-2) faster and stronger than imiquimod. The former ligand induced a significantly higher MDA-5 expression level at 12 hps ($p < 0.01$) and a significantly higher LGP-2 expression level at 6 hps ($p < 0.05$), 12 hps ($p < 0.01$). The PKR response was quicker and stronger after poly (I:C) treatment in comparison with imiquimod treatment. Accordingly, IRF-3 and IRF-7 expression was quicker and more robust after poly (I:C) treatment and reached a maximum at 6 hps-1 dps, whereas the IRFs did not reach a maximum until 2 dps after imiquimod treatment. Strong stimulation of type I IFN in the poly (I:C) group (at 6 and 12 hps) was evident; however, there was no significant impact of imiquimod. Mx gene expression was highly elevated in olive flounder treated with poly (I:C) from 12 hps (120-fold) to 1 dps (145-fold), while the impact of imiquimod was visible at 2 dps with a 47-fold increase. Poly (I:C) induced the upregulation of IL-1β at 12 hps (462-fold), which was significantly higher than the imiquimod treated group, which caused a late (2 dps) and milder (12-fold) response. Poly (I:C) induced a higher caspase-3 level at 1 dps. This experiment demonstrated that poly (I:C) caused immunostimulation is 12 h to 1d post stimulation while imiquimod did not induce a response until 1–2 d post stimulation.

3.3. Immune gene expression kinetics of pre-ligand treated/VHSV infected olive flounder

Olive flounder were infused with imiquimod or poly (I:C) and challenged with VHSV after two days. The immune response of these groups was compared with the group that was infected but not pre-ligand treated. Sampling was conducted to study viral amplification in the host and host immune response at regular intervals. Samples were taken from the time of VHSV challenge, which is referred to as 0 days post infection. Viral transcripts were at the highest level in group-V followed by group-I and group-P. Poly (I:C) treatment caused a significant reduction in VHSV transcription from 3.7 log at 1 dpi to 1 log at 5 dpi. However, in group-I, viral transcription during the experimental period was 5.3 log at 1 dpi and remained 4.6 log at 5 dpi; it remained higher, reaching 6.0 log at 1 dpi and 5.6 log at 5 dpi in group-V. It is evident from Fig. 3 that poly (I:C) treated olive flounder had the least viral transcripts, while imiquimod treated or untreated had a significantly larger quantity.

Immune gene expression data is shown in Table 3 and is graphically presented in Fig. 4. Interestingly, group-I and group-V had higher gene expression than group-P, though the other two groups had a higher viral load. Group-P had significantly higher expression of TLR-7, PKR, IRF-3, Mx and IL 1β than group-V two days post stimulation and at the time of VHSV infection. On the other hand, group-I only had an increase in the expression of TLR-7 and PKR. However, at later time points VHSV infection, group-P had significantly lower expression of these genes than group-I or group-V. After VHSV infection, the expression of

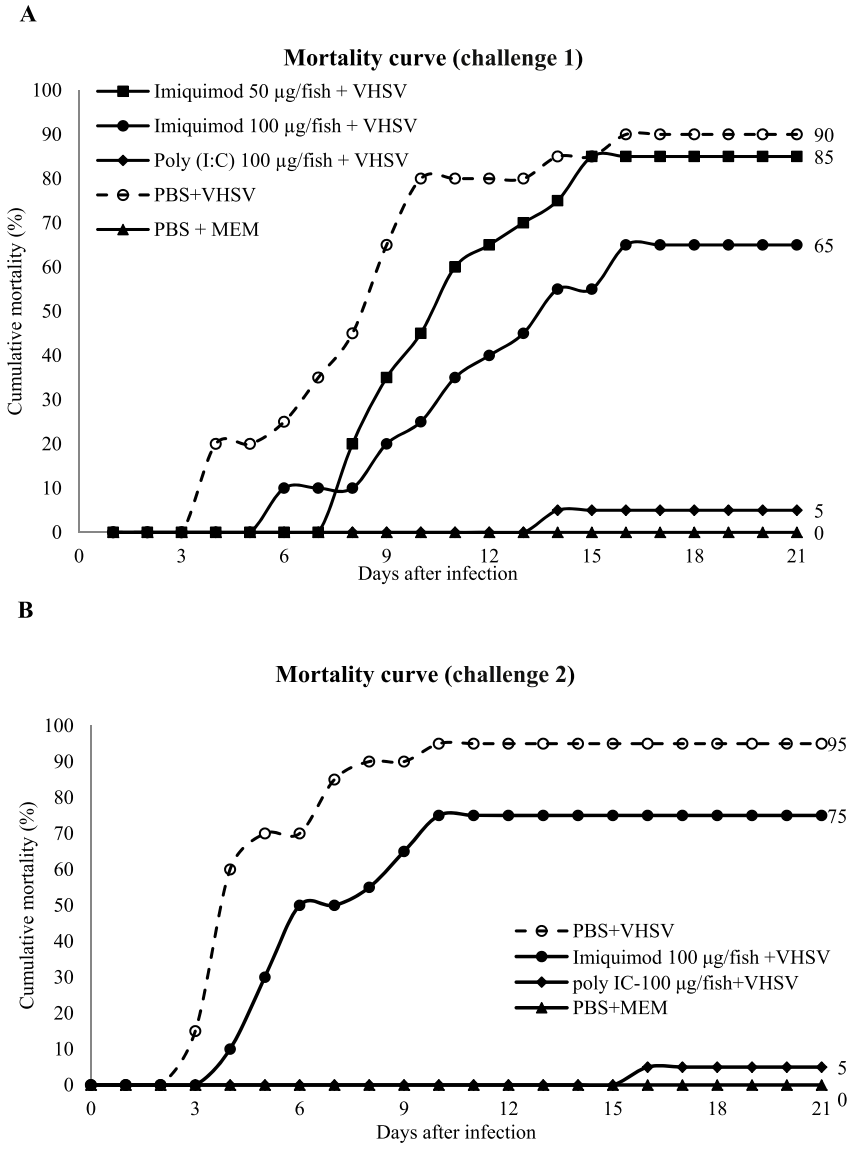

Fig. 1. Mortality curve of the imiquimod or poly (I:C) pre-stimulated olive flounder infected with viral haemorrhagic septicaemia virus (VHSV). (A) Olive flounder (n = 20/group) were intra-peritoneally injected with imiquimod (50 or 100 µg/fish), poly (I:C) (100 µg/fish) or PBS and reared at 15 °C. (B) The experiment was repeated with pre-stimulation of olive flounder by injecting 100 µg/fish with imiquimod or poly (I:C). Two days post stimulation; fish were injected with VHSV (10^7 $TCID_{50}$/fish) or virus culture medium and observed for mortality for 21 days.

immune genes had a similar trend in both group-I and group-V. Combined with the results of the previous experiment, we can state that poly (I:C) stimulated a wider range of innate immune system responses at very early stages after the host was infected with VHSV, which allowed the host to contain viral replication more effectively than group-I. The elevated expression of genes such as Mx seems to be a simple reaction to viral transcription in the olive flounder and is not sufficient to overcome infection.

Table 2
Relative expression of immune gene transcripts in the head kidney of olive flounder (n = 3) treated with imiquimod or poly (I:C). Mean ± standard error analysed per time point are displayed.

Time	TLR 3	TLR 7	MDA 5	LGP2	PKR	IRF 3	IRF 7	IFN type I	Mx	IL 1β	Caspase 3
Poly IC											
6h	8.7 ± 0.9	2.9 ± 0.1	1.4 ± 1.0	35.9 ± 5.6^{ab}	3.8 ± 1.6	3.2 ± 0.9	5.1 ± 2.0^{ab}	74.1 ± 70.2	18.5 ± 13.2	20.9 ± 5.8^{b}	0.7 ± 0.9
12h	30.3 ± 13.7	2.5 ± 0.3	5.1 ± 0.6	77.5 ± 7.4^{a}	6.1 ± 2.4	9.9 ± 4.2	11.1 ± 2.5^{a}	68.5 ± 32.6	120 ± 64.6	461.8 ± 147.3^{a}	0.8 ± 0.4
1d	5.7 ± 2.9	6.4 ± 3.3	13.2 ± 5.9	41.4 ± 21.5^{ab}	5.8 ± 2.0	3.4 ± 1.4	5.9 ± 2.3^{ab}	2.2 ± 1.6	145.2 ± 50.4	20.4 ± 11.6^{b}	2.1 ± 0.9
2d	4.6 ± 2.2	6.4 ± 1.8	6.1 ± 3.6	8.1 ± 2.5^{b}	3.7 ± 1.2	1.2 ± 0.4	4.1 ± 1.6^{ab}	0.9 ± 0.6	13.7 ± 5.4	17.8 ± 6.7^{b}	1.3 ± 0.7
4d	4.1 ± 2.0	2.1 ± 0.7	1.3 ± 0.8	0.8 ± 0.4^{b}	1.9 ± 0.7	1.3 ± 0.9	2.0 ± 0.9^{b}	0.6 ± 0.4	4.4 ± 2.5	19.9 ± 12.9^{b}	1.2 ± 0.4
Imiquimod											
6h	2.3 ± 1.2	1.9 ± 1.2^{b}	1.2 ± 0.8^{b}	1.9 ± 1.2^{b}	1.1 ± 0.5	0.4 ± 0.2	1.3 ± 0.9	2.1 ± 1.6	5.8 ± 4.6^{b}	7.8 ± 2.6	0.7 ± 0.1
12h	1.3 ± 0.4	2.2 ± 0.9^{b}	0.8 ± 0.3^{b}	2.2 ± 0.2^{b}	0.8 ± 0.2	0.4 ± 0.3	1.1 ± 0.6	1.2 ± 0.6	3.1 ± 1.0^{b}	3.2 ± 0.7	1.1 ± 1.0
1d	0.7 ± 0.4	8.2 ± 3.3^{ab}	2.5 ± 1.2^{ab}	10.2 ± 2.9^{ab}	2.6 ± 1.0	1.1 ± 0.5	3.3 ± 0.7	2.5 ± 0.9	12.9 ± 6.0^{ab}	1.8 ± 0.4	1.0 ± 0.1
2d	0.8 ± 0.2	11.6 ± 2.6^{a}	14.7 ± 5.8^{a}	20.2 ± 7.5^{a}	4.9 ± 1.8	5.3 ± 3.2	7.7 ± 4.0	2.4 ± 1.2	46.9 ± 17.5^{a}	11.8 ± 8.3	1.7 ± 1.1
4d	0.9 ± 0.5	1.7 ± 0.5^{b}	1.4 ± 0.7^{b}	1.1 ± 0.5^{b}	2.4 ± 1.1	0.7 ± 0.4	1.4 ± 0.7	0.7 ± 0.4	1.6 ± 0.9^{b}	2.1 ± 0.7	2.4 ± 1.1

Superscript lower case letters denote significant differences at different sampling time within the group, according to the Tukey's Studentized Range (HSD) Test (α = 0.05).

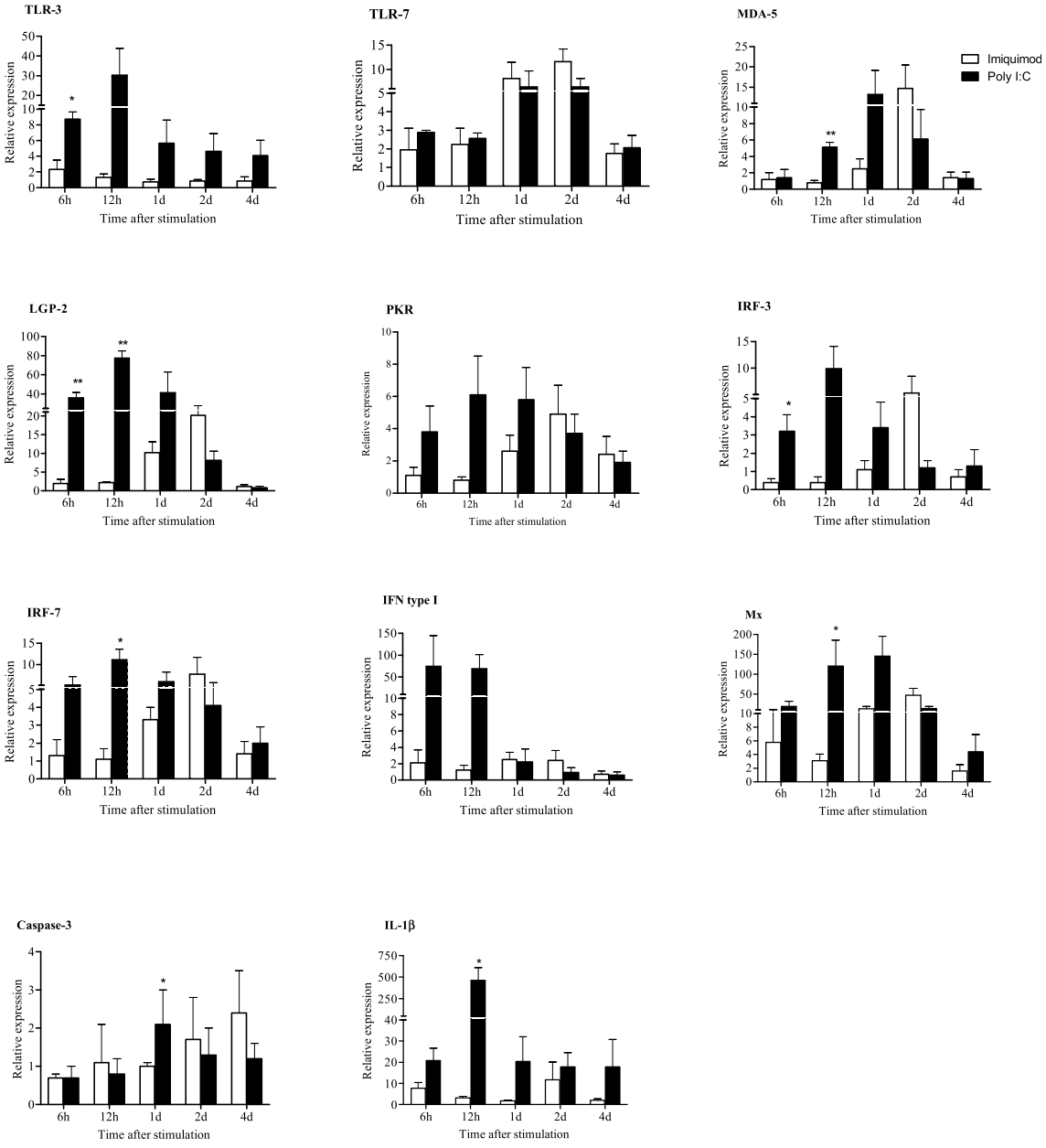

Fig. 2. Relative expression of immune gene transcripts in anterior kidney of olive flounder (n = 3) treated with 100 μg imiquimod or 100 μg poly (I:C). Relative expression of immune genes was normalised with β-actin. The mean expression values are plotted with standard error bar. An unpaired *t*-test was performed to determine the statistical significance of the differences in immune gene expression between the two groups at each time point ($p < 0.05$ indicated by *; $p < 0.01$ indicated by **).

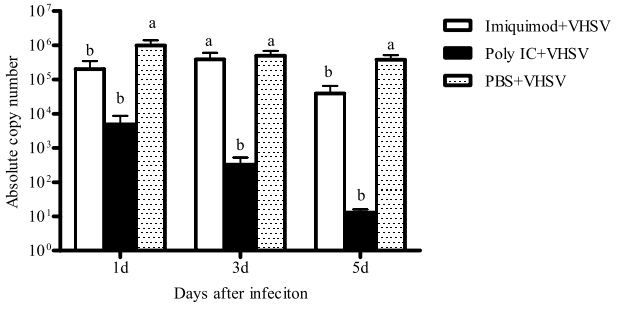

Fig. 3. VHSV copy number coding nucleoprotein of VHSV, in pre-treated olive flounder infected with VHSV. The mean viral copy number (n = 5) was plotted with standard error bar. Two-way ANOVA with Tukey's Studentized Range (HSD) Test (α = 0.05) was used to determine the significance ($p < 0.05$ indicated by lower case letters) of the differences in viral copy number.

4. Discussion

Innate immunity plays a crucial role in the fight against viral pathogens; therefore pre-stimulation of PRRs to activate the innate immune response has been used as a prophylactic/therapeutic method in mammals and is effective in fishes, including the olive flounder [13,14,18–20,32–36]. Poly (I:C) pre-stimulated olive flounder had a 100% survival rate at 15 °C, whereas fish without poly (I:C) failed to survive from the VHS [29]. We tested the ability of imiquimod as well as poly (I:C) to stimulate the immune response in olive flounder, though imiquimod was previously thought to induce milder or delayed immune response. Imiquimod induced IFNs and ISGs in salmon [37] but the enhancement of immunity in olive flounder using imiquimod was not strong enough to provide protection from VHSV. In our challenge experiment, olive flounder treated with 50 or 100 μg imiquimod/fish experienced a delay in mortality, and their mortality was lower than the untreated group. Nevertheless, the poly (I:C) pre-stimulated group

Table 3
Pre-ligand treated/VHSV infected group. Relative expression of immune gene transcripts in the kidney of olive flounder (n = 5) at 0d (2 d after ligands treatment, just before virus infection), 1d, 3d and 5d after VHSV infection.

Gene	Time	IQ + VHSV	Poly IC + VHSV	PBS + VHSV
TLR-3	0d	3.8 ± 1.4	3.0 ± 0.9	1.3 ± 0.4
	1d	8.2 ± 2.2	0.8 ± 0.3*	24.1 ± 7.2
	3d	16.6 ± 9.2	1.3 ± 0.4**	18.4 ± 10.0
	5d	4.4 ± 1.7	2.1 ± 0.5	6.0 ± 3.0
TLR-7	0d	2.5 ± 0.4**	5.1 ± 0.5^{a}**	1.0 ± 0.2
	1d	3.5 ± 1.2	5.4 ± 0.9^{a}*	3.0 ± 0.4
	3d	3.0 ± 1.0	1.5 ± 0.3^{b}	2.1 ± 1.3
	5d	2.1 ± 0.6	1.8 ± 0.1^{b}*	0.8 ± 0.3
MDA-5	0d	3.2 ± 0.8	3.0 ± 0.8^{b}	1.2 ± 0.5^{b}
	1d	10.9 ± 4.9	5.9 ± 1.1^{a}	4.9 ± 1.3^{a}
	3d	4.5 ± 4.0	1.5 ± 0.3^{b}	3.0 ± 0.8^{ab}
	5d	2.7 ± 0.3	1.4 ± 0.2^{b}**	3.2 ± 0.5^{b}
PKR	0d	3.0 ± 0.6^{b}*	3.8 ± 0.8^{ab}**	1.1 ± 0.2^{b}
	1d	9.1 ± 1.8^{a}	6.8 ± 1.4^{a}	6.1 ± 1.6^{a}
	3d	5.1 ± 1.5^{ab}	0.9 ± 0.1^{b}**	4.7 ± 1.1^{ab}
	5d	1.4 ± 0.2^{b}	1.0 ± 0.1^{b}	1.1 ± 0.4^{b}
IRF-3	0d	2.6 ± 0.7^{b}	6.7 ± 1.6^{b}**	1.1 ± 0.3^{c}
	1d	36.7 ± 14.1^{a}	17.5 ± 3.7^{a}	19.7 ± 5.2^{a}
	3d	12.2 ± 2.4^{ab}	2.4 ± 0.7^{b}**	13.9 ± 1.2^{ab}
	5d	7.7 ± 1.5^{ab}	3.9 ± 0.8^{b}*	6.6 ± 0.6^{bc}
IRF-7	0d	1.6 ± 0.4^{b}	1.1 ± 0.1^{b}	1.1 ± 0.3^{b}
	1d	8.6 ± 3.1^{a}	3.2 ± 1.1^{ab}	11.6 ± 4.6^{a}
	3d	1.1 ± 0.2^{b}	3.9 ± 0.6^{a}**	1.0 ± 0.2^{b}
	5d	3.2 ± 0.5^{ab}	2.9 ± 0.3^{ab}	2.5 ± 0.5^{ab}
IFN-I	0d	4.4 ± 3.5	1.0 ± 0.2	1.2 ± 0.4
	1d	12067.4 ± 9152.2	311.8 ± 307.3	3982.3 ± 2162.3
	3d	1.0 ± 0.3	0.5 ± 0.1	0.7 ± 0.1
	5d	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
Mx	0d	4.5 ± 2.1^{b}	69.9 ± 12.5^{b}**	1.1 ± 0.2^{b}
	1d	1015.9 ± 327.2^{a}	497.7 ± 110.1^{a}	528.4 ± 198.1^{a}
	3d	399.6 ± 115.7^{ab}	30.5 ± 14.1^{b}**	343.1 ± 46.4^{ab}
	5d	66.6 ± 10.9^{b}	16.2 ± 5.3^{b}**	91.0 ± 15.8^{b}
IL-1β	0d	2.6 ± 0.7^{b}	2.2 ± 0.4^{ab}*	1.1 ± 0.2
	1d	22.3 ± 8.5^{a}	2.1 ± 0.2^{ab}*	22.5 ± 10.5
	3d	3.5 ± 0.4^{b}	0.8 ± 0.2^{b}**	4.4 ± 0.9
	5d	4.2 ± 1.6^{b}	3.9 ± 1.2^{a}	2.0 ± 0.5

Superscript lower case letters denote significant at different sampling time within the group according to the Tukey's Studentized Range (HSD) Test (α = 0.05). An unpaired *t*-test was used to ascertain the significance of the differences in gene expression. Asterisk indicates significant difference in expression of PBS + VHSV group with imiquimod treated and poly (I:C) treated group; * and **indicate confidence limit of 95% and 99% respectively.

showed no signs of VHS. This drastic difference could be due to the stimulation efficiency of the two ligands. It has been observed that poly (I:C) is a potent stimulator of TLR-3, protein kinase receptors and RLRs to initiate robust type I IFN production [38–40]. Our observation of the immune response stimulation by these two ligands demonstrated that poly (I:C) has a greater and quicker immune modulation, which provides a window of opportunity for the host to fight VHSV, while the slower response induced by imiquimod could affect the fate of the host.

The head kidney is the primary haematopoietic organ in fish and was selected as a target organ. Poly (I:C), imitating dsRNA, significantly elevated TLR-3 expression, but imiquimod, which imitates ssRNA, failed to do so. However, both imiquimod and poly (I:C) triggered equal levels of TLR-7 expression, which may be attributed to the type I IFN mediated enhancement of TLRs that has been observed in poly (I:C) treated human leukocytes [41,42]. Poly (I:C) stimulated TLR-3, MDA-5 and LGP-2 in a short time span at a higher level than imiquimod, indicating that it is a potential PRR ligand. Poly (I:C) also induced quicker and stronger IRF-3 and IRF-7 expression, causing robust type I IFN signalling. Comparatively, the imiquimod group had a delay in the enhancement of IRFs and very low type I IFN. Even though imiquimod and other imidazoquinolines can induce type I IFN in Atlantic salmon and rainbow trout [24,37], it induces a lower expression level in the olive flounder. Poly (I:C) enhanced type I IFN, which elevated the expression of Mx at 12–24 hps, while imiquimod was unable to elevate Mx expression until 2 dps. IFN independent activation of ISGs may explain the higher Mx levels found in the imiquimod treated group [43].

We extended the experiment to determine why poly (I:C) treated animals do not suffer from VHSV but imiquimod treated fish are highly susceptible by studying the level of immune response in each group. As expected, poly (I:C), but not imiquimod, protected the host. The response of PRRs to different PAMPS varies depending on the type of PAMP or ligand [44]. This study shows that poly (I:C) has a stronger immunomodulatory effect in olive flounder than imiquimod and efficiently controls the transcription of VHSV. Tafalla et al. [45] observed a reduction and delay in transcription as well as translation of VHSV protein in poly (I:C) treated RTS-11 cells. The imiquimod treated group and untreated group had similarly high expression of Mx throughout the study, and both groups had high mortality. Though olive flounder kept at 15 °C had significantly higher levels of ISG-15 and Mx expression, the mortality and viral copy number remained high. On the other hand, flounder at 20 °C had a quick immune response and contained VHSV transcription at a very early stage. Therefore, Mx expression is probably essential early after infection period rather than later [27]. In our experiment, poly (I:C) induced a robust and efficient innate immune response through TLRs and RLRs at a very early stage of treatment and efficiently controlled VHSV before reaching the breaking point of the host immune system. Therefore, Mx expression was low at later stages when viral replication was already contained. Hansen et al. [46] and Purcell et al. [47] observed a high correlation between expression of Mx and viral load in the host, suggesting that the Mx expression level corresponds to the host viral load and does not necessarily protect the host. Therefore, response time is more important for successful viral replication containment than the quantum of response. A similar approach may hold true in the case of high Mx and ISG-15 expression in olive flounder infected with VHSV at 15 °C while they remain highly susceptible to VHSV, in contrast to fish kept at 20 °C, which have a quicker immune response and can efficiently control VHSV amplification. It is also evident that the quicker immune response induced by poly (I:C) treatment resulted in efficient control of VHSV transcription and better host survival. Olive flounder infected with VHSV at 20 °C had a quick and robust immune response that contained viral replication and prevented mortality, unlike at 15 °C, where a significant number of the host fish died when infected with VHSV [27]. Kim et al. [48] observed that poly (I:C) treated olive flounder had significantly lower mortality and could efficiently control viral multiplication. This is also true in the case of tongue sole (*Solea senegalensis*), where poly (I:C) treated fish had a faster immune response and higher immune gene expression and could contain viral transcription at a very early stage [49].

In this study, we compared the immune modulatory ability of poly (I:C) and imiquimod in olive flounder and their efficacy in fighting VHSV infection. Poly (I:C) was observed to be a quicker and stronger inducer of host immunity than imiquimod. In both experiments, the imiquimod response lagged behind the poly (I:C) response. Despite the elevated immune response induced by imiquimod, VHSV growth was uncontrolled and the host succumbed to the disease. Increased levels of interferon related immune genes in response to imiquimod or to VHSV was not enough to impart a higher survival rate. The viral copy number and immune gene kinetics demonstrate that a quicker immune response is more important for host survival after VHSV infection than a higher level of immune response.

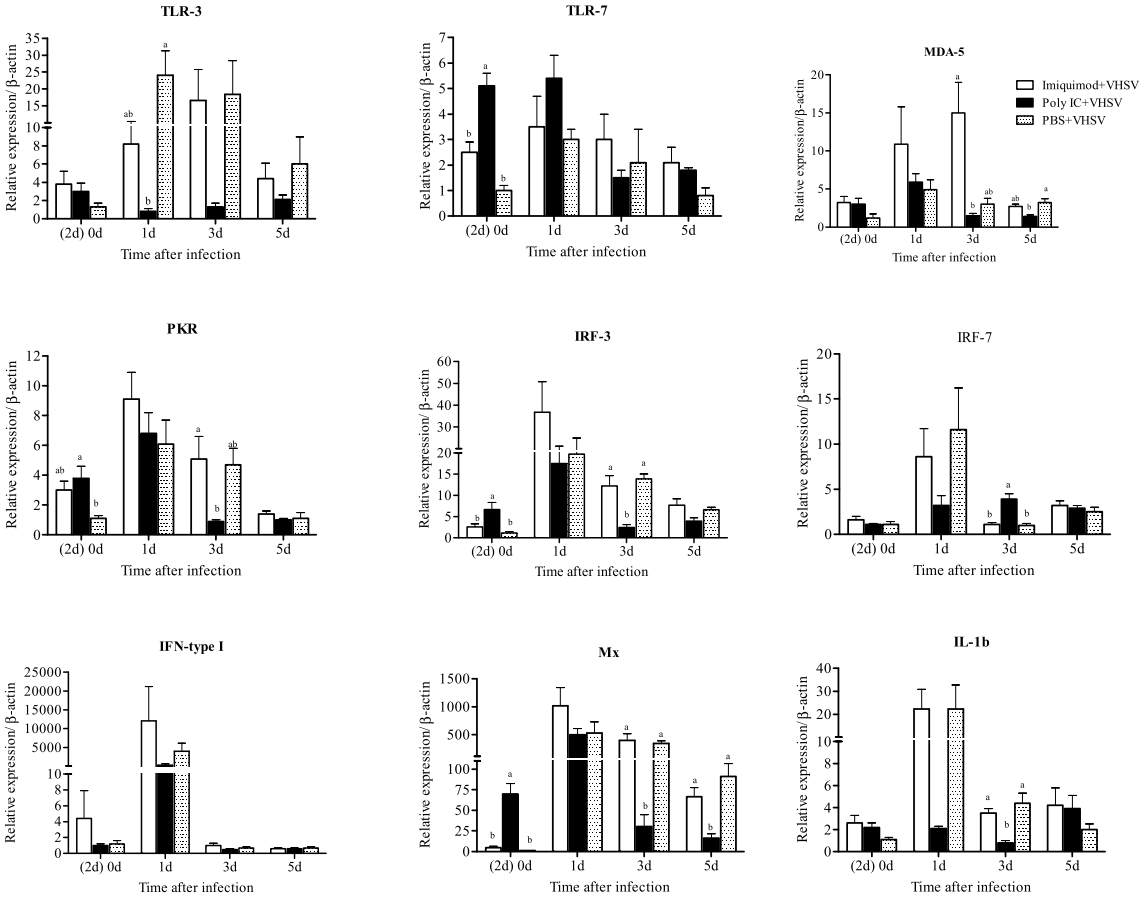

Fig. 4. Relative expression of immune gene transcripts in olive flounder infected with VHSV; that were pre-treated with poly (I:C), imiquimod or PBS. The experimental groups were infected with VHSV two days post ligand treatment and considered as 0d after infection. Two-way ANOVA with Tukey's Studentized Range (HSD) Test ($\alpha = 0.05$) was used to determine the significance ($p < 0.05$ indicated lower case alphabets) of the differences in gene expression.

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