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Inactivated ostreid herpesvirus-1 induces an innate immune response in the Pacific oyster, *Crassostrea gigas*, hemocytes

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Infectious diseases are a major constraint to the expansion of shellfish production worldwide. Pacific oyster mortality syndrome (POMS), a polymicrobial disease triggered by the Ostreid herpesvirus-1 (OsHV-1), has devastated the global Pacific oyster (*Crassostrea gigas*) aquaculture industry. Recent ground-breaking research revealed that *C. gigas* possess an immune memory, capable of adaption, which improves the immune response upon a second exposure to a pathogen. This paradigm shift opens the door for developing 'vaccines' to improve shellfish survival during disease outbreaks. In the present study, we developed an *in-vitro* assay using hemocytes – the main effectors of the *C. gigas* immune system – collected from juvenile oysters susceptible to OsHV-1. The potency of multiple antigen preparations (e.g., chemically and physically inactivated OsHV-1, viral DNA, and protein extracts) to stimulate an immune response in hemocytes was evaluated using flow cytometry and droplet digital PCR to measure immune-related subcellular functions and gene expression, respectively. The immune response to the different antigens was benchmarked against that of hemocytes treated with Poly (I:C). We identified 10 antigen preparations capable of inducing immune stimulation in hemocytes (ROS production and positively expressed immune-related genes) after 1 h of exposure, without causing cytotoxicity. These findings are significant, as they evidence the potential for priming the innate immunity of oysters using viral antigens, which may enable cost-effective therapeutic treatment to mitigate OsHV-1/POMS. Further testing of these antigen preparations using an *in-vivo* infection model is essential to validate promising candidate pseudo-vaccines.

KEYWORDS

immune priming, Ostreid herpesvirus-1, pseudo-vaccination, innate immune memory, flow cytometry, reactive oxygen species, droplet digital PCR

1 Introduction

The occurrence of mass mortality events and the emergence of infectious diseases affecting marine organisms have increased dramatically in recent years, exacerbated by a changing environment (1–3). These outbreaks can have disastrous consequences on biodiversity and cause rapid population declines, particularly in cultured livestock (4, 5). Diseases are the major limiting factor for the expansion of the aquaculture industry, with losses attributed to infectious microbial diseases alone exceeding US $ 6 billion per annum (6). One striking example is Pacific oyster mortality syndrome (POMS), which is associated with the detection of the Ostreid Herpes virus-1 (OsHV-1) and its variants. Over the last 15 years, OsHV-1 has decimated Pacific oysters, *Crassostrea gigas*, worldwide (7; see for review 8–10). The virus induces an immune-compromised state in infected oysters, which evolves toward subsequent bacteremia by opportunistic bacterial pathogens, leading to mortality rates of up to 100% in juveniles (11). Recorded for the first time in France in 2008, OsHV-1 µvar rapidly spread along the European coastline (9, 10, 12), and closely related variants of the virus were further detected during mortality events in Australia (13), New Zealand (14), Korea (15), and more recently in California (16). The inability to contain the rapid spread of the virus combined with an absence of therapeutic treatments resulted in huge losses of aquaculture stocks. Selective breeding to improve resistance to POMS (or OsHV-1) has shown potential as a prevention strategy, with moderate to high heritability for survival achieved during OsHV-1 infection (17–20). Implementation of a breeding program and access to selectively bred stocks can, however, be economically challenging for many end-users, prompting the need for new, accessible, and complementary mitigation strategies to reduce the impact of diseases.

For example, vaccination and immune priming have proven to be an effective preventative measure for many major diseases affecting livestock, including fish, and more recently invertebrates, such as crustaceans (21–25).

Invertebrates lack a conventional adaptive immune system (i.e., lymphocytes or antibodies) and instead rely on innate immunity to prevent the infection of invading pathogens (26, 27). Numerous studies have reported that invertebrates also possess diverse forms of immune 'memory' in which a potentiated immune response (resulting in a reduction of host susceptibility to the infection) has been recorded following a secondary exposure to a pathogen (28–31). For instance, in the scallop *Chlamys farreri*, a first short exposure to the pathogen *Vibrio anguillarum* increased phagocytosis, acid phosphatase activity, and survival following a second encounter to the pathogen (32). Pacific oysters stimulated primarily by heat-killed *Vibrio splendidus* also displayed stronger immune responses at cellular and molecular levels when they were subjected to a secondary challenge with the live bacteria (33). (34, 35) showed that injection of Polyinosinic: polycytidylic acid or Poly (I:C), a synthetic analog of double stranded RNA with immunostimulant properties, induced a long-lasting antiviral response in Pacific oysters, protecting them against subsequent OsHV-1 infection in natura. More recently, Fallet et al. (36) showed that early life exposure of *C. gigas* to 'microorganisms' provided inter-generational protection against recurring OsHV-1 infections, indicating a potential trained immunity *via* epigenetic modifications. In bivalves, hemocytes play a central role in immunity, identifying and destroying pathogens through phagocytosis, oxidative stress, apoptosis, and autophagy – functions that can be characterized using flow cytometry and molecular analyses (37–43). Transcriptome analyses in oysters primed with Poly (I:C) have identified several pattern recognition receptors (PRRs) involved in antiviral signaling. These include, for example, retinoic acid-inducible-gene-I and Toll-like-receptors homologs of the Jak-Stat pathway, stimulator of interferon genes, interferon regulatory factors, and many IFN-stimulated genes (i.e., Viperin or ADAR), which are all implicated in the detection of virus and antiviral functions (44–48).

We aim to evaluate the potency of inactivated OsHV-1 preparations (antigens) to elicit an antiviral response in *C. gigas* hemocytes. This research is undertaken in the context of developing new strategies of immune priming to improve oyster resilience to POMS.

2 Material and methods

2.1 Preparation of OsHV-1 antigens

In June 2021, the experiment was conducted to (1) screen multiple preparations of inactivated OsHV-1 (hereafter referred to as antigens) by measuring immune-related functions of hemocytes using flow cytometry (FCM, repeated three times), and (2) confirm the potency of a selected subset of antigen preparations to stimulate immunity using FCM (cytotoxicity and ROS) and molecular analyses (immune- related gene expression, repeated three times, Figure 1).

2.1.1 OsHV-1 stock

The OsHV-1 suspension stock was produced in October 2019, as described in Camara et al. (18), from diseased oysters infected with OsHV-1 during a lab challenge (49). Briefly, tissue from high virus load oysters was homogenized, purified by serial filtrations down to 0.22 µm and cryopreserved according to Kirkland et al. (50). On the 3 June 2021, cryopreserved OsHV-1 stock was defrosted by dipping in a 22°C water bath for 10 min. Viral suspension was titrated using qPCR (51) and diluted in 0.22 µm-filtered sterile seawater (SSW) to reach a final concentration of $9.0.10^5$ copies. μl^{-1}. Prepared virus was then inactivated using the methods described below.

2.1.2 BEI inactivation

A 0.2M Binary ethylenimine (BEI) solution was prepared by cyclization of 0.2M 2-bromoethylamin-HBr in 0.2M NaOH at 37°C for 1 h (52). The BEI solution was added to OsHV-1 suspension to either a concentration of 0.1% (v/v) and incubated at 22°C for 1 h, 4 h, or 6 h, or to a concentration of 0.04% (v/v), and incubated at

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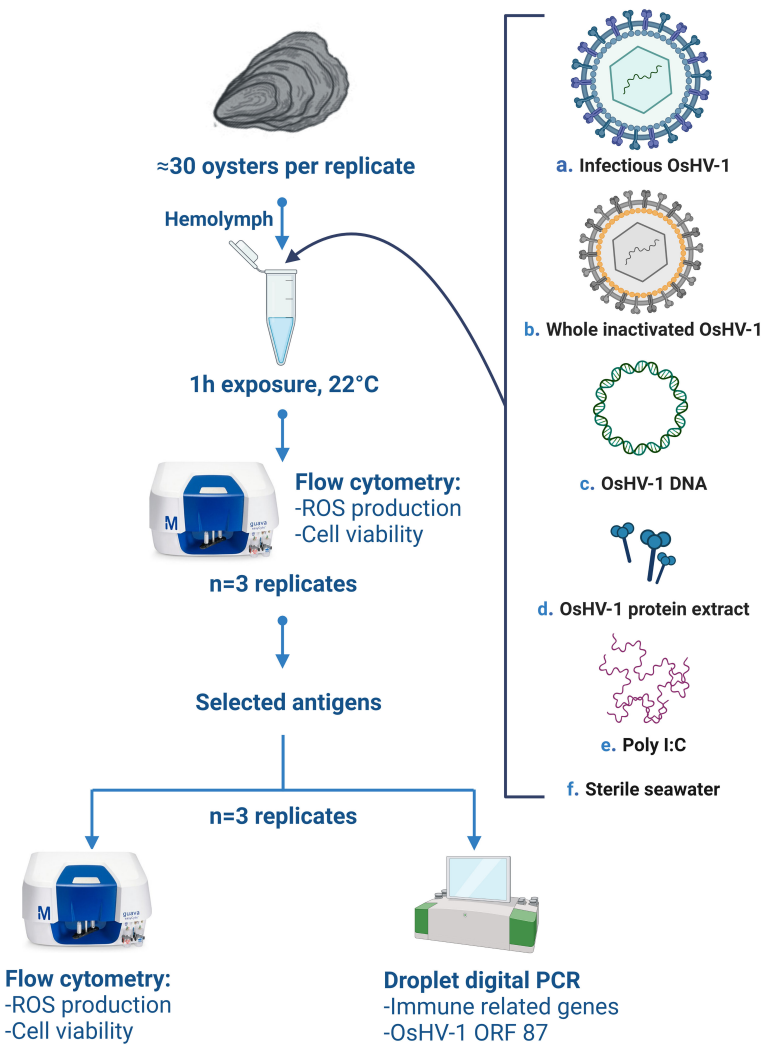

FIGURE 1
Schematic overview of the *in-vitro* experimental design, **(A)** refers to the live infectious OsHV-1, **(B)** whole virus killed by chemical (BEI, formaldehyde), heat inactivated, or exposed to freeze-thaw cycles, **(C)** OsHV-1 DNA, **(D)** OsHV-1 whole protein extract, **(E)** Poly (I:C, positive control), **(F)** sterile filtered seawater (negative control). ROS stands for 'reactive oxygen species', ORF to 'open reading frame'. Figure was created using BioRender.com.

22°C for 4 h, 6 h, 9 h, 18 h, or 22 h. Inactivation reactions were stopped by addition of sodium thiosulphate 1M (neutralizing agent) to reach a final concentration of 10% (v/v). Obtained inactivated viral suspensions were stored at 4°C until use. Suspension of neutralized 0.1% (v/v) BEI diluted in filtered (0.22 µm) SSW was used as the vehicle control (VC), (Supplementary Table 1).

2.1.3 Formaldehyde inactivation

Formaldehyde solution 37% (w/v) was added to the OsHV-1 suspension to a final concentration of 5%, 0.3%, or 0.01%, and incubated at 22°C for 2 h, 4 h, or12 h (5%); for 4 h, 8 h, 12 h, 24 h, 48 h, or 60 h (0.3%); or for 12 h, 24 h, or 60 h (0.01%), respectively. Virus inactivation was stopped by addition of 35% sodium bisulphite to reach a final concentration of 0.035%, and suspensions were then stored at 4°C. Suspension of 5% (v/v) neutralized formalin diluted in filtered (0.22 µm) SSW was used as the VC (Supplementary Table 1).

2.1.4 Heat inactivation

For preparation of heat-inactivated OsHV-1, viral suspensions were incubated for 1 h at 45°C, 50°C, or 52°C, or for 30 min at 54°C, 56°C, and 60°C using a dry bath, and then stored at 4°C until use (Supplementary Table 1).

2.1.5 Freeze-thaw cycles

Viral suspensions were placed at -80°C for 12 h, transferred to -20°C for 12 h, and then maintained at 4°C. After complete thawing, viral suspensions were immediately re-frozen at -80°C for 12 h. The freeze-thaw cycles were repeated twice (for Thawing 1) or three times (for Thawing 2), and the antigen suspensions were stored at 4° C until use (Supplementary Table 1).

2.1.6 OsHV-1 DNA

Total viral DNA was extracted from 1 ml of the OsHV-1 stock suspension using blood and tissues kit (QIAGEN)

according to the manufacturer's protocol. Extracted DNA was resuspended in 10 mM Tris-HCl buffer (pH 7.4) to reach a final concentration of 10 ng DNA μl^{-1} and stored at -20°C until use. The solution 10 mM Tris-HCl buffer (pH 7.4) was used as a VC (Supplementary Table 1).

2.1.7 Viral proteins

Total viral proteins were extracted from 5 ml of the OsHV-1 stock suspension *via* bead beating for 10 min at 1500 rpm and 4°C using a 1600 MiniG automated tissue homogenizer (SPEX Sample Prep, Metuchen, NJ). Proteins were then solubilized for 45 min by adding 2 ml of extraction buffer (100 mM potassium phosphate, 50 mM NaCl, 0.1 mM EDTA-Na2, 1% polyvinyl pyrrolidone, 2 mM phenylmethylsulfonyl fluoride and 0.1% TritonX-100; pH 7.5 at 4° C). Solubilized proteins were extracted by centrifugation at 13,500 rpm for 15 min at 4°C and ultra-filtered (10 kDa molecular weight cut-off, Amicon Ultra-0.5 10K, Merk-Millipore, Burlington, USA) following manufacturer's specifications. The semi-purified proteins were reconstituted in 200 µl of sterile Phosphate Buffer Saline (PBS). Total protein content of the lysate was quantified by the Lowry protein assay (53), diluted in sterile PBS to reach a final concentration of 0.2 $mg.ml^{-1}$, and stored at -80°C until use. Protein extracts were either pure (Protein 1) or diluted at 1:10 (v/v) in PBS (Protein 2). Sterile PBS was used as a vehicle control (Supplementary Table 1).

2.2 Oysters

The experiment was performed using hemolymph of hatchery-bred juvenile *C. gigas* [8 months old, mean live weight 6.7 ± 3.1 g]. These oysters were the offspring of naïve wild stocks and therefore were expected to be highly susceptible to POMS. Prior to sampling, oysters were maintained in flow-through seawater (10 µm filtered) at ambient conditions (10 – 22°C and a salinity of 35 ± 1) and fed ad libitum with hatchery-grown algal food. Experimental oysters were considered naïve to POMS/OsHV-1 due to their rearing with continuous supply of UV-sterilized seawater (80 mJ cm^{-2}) and maintenance under strict biosecurity management to ensure they remained OsHV-1-free. This status was confirmed prior to the experiment by the absence of significant mortality and OsHV-1 DNA detection in tissue (n = 10) using qPCR (51). Oysters were starved for 24 h prior to hemolymph collection to minimize algal contamination of the hemolymph.

2.3 Hemolymph collection

Hemolymph was sampled from between 22 and 35 oysters collected and pooled daily for experimental exposure. A small notch was made in the shell using wire cutters, and oysters were bled from the adductor muscle sinus using a 25G 1.5-inch needle with 1 mL syringe, previously kept on ice. Between 150 and 1500 µl of hemolymph was withdrawn from each individual and immediately added to a 1.5 mL Eppendorf, previously kept on ice. Individual samples were checked under a light microscope (40X magnification) to confirm purity. When ≈16 mL of pure, clean hemolymph had been collected from multiple individuals, hemolymph samples were pooled, diluted (1:4 v/v) with autoclaved 0.2 µm-filtered sterile seawater (FSSW) for flow cytometry or kept undiluted for molecular analyses, and stored on ice until exposure.

2.4 *In-vitro* exposure

Before experimental exposure, each antigen preparation and vehicle control were diluted (1:100 v/v) with FSSW. Polyinosinic-polycytidylic acid [Poly (I:C)], a synthetic analogue of double-stranded RNA (dsRNA), was used as positive control (0.05 mg. ml^{-1} in SSW; 54) and FSSW was used as a negative control. For flow cytometry, antigens preparations or controls were added (1:80 v/v) to (1:4 v/v) diluted hemolymph (detailed above) and incubated at room temperature (22°C) for 1 h. The expression of five immune-related genes and the viral gene ORF 87 was evaluated by adding 20 µl of the diluted antigen preparations (1:100 (v/v) or controls to 400 µl of pure hemolymph in 1.5 ml Eppendorf tubes and incubated at 22°C for 1 h.

Following experimental exposure, hemocyte reactive oxygen species (ROS) production and viability were determined (detailed below). This process was repeated daily for 3 consecutive days to obtain n = 3 independent replicates.

Based on ROS production in hemocytes and an absence of cytotoxicity, a subset of promising antigens was selected for further FCM validation and molecular analyses in a second experiment. Specific antigen preparations which induced significantly less ROS production were also included in the second experiment to maintain a range of contrasted immune responses and improve validation. This second *in vitro* challenge was repeated daily for 3 consecutive days to obtain n = 3 independent replicates.

2.5 FCM assessments

Hemolymph samples that had been exposed to different antigen preparations for 1 h were analyzed using a Guava® EasyCyte™ 5HT flow cytometer equipped with a blue laser (488 nm) and green (525/30 nm), yellow (583/26 nm), and red (695/50) detectors (EMD Millipore, USA). Samples were mixed at medium speed and acquired at a flow rate of 0.24 µL s^{-1} for 30s.

The production of intracellular ROS was measured using 2',7'-Dichlorodihydro-fluorescein diacetate (DCFH-DA, Sigma Aldrich, D6883) according to Donaghy et al. (55). Following 30 min of experimental treatments incubation, DCFH-DA was added at a final concentration of 10 µMand incubated in the dark at room temperature (22°C) for another 30 min until analysis (= total 1 h exposure to treatments). Relative ROS production was expressed as the level of green (FL1) fluorescence.

The viability of hemocytes was measured using Fluorescein diacetate (FDA, Invitrogen, F1303) according to Rolton et al. (56). Following 50 min of experimental treatments incubation, FDA was added at a final concentration of 1.25 mg L^{-1} and incubated in the dark at room temperature for 10 min until analysis (= total 1 h exposure to treatments). Hemocytes were divided into those with high FL1 (corresponding to metabolically active/viable cells) and those with low green fluorescence (non-viable).

2.6 Molecular analyses

RNA was extracted from 400 µl of hemolymph previously exposed to 20 µl of antigen preparation, SSW (negative control), Poly I:C (positive control), or live infectious OsHV-1 using the Quick RNA/DNA Miniprep plus kit (Zymo Research) according to the manufacturer's protocol. RNA was eluted in 50 µl DNAse/RNase-free water. As described in Delisle et al. (49), samples were treated with DNAse I (TURBOTM DNase, Invitrogen), the absence of DNA in the samples was confirmed by a 16S PCR assay, purity of the isolated RNA was assessed, and DNAse-treated RNA was transcribed into cDNA. Finally, droplet digital PCR (ddPCR) was conducted in an automated droplet generator (QX200 Droplet Digital PCR SystemTM, Bio-Rad) to determine the expression of five genes (Jak, Stat6, Viperin, IRF2, Myd88) related to oyster innate immunity (44, 57, 58), as well as the ORF 87, an OsHV-1 gene selected from the 39 ORFs described by Segarra et al. (59). Each ddPCR reaction included 1 µl of 3 µM of the primers (Jak, Stat, Viperin, Myd88) or 10 µM (IRF2, ORF87), 10 µl ddPCR Supermix for Evagreen (Bio-Rad), 2 µl cDNA, and 8 µl sterile water for a total reaction volume of 21 µl. As described in Delisle et al. (49), ddPCR was performed using the following cycling protocol: hold at 95°C for 5 s, 45 cycles of 95°C for 30 s, 60°C for 1 min, 4°C for 5 min, and a final enzyme deactivation step at 90°C for 5 min. The plate was then analyzed on the QX200 instrument (Bio-Rad). For each ddPCR plate run, at least one negative control (RNA/DNA-free water; Life Technologies), and one positive control (*C. gigas* DNA or Gblock for ORF87 diluted 1/10,000) were included.

2.7 Statistical analyses

Statistical analyses were computed using R 4.2.1 (https://www.r-project.org/) and the packages 'ggpubr' (60) and 'rstatix' (61). One way ANOVA and t tests were performed to evaluate the effects of each antigen preparation on ROS production, hemocytes viability, and gene expression, in comparison to the effects of SSW exposure (negative control); p-values were adjusted with Holm correction. For gene expression, a heatmap was constructed using Multiple Experiment Viewer software (62; http://mev.tm4.org/#/datasets/upload). For all analyses, the threshold significance level was set at 0.05.

2.8 Ethics approval

The study was conducted according to the guidelines of the Declaration of Helsinki and approval of the Animal Ethics Committee was not applicable for the use of oysters.

3 Results

3.1 79% of the antigenic preparations did not affect the viability of oyster hemocytes

Of the 33 antigenic preparations tested, 26 had no cytotoxic effect on oyster hemocytes (as measured by viability). However, hemocytes exposed for 1 h to the following antigen preparations – OsHV-1 previously inactivated using BEI 0.04% for 4 h and 6 h (Figure 2A), formaldehyde 5% for 12 h, formaldehyde 0.3% for 8 h or 24 h, or formaldehyde 0.01% for 12 h (Figure 2C), and heated at 52°C for 1 h (Figure 2D) – showed a significant reduction in viability compared to hemocytes exposed to SSW. The viability of hemocytes that had been heat killed (negative control) was very low (10.2 ± 5.2% mean ± SD, n = 3) compared to hemocytes exposed to SSW (91.3 ± 8.0%, p = 1.7e-13) (Figure 2).

3.2 Antigenic preparations induced ROS production in hemocytes

Hemocytes of C. gigas that had been exposed to 10 antigenic preparations of inactivated OsHV-1 and to the positive control (Poly I:C) showed increased ROS production (Figure 3). Specifically, preparations of virus inactivated using BEI 0.04% at 9, 18, and 22 h (Figure 3A), formaldehyde 5% for 4 h, formaldehyde 0.01% for 24 h (Figure 3B), heat shock at 50°C for 1 h, at 60°C for 0.5 h, or three freeze-thaw cycles (Figure 3C), as well as viral protein extracts pure or diluted 1/10 (v/v, Figure 3D), all significantly increased hemocyte ROS production compared to those exposed to SSW ($p < 0.001$).

Based on an absence of cytotoxicity and high levels of ROS production (using FCM), 10 antigen preparations, as well as eight additional antigen preparations that induced a limited subcellular immune response (Figures 2, 3), were selected for validation (using FCM and molecular analysis).

3.3 All the selected antigen preparations induced upregulation of immune-related genes

All the selected antigen preparations induced the upregulation of at least one of the immune- related genes of *C. gigas*. Exposure of hemocytes to the Poly (I:C) at 0.05 mg. ml^{-1} for 1 h induced a significant upregulation of MyD88, Viperin, and Stat6 (Figure 4). Exposure of hemocytes to OsHV-1, which had been inactivated using BEI 0.04% for 22 h, resulted in a significant upregulation of all five of the immune- related genes analyzed. Vehicle controls did not induce the expression of the immune- related genes in hemocytes, except the phosphate buffered saline (VC protein, Supplementary Table 1), which induced a significant upregulation of Stat6, Viperin, IRF2, and MyD88. As expected, the expression of ORF87 was only detected in hemocytes exposed to live infectious OsHV-1 (Figure 4).

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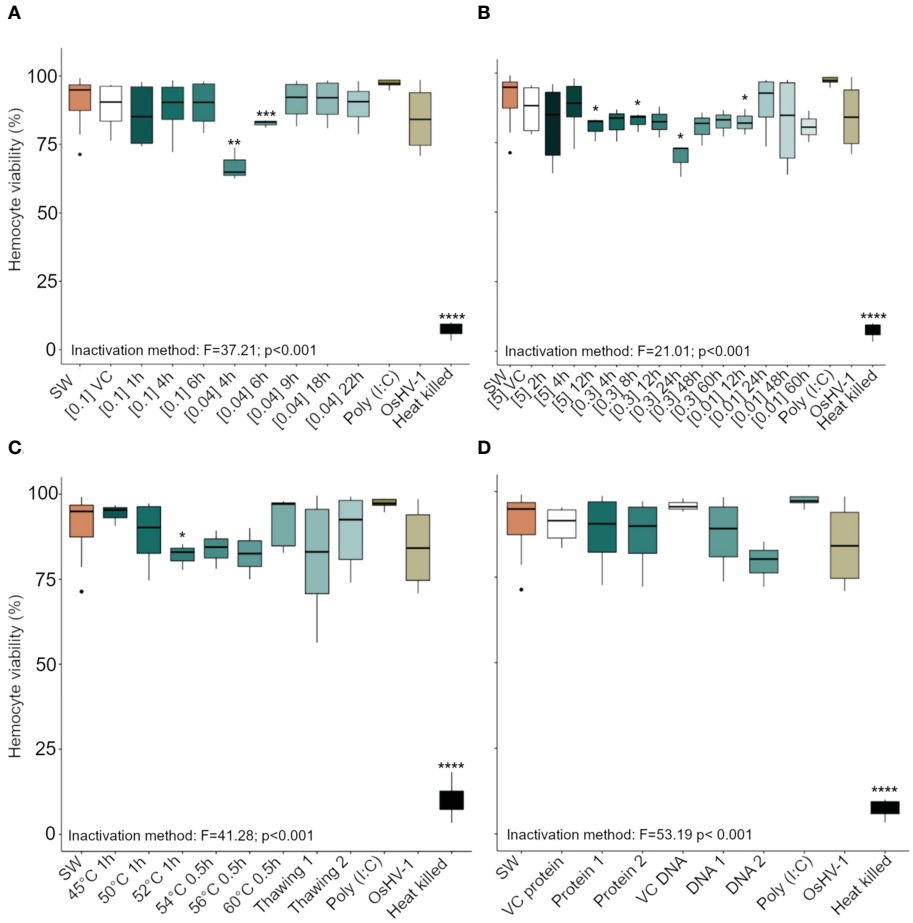

FIGURE 2
Viability (in percent) of *Crassostrea gigas* hemocytes exposed for 1 h to OsHV-1, which had been inactivated with different preparations of **(A)** Binary ethylenimine BEI, **(B)** formaldehyde, and **(C)** temperature treatments. **(D)** shows viability of hemocytes exposed to two viral protein concentrations and purified viral DNA (see Supplementary Table 1 for details of the different treatments). Box plots indicate the median, upper, and lower quartiles. Whiskers indicate the highest and the lowest percent viable hemocytes and dots indicate outliers. Significance levels are expressed by asterisks: * ($p < 0.05$), ** ($p < 0.01$), ***($p < 0.001$), **** ($p < 0.0001$). N = 3/6 replicates. 'SW' stands for seawater, 'VC' for vehicle control.

4 Discussion

In the present study, we were able to stimulate an antiviral response in juvenile *Crassostrea gigas* hemocytes using inactivated OsHV-1 and viral extracts, as shown by ROS induction and upregulation of antiviral response-related genes. A previous study, in which *C. gigas* hemocytes were exposed to live OsHV-1, also resulted in the expression of genes involved in immune-related functions (42); however, this study is the first time inactivated OsHV-1 preparations have been shown to elicit an immune response *in-vitro*.

Virus inactivation transforms antigens from being infectious to non-infectious, and it is important to determine any cytotoxicity of the antigen preparations prior to determining their potency (52). In the absence of bivalve cell line cultures in which to determine cytotoxicity, here, we used a FCM-based assessment of hemocyte viability for rapid ***in-vitro*** screening of the OsHV-1 inactivated preparations. Morga et al. (63), similarly used FCM to determine hemocyte viability following an ***in-vitro*** exposure of *C. gigas* hemocytes to the protozoan parasite *Bonamia ostreae*. Among the 33 antigen preparations tested in our study, only seven induced a significant decline in hemocyte viability. Incomplete neutralization of the denaturing agent could explain this observed cytotoxicity. The residual infectivity post-inactivation of the preparations tested was verified by the absence of the open reading frame 87 (ORF87) expression in the hemocytes following 1h of antigen exposure. This ORF codes for an apoptosis inhibitor protein and is expressed during the first few hours post OsHV-1 infection in oysters (42, 59).

A 1-hour exposure of hemocytes to 0.5 $mg.ml^{-1}$ of Poly (I:C) was sufficient to induce cellular ROS production – a proxy of immune response in bivalves (64) – and an upregulation of the genes coding key antiviral effectors: MyD88, Stat 6, and Viperin, validating our screening approach. Increased ROS indicates a stimulation of an immune response, as ROS production is associated with internal chemical destruction of engulfed pathogens or foreign particles within hemocytes (65, 66). The mechanisms of hemocyte activation in oyster immune defense, however, are still largely unknown. In response to foreign particles, oyster hemocytes can: secrete effectors extracellularly; phagocytose particles, where they are destroyed by ROS and

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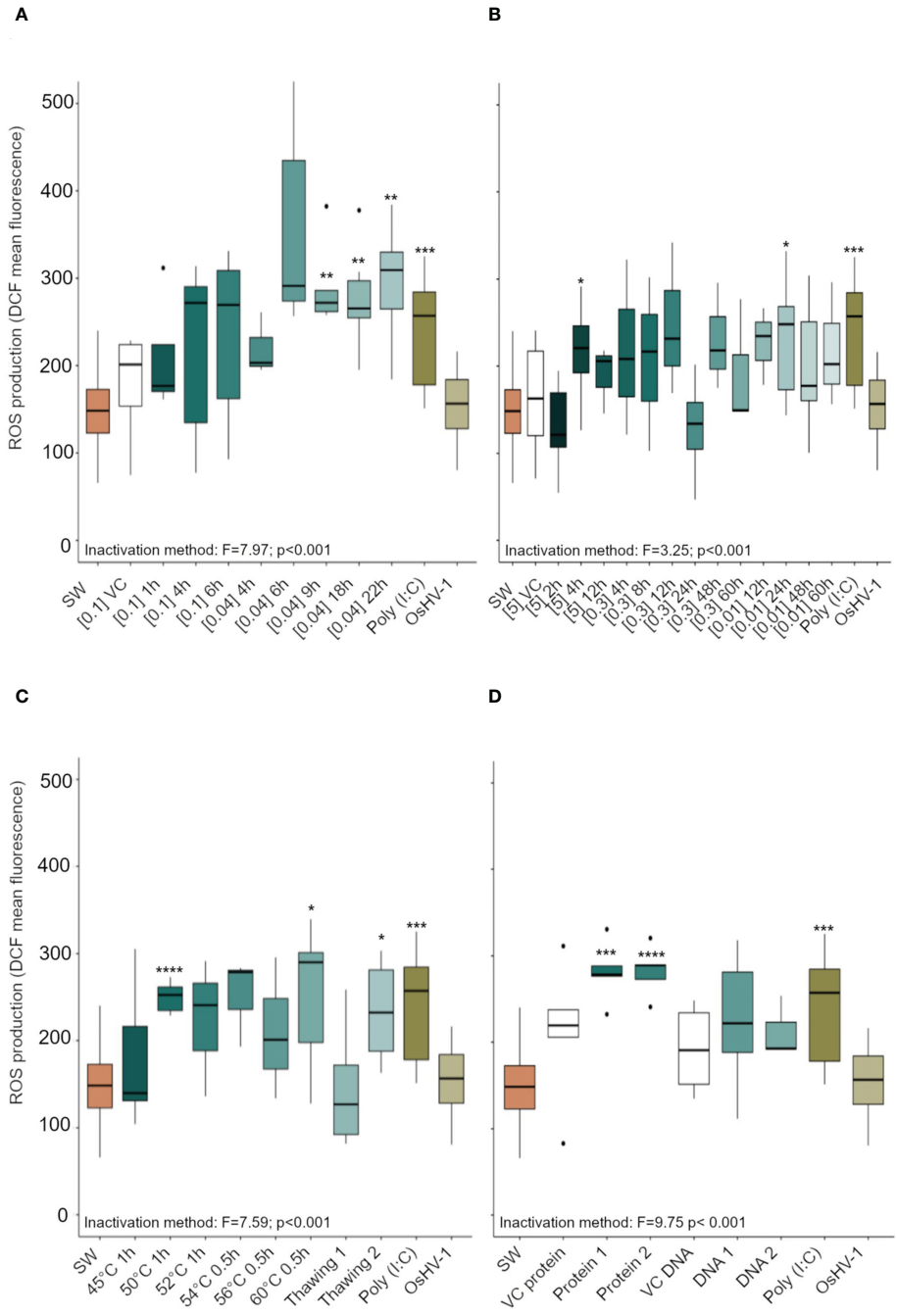

FIGURE 3
Reactive oxygen species (ROS) production (expressed in arbitrary unit, a.u) in *Crassostrea gigas* hemocytes exposed for 1 h to OsHV-1, which had been inactivated with different preparations of **(A)** Binary ethylenimine BEI, **(B)** formaldehyde, and **(C)** temperature treatments. **(D)** shows ROS production in hemocytes exposed to two viral protein concentrations and purified viral DNA (see Supplementary Table 1 for details of the different treatments). N = 3/6 replicates, box plots indicate the median, upper and lower quartiles; whiskers indicate the highest and the lowest values of the dataset and dots indicates outliers. Significance levels are expressed by asterisks: * ($p < 0.05$), ** ($p<0.01$), ***($p<0.001$), **** ($p<0.0001$). 'SW' stands for seawater, 'VC' for vehicle control.

defense molecules stored in granules; and following stress or recognition of foreign particles by soluble and cellular pattern recognition receptors, induce the expression of specific immune genes (67). An induction of MyD88 gene, an essential signal transducer in the interleukin-1 and Toll-like receptor signaling pathways, was also observed in mussel (*Mytilus galloprovincialis*) and scallop (*Pecten maximus*) hemocytes that had been stimulated for 8 h and 3 h, respectively, with 50 $\mu g.ml^{-1}$ of Poly (I:C), a much higher concentration than was used in the present study (68, 69). Upregulation of Viperin – an interferon-inducible antiviral protein – and Stat 6 – a signal transducer and activator of transcription – have also been observed in oyster hemocytes primed with Poly (I:C), in comparison to those exposed to seawater (46, 70).

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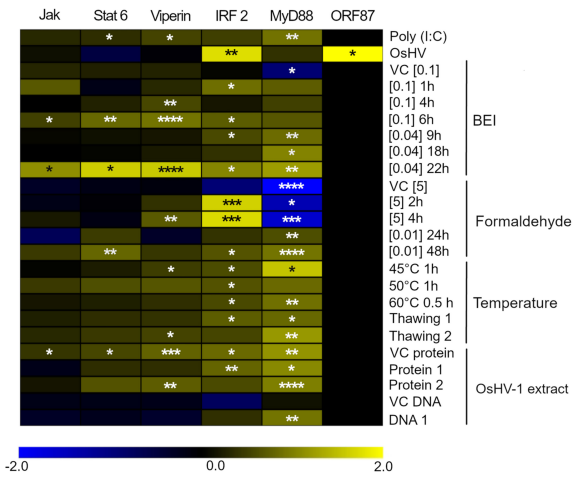

FIGURE 4
Heatmap focusing on the expression of 5 genes related to the innate immune response in *Crassostrea gigas* and ORF87, an OsHV-1 ORF expressed early after infection. The intensities of the colors indicate the magnitude of the differential expression (log2 fold-change), N = 3 replicates. The fold-changes were calculated by comparing the expression of each gene in hemocytes exposed to Poly (I:C), live infectious OsHV-1, or OsHV-1 inactivated, with its expression in negative control (hemocytes exposed to SSW). Significance levels are expressed by asterisks: * ($p < 0.05$), ** ($p < 0.01$), ***($p < 0.001$), **** ($p < 0.0001$).

The interaction between OsHV-1 and the oyster host cells has not been fully elucidated, and the functions of proteins coded by OsHV-1 genome are largely unknown. However, antibody blocking and pull-down assays suggest the potential implication of three putative OsHV-1 membrane proteins (ORF 25, ORF 41, and ORF 72) in the virus/host interaction by binding of host cytoskeleton (71, 72). Interestingly, antigen preparations resulting from BEI inactivation induced the strongest immune and antiviral response. Binary ethylenimine is an aziridine preparation commonly used in veterinary vaccine production as an inactivating agent (73). At 1 mM, BEI induces an alkylation of the nucleic acids without damaging proteins (74). In the present work, high ROS production coupled with upregulation of the five tested genes were recorded when using OsHV-1 inactivated with BEI at [0.04%] for 22h, supporting the role of viral proteins in the stimulation of antiviral response in oysters. Conversely, we observed a reduced potency of antigen preparations that were inactivated by formaldehyde, one of the most widely used inactivating agents, for an extended exposure time (12 and 48h), suggesting an irreversible denaturation of proteins induced by formaldehyde (75).

Three antigen preparations inactivated with BEI induced a strong upregulation of the transcript coding for Viperin. Viperin is a highly conserved evolutionary host protein (76), which restricts the replication of a range of RNA and DNA viruses [e.g., human cytomegalovirus (77), immunodeficiency virus (78), and Hepatitis C virus (79)], by interacting with viral protein and altering the site of virus budding. In *C. gigas*, Viperin has been reported to be one of the earliest and most regulated genes in response to OsHV-1 exposure (54, 80), and it also exhibits the same level of antiviral activity as human Viperin against Dengue virus ***in-vitro*** (46, 80). Consequently, its expression in hemocytes could constitute a good indicator of the potency of the antigen preparations.

Besides chemical agents, physical methods were used to inactivate OsHV-1 in this study. Thermal inactivation of OsHV-1 at 50°C and 60°C and long thawing cycles caused an increase in ROS production, while the associated molecular responses appeared moderate. These variations in the results could probably be explained by the broad range of stressors able to induce ROS production in bivalves (81), in comparison to the specificity of the antiviral response. Nonetheless, we obtained a good correlation overall between immune-related markers measured *via* flow cytometry (ROS production) and qPCR analyses (immune-gene expression), with the ranking of the 10 best antigen preparations maintained when upregulation of immunity genes was considered.

Of note, protein extracts obtained from OsHV-1 and resuspended in PBS also induced a marked positive expression of MyD88, IRF2, and Viperin; however, the confounding effect of the vehicle control PBS on the hemocytes cannot be excluded. Similar induction of immune response following PBS exposure has been reported (82, 83).

To conclude, these findings are significant as they evidence for the first time the potential for stimulating oyster's innate immunity using OsHV-1 antigens, which may enable cost-effective therapeutic treatment to mitigate the economic impacts of OsHV-1/POMS. For instance, chemical inactivation using the Binary ethylenimine at 0.04% for 22 h was identified as the best candidate preparation requiring additional research. However, it is essential to test these antigen preparations further using *in-vivo* infection models to validate promising candidate pseudo-vaccines. Nonetheless, we demonstrated that the use of flow cytometry-based cellular assays was an effective and rapid screening tool to select treatments for 'pseudo-vaccine' development.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding authors.

Author contributions

LD, AR, JV, conception and design. LD and AR lab trial and data analyses. All authors, writing, revision/editing. LD, JV funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1161145/full#supplementary-material

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