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RESEARCH ARTICLE

Differential response of RTGUTGC and RTGILL-W1 rainbow trout epithelial cell lines to viral stimulation

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Abstract

Mucosal surfaces constitute the main route of entry of pathogens into the host. In fish, these mucosal tissues include, among others, the gastrointestinal tract, the gills and the skin. However, knowledge about the mechanisms of regulation of immunity in these tissues is still scarce, being essential to generate a solid base that allows the development of prevention strategies against these infectious agents. In this work, we have used the RTgutGC and RTgill-W1 epithelial-like cell lines, derived from the gastrointestinal tract and the gill of rainbow trout (*Oncorhynchus mykiss*), respectively, to investigate the transcriptional response of mucosal epithelial cells to a viral mimic, the dsRNA poly I:C, as well as to two important viral rainbow trout pathogens, namely viral haemorrhagic septicaemia virus (VHSV) and infectious pancreatic necrosis virus (IPNV). Additionally, we have established how the exposure to poly I:C affected the susceptibility of RTgutGC and RTgill-W1 cells to both viruses. Our results reveal important differences in the way these two cell lines respond to viral stimuli, providing interesting information on these cell lines that have emerged in the past years as useful tools to study mucosal responses in fish.

KEYWORDS
infectious pancreatic necrosis virus (IPNV), poly I:C, rainbow trout, RTgill-W1, RTgutGC, viral haemorrhagic septicaemia virus (VHSV)

1 | INTRODUCTION

Mucosal surfaces constitute the main route of entry for viral agents, as they constitute the physical and chemical barriers that separate the host from the external environment. In the case of fish, mucosal tissues include, among others, the skin, the nose, the gills and the intestinal epithelium (Roberts, 2012). These barriers are formed by epithelial cells, mucus-producing cells, neuroendocrine cells and dispersed immune cells that include mainly B and T cells, macrophages, mast cells and dendritic cells.

Importantly, mucosal epithelial cells not only form a tight cellular barrier that controls the diffusion of different molecules across the paracellular compartments but also play a key role in maintaining homeostasis and regulating innate and adaptive immune responses, thereby influencing the response to pathogens and commensal colonization (Gomez et al., 2013). Hence, epithelial cells are able to secrete molecules such as cytokines, chemokines and growth factors, similarly to immune cells (Peterson & Artis, 2014). Additionally, epithelial cells interact directly with pathogens and commensals through pattern recognition receptors (PRRs). Pattern recognition

receptors include the family of Toll-like receptors (TLRs) that sample the extracellular and endosomal compartments, whereas cytoplasmic PRRs also include NOD (nucleotide-binding oligomerization domain)-like receptors (NLRs) that protect the cytoplasmic compartment (Rebl et al., 2010). In the case of viruses, PRR signalling results in the expression of type I interferon (IFN) that induces an antiviral state in affected and surrounding cells through the activation of IFN-induced genes that code for a wide range of proteins, many of which have direct antiviral effects (O'Farrell et al., 2002; Pestka et al., 2004). Activation of IFN is a conserved innate mechanism to combat viral infections across vertebrates. Considerable efforts to identify and characterize IFN genes in fish have been undertaken in the past years, and several excellent reviews describing the complexity of IFN signalling in fish have been published on this issue (Langevin et al., 2013, 2019; Poynter & DeWitte-Orr, 2016).

As mentioned above, mucosal surfaces are often initial replication sites for pathogenic agents. Therefore, how epithelial cells in these mucosal tissues respond to infectious agents is an important line of research. In this context, in this work, we have used established rainbow trout intestinal and gill epithelial cell lines (RTgutGC and RTgill-W1, respectively) (Bols, 1994; Kawano et al., 2011) as in vitro models to better understand how these mucosal epithelial cells directly respond to viral infections. As viral models, we have used two important rainbow trout pathogens, namely viral haemorrhagic septicaemia virus (VHSV) and infectious pancreatic necrosis virus (IPNV). Viral haemorrhagic septicaemia virus is an enveloped virus belonging to the genus *Novirhabdovirus* within the family *Rhabdoviridae*. Its genome consists of a single-stranded negative-sense RNA of approximately 11,200 nucleotides (Schuetze et al., 1999). Infectious pancreatic necrosis virus, on the contrary, belongs to the family *Birnaviridae* and the genus Aquabirnavirus. It is a non-enveloped, icosahedral virus, with a bi-segmented dsRNA genome that codifies five viral proteins (Dopazo, 2020).

The capacity of VHSV to replicate in the RTgill-W1 has already been established (Al-Hussinee et al., 2016; Pham et al., 2013), whereas another study also characterized some transcriptional effects of VHSV infection in this cell line (Misk et al., 2022). By contrast, the capacity of this virus to replicate in RTgutGC cells or the susceptibility of both epithelial cell lines to IPNV has never been established, and this is something we have confirmed for the first time in this work. Interestingly, a previous study from Poynter and colleagues demonstrated that the mechanisms through which RTgutGC and RTgill-W1 cell lines responded to poly I:C, a synthetic analogue of double-stranded RNA (dsRNA), differ significantly (Poynter et al., 2015). As a consequence, poly I:C induced a stronger response in RTgutGC cells than in RTgill-W1 cells (Poynter et al., 2015). Hence, in this work, we have followed this line of work, further investigating not only the response of both cell lines to poly I:C but also in response to VHSV or IPNV, or to the combination of viruses and poly I:C. Finally, we have also established how poly I:C prestimulation affected viral replication in these cell types. Our results provide us with novel valuable information regarding how these two mucosal epithelial cell lines differentially respond to viral stimuli.

2 | MATERIALS AND METHODS

2.1 | Fish cell lines

The fish cell lines used in this study were the intestinal epithelial cell line RTgutGC (Rainbow Trout gut Guelph Canada), isolated from the distal portion of the intestine of a female rainbow trout (*Oncorhynchus mykiss*) (Kawano et al., 2011); the epithelial RTgill-W1 (Rainbow Trout gill) cell line that was originally developed from a primary gill cell culture established from pieces of gill filaments of a rainbow trout (Bols, 1994); the RTG-2 (Rainbow Trout Gonad-2) cell line that is an established line of fibroblasts from rainbow trout gonads (Wolf & Quimby, 1962); and the epithelial cell line EPC (Epithelioma Papulosum Cyprini) that was originally reported to be from carp (*Cyprinus carpio*) epidermal herpes virus-induced hyperplastic lesions, but was more recently found to be derived from fathead minnow (*Pimephales promelas*) (Winton et al., 2010). All these fish cell lines were routinely cultivated in normal atmosphere following the protocols described by Kawano et al. (2011). In short, cells were cultured in Leibovitz 15 medium (L-15) with phenol red (Gibco) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and 10% foetal calf serum (FCS, Gibco). The cells were grown in 75 cm^2 culture flasks (ThermoFisher Scientific) at 19°C, and split 1:2 when confluent after detaching the cells with 0.25% trypsin-EDTA in PBS (Phosphate Buffer Saline) (Gibco). Dye exclusion test using trypan blue (Sigma) was used to determine the number of viable cells. For the experiments, cells were adjusted to 1×10^6 cells/ml, and depending on the experiment, the cells were distributed in 24-well plates (1 ml per well) or 96-well plates (100 μl per well) (Corning Costar) and were cultured at 19°C for 24 h to reach at least 80% confluency before use.

2.2 | Viral production

Viral haemorrhagic septicaemia virus (strain 3592B, genotype Ia) was propagated in the EPC cell line, whereas IPNV (Sp strain) was propagated in the RTG-2 rainbow trout cell line. In both cases, the complete culture media was removed from 80% confluent cell cultures in 75 cm^2 culture flasks, and viruses added to the cells at a multiplicity of infection (moi) of 1. Immediately after, L-15 medium with antibiotics and 2% FCS was added to the cells that were cultured at 14°C for approximately 5–7 days. When cytophatic effect was extensive, the supernatant was harvested and centrifuged to eliminate cell debris (2000 × *g* for 15 min at 4°C). Clarified supernatants were used for the experiments. All virus stocks were titrated in 96-well plates according to the procedure described by Reed and Muench (1938).

2.3 | Replication of VHSV and IPNV on the RTgutGC and RTgill-W1 cell lines

To verify whether both viruses had the ability to fully replicate in RTgutGC and RTgillW1, 80% confluent RTgutGC and RTgill-W1 cells

disposed on 24-well plates were infected with the corresponding viral supernatants (10 μl of a viral stock at 1×10^8 $TCID_{50}$/ml to achieve a final concentration of 1×10^6 $TCID_{50}$/ml) in L-15 medium supplemented with 2% FCS and maintained at 14°C for 7 days. Cytopathic effects were visualized at different days post-infection (1, 2, 5 and 7 days) under an inverted light microscope (Olympus). Three replicates were made per condition, and non-infected cells were included as controls. Images were processed using Adobe Photoshop® PSC6.

2.4 | Immune gene transcription elicited by poly I:C

The dsRNA poly I:C (P0913, Sigma) was used to establish the transcriptional response of RTgutGC and RTgill-W1 cell lines to this viral mimic. For this, cells disposed on 24-well plates in complete medium were stimulated with poly I:C (10, 20 and 100 μg/ml) or left untreated. After 24 h at 19°C, the cells were harvested for RNA extraction as described later. In other experiments, cells were stimulated with 100 μg/ml poly I:C or left untreated in the presence or absence of VHSV and IPNV (1×10^6 $TCID_{50}$/ml). Poly I:C and viruses were simultaneously added to cells. In this case, the medium used was L15 supplemented with 2% FCS and antibiotics, and cells were incubated for 24 h at 14°C, prior to RNA extraction. In all cases, each experimental condition was carried out in quadruplicate.

2.5 | RNA isolation, cDNA synthesis and real-time PCR

Total RNA was extracted from RTgutGC and RTgill-W1 cells using the TRI Reagent solution (Invitrogen) following the manufacturer's instructions. RNA was then quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and treated with DNase to remove genomic DNA that might interfere with the PCR reactions using the rapid Out DNA Removal kit (Thermo Scientific). Reverse transcription was then performed using the Revert Aid RT kit (Thermo Scientific) following the manufacturer's instructions. The resultant cDNA was diluted in nuclease-free water and stored at −20°C until use. The levels of transcription of the different immune genes (Table S1) were determined by real-time PCR and were normalized to the relative expression of the rainbow trout elongation factor 1α (EF-1α) gene. Expression levels were calculated using the 2-ΔCt method, where ΔCt is determined by subtracting the EF-1α value from the target cycle threshold (Ct cut-off set to 38). EF-1α was selected as reference gene according to the MIQE guidelines (Bustin et al., 2009) given that no statistical differences were detected among Ct values obtained for EF-1α in the different samples. Negative controls with no template and *minus*-reverse transcriptase (−RT) controls were included in all experiments.

2.6 | Poly I:C induced antiviral effects

To establish whether poly I:C could elicit an antiviral state in the epithelial cells that could limit viral replication, RTgutGC and RTgill-W1 cells were disposed in 96-well plates and prestimulated with poly I:C at different concentrations (10, 20 and 100 μg/mL) for 24 h at 19°C. Untreated cells were also included. At this point, cells were infected with different serial dilutions of IPNV or VHSV and viral titres estimated by the protocol described by Reed and Muench (1938) after 7 days at 14°C. In all conditions, the tests were repeated six times.

To clearly visualize the cytopathic effect provoked by VHSV or IPNV, in some cases, cells were stained with a crystal violet (Sigma) solution containing 0.25 g of crystal violet, 40 ml of distilled water and 10 ml of methanol. For this, the culture medium was discarded and the crystal violet solution added to each well (100 μl/well). After 2 h at room temperature, the crystal violet solution was removed, and the wells washed carefully with tap water. The wells were allowed to dry for 24 h at room temperature. Photographs were taken with an inverted optical microscope (Olympus) and images were processed using Adobe Photoshop® PSC6.

2.7 | Statistics

Data handling, statistical analyses and graphic representation were performed using Microsoft Office Excel 2010 and GraphPad Prism version 7.02 (GraphPad Software). Data were expressed as means ± SE. Statistical analyses were performed to compare values obtained in each experimental group using a Student's unpaired *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. The differences between the mean values were considered significant on different degrees, where * means $p \le 0.05$, ** means $p \le 0.01$ and *** means $p \le 0.005$.

3 | RESULTS

3.1 | Transcriptional effects of poly I:C on the RTgutGC and RTgill-W1 cell lines

To investigate the capacity that both cell lines had to respond to a viral stimuli, we first studied their transcriptional response to poly I:C. After 24-h incubation with three doses of poly I:C (10, 20 and 100 μg/ml) or media alone, we evaluated the levels of transcription of genes involved in the antiviral response (IFN1, IFN2 and Mx1), antimicrobial peptides (AMPs) (cathelicidin 1, cathelicidin 2 and hepcidin), genes that code for innate receptors (MDA5, LGP2a, TLR2, TLR3, TLR9 and TLR22) and a representative pro-inflammatory cytokine (IL-1β).

We found that, in RTgutGC cells, poly I:C induced a significant upregulation of the levels of transcription of all genes analysed with exception of TLR2, TLR9 and IL-1β (Figure 1a). Intriguingly, the highest poly I:C concentration used induced a significant downregulation of IL-1β mRNA levels in these cells (Figure 1a).

The transcriptional response to poly I:C was much lower in the case of RTgill-W1 cells. Thus, significantly increased transcription levels were only observed in the case of IFN1 (with 100 μg/ml poly I:C), IFN2 (with 20 μg/ml poly I:C), TLR2 (with 10 μg/ml poly I:C),

TLR3 (with 100 μg/ml poly I:C), cathelicidin 2 (with poly I:C at 100 μg/ml) and hepcidin (with the three concentrations of poly I:C tested) (Figure 1b). Additionally, a significant downregulation of TLR9 and IL-1β levels of transcription was also detected with the highest and the lowest poly I:C doses, respectively. Interestingly, no TLR22 transcription was ever detected in these cells (Figure 1b).

3.2 | Transcriptional effects of poly I:C in combination with VHSV or IPNV on the RTgutGC and RTgill-W1 cell lines

We also determined the transcriptional response of RTgutGC and RTgill-W1 cell lines when poly I:C was combined with either VHSV or IPNV. As shown in Figure 2a, a significant upregulation of IFN1, IFN2, MDA5, Mx1, TLR3, TLR22, IL-1β and cathelicidin 2 transcription was observed in RTgutGC cells stimulated for 24 h with VHSV alone compared with non-infected cells. In the presence of poly I:C, significantly higher transcription levels were reached for all these genes (Figure 2a). Furthermore, LGP2a, TLR9 and hepcidin mRNA levels also reached significantly higher transcription levels in cells stimulated with VHSV and poly I:C when compared to those stimulated with VHSV alone (Figure 2a).

Infectious pancreatic necrosis virus significantly increased the levels of transcription of IFN1, IFN2, MDA5, Mx1, LPG2a, TLR3, TLR22, IL-1β and CATH2 in RTgutGC cells (Figure 2b). As occurred with VHSV, when cells were incubated with poly I:C in addition to IPNV, the levels of transcription of all these genes significantly increased in comparison with the levels observed in cells exposed to IPNV alone (Figure 2b). Additionally, TLR9 and hepcidin mRNA levels reached in response to IPNV and poly I:C combination were also significantly higher than those observed in response to IPNV alone (Figure 2b).

When RTgill-W1 cell cultures were stimulated with poly I:C in the presence of VHSV or IPNV, again their transcriptional response was much weaker than that observed in RTgutGC cells. As shown in Figure 3a, IFN1 was the only gene that significantly increased its transcription in cells exposed to VHSV alone in comparison with unstimulated cells (Figure 3a). In the presence of both VHSV and poly I:C, RTgill-W1 cells significantly upregulated the levels of transcription of IFN1, IFN2, MDA5, IL-1β and CATH2 when compared to the levels reached in response to VHSV alone (Figure 3a). Surprisingly, TLR22 transcription was downregulated in co-stimulated cells than in cells infected only with virus (Figure 3a).

On the contrary, RTgill-W1 cells significantly upregulated IFN1, IFN2, MDA5, TLR9 and TLR22 transcription in response to IPNV

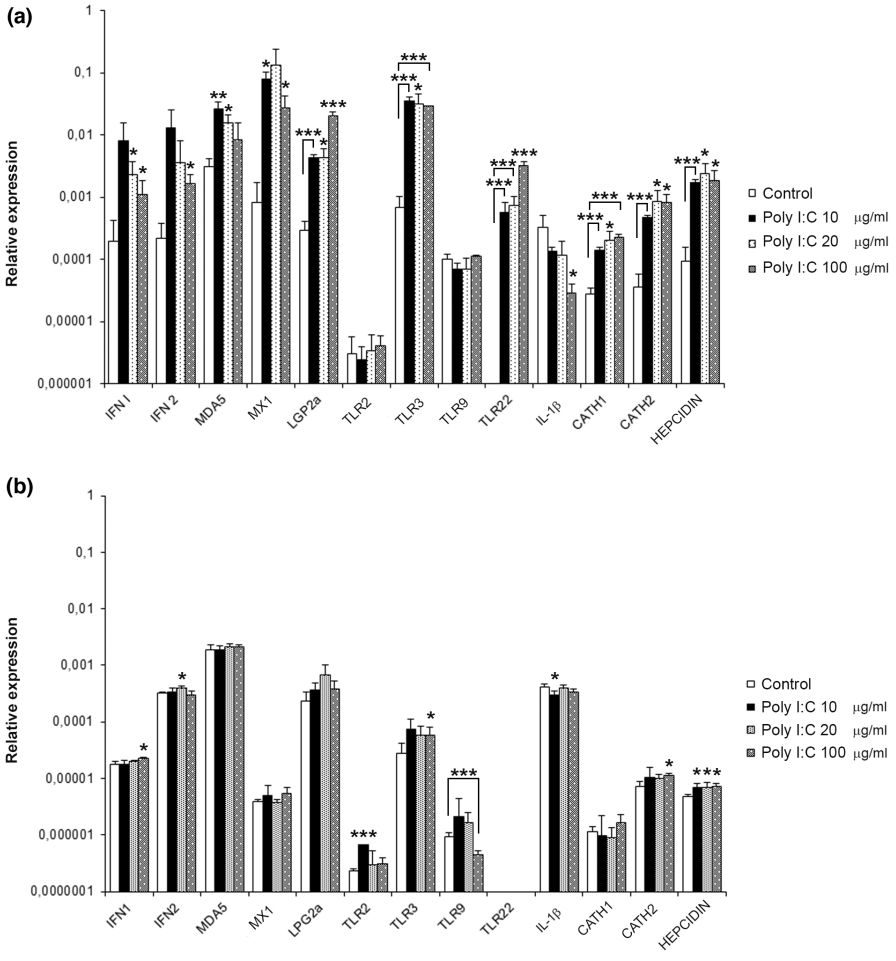

FIGURE 1 Transcriptional analysis of RTgutGC and RTgill-W1 cells treated with different doses of the immunostimulant poly I:C. confluent monolayers of RTgutGC and RTgill-W1 cells (24-well plate format) were treated with poly I:C at 10, 20 and 100 μg/ml or non-treated (control) for 24 h at 19°C. After that time, RNA from RTgutGC (a) and RTgill-W1 (b) cells was extracted as described in 'Materials and Methods' section to study the transcription levels of different marker genes by real-time PCR. Results are shown as relative expression values to endogenous control EF-1α (mean ± SD; $n = 4$). Asterisks denote significantly different values between treated and control cells (*$p \leq 0.05$ and ***$p \leq 0.005$).

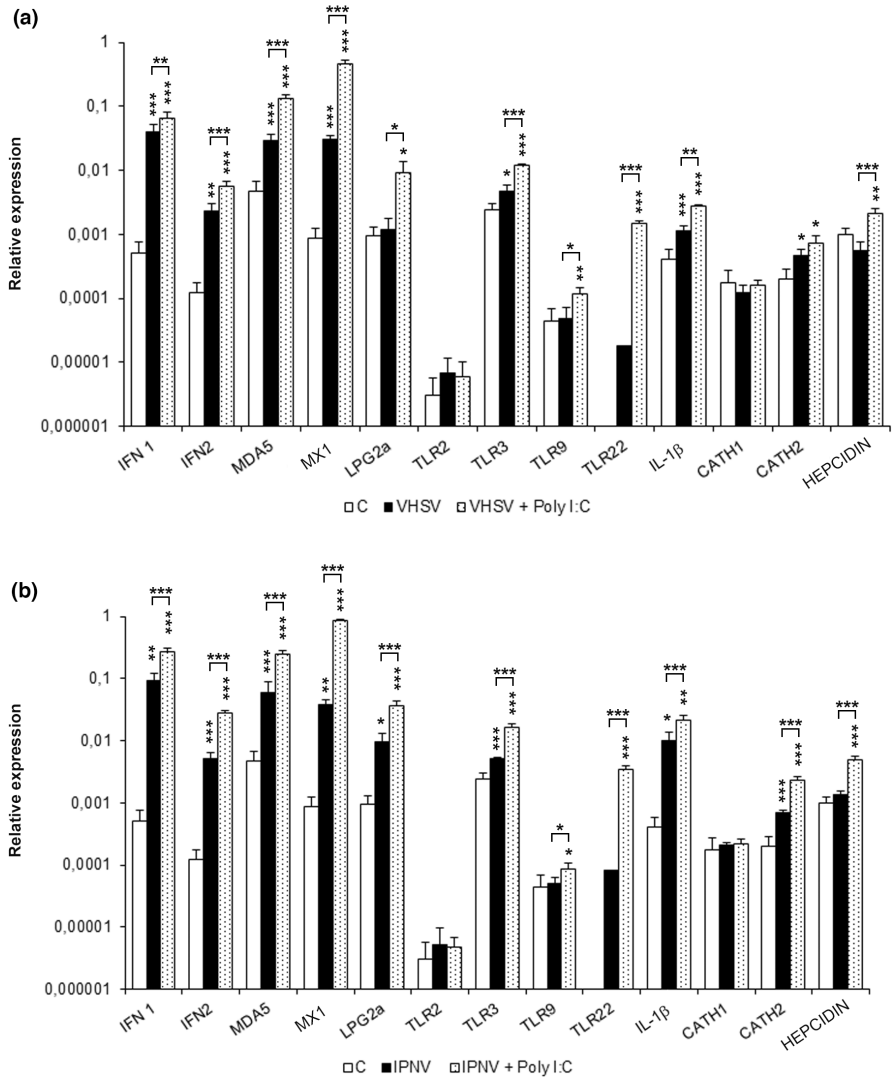

FIGURE 2 Transcriptional analysis of RTgutGC cells co-stimulated with VHSV or IPNV and poly I:C. confluent cell cultures of RTgutGC cells (24-well plate format) were co-stimulated with VHSV (1×10^6 $TCID_{50}$/ml) and poly I:C (100 μg/ml) (a) or with IPNV (1×10^6 $TCID_{50}$/ml) and poly I:C (100 μg/ml) (b) for 24 h at 14°C. In both experiments, well plates with only virus infected cells and non-stimulated cells (control) were also included. After 24 h of incubation, RNA from treated or non-treated RTgutGC cells was extracted as described in 'Materials and Methods' section to study the transcription levels of different marker genes by real-time PCR. Results are shown as relative expression values to endogenous control EF-1α (mean ± SD; $n = 4$). Asterisks above bars denote significantly different values between treated and control cells, whereas asterisks above brackets indicate significant differences between groups, as indicated (*$p \le 0.05$; **$p \le 0.01$ and ***$p \le 0.005$).

when compared to cells not exposed to the virus (Figure 3b). All these immune genes were further significantly upregulated in co-stimulated RTgill-W1 cell cultures (Figure 3b), with exception of TLR22 which was downregulated, when compared to cells only exposed to the virus. Additionally, Mx1, LPG2a, TLR3 and CATH2 genes were also upregulated in co-stimulated cells when compared to cells stimulated with the virus alone. Nevertheless, all these results confirm that the transcriptional response of RTgill-W1 cells to poly I:C or to a viral agent is much lower than that of RTgutGC cells.

3.3 | Induction of antiviral effects induced by poly I:C on RTgutGC and RTgill-W1 cells

Having established that poly I:C is able to regulate the transcription of different genes involved in the antiviral response, we wanted to determine whether preincubation of RTgutGC and RTgill-W1 cells with poly I:C was capable of inducing an antiviral state that then limited the capacity of both viruses to replicate in these cell lines. Prior to conducting these experiments, we visually confirmed that VHSV and IPNV were capable of fully replicating in RTgutGC and RTgill-W1 cell lines. For this, both cell types were infected with VHSV or IPNV and analysed under the inverted microscope at different times post-infection searching for cytopathic effects. Non-infected cells treated in the same conditions were included as controls. After 5 days of infection, cytopathic effects in response to both viruses were clear in both cell lines, although the cytopathic effect provoked by VHSV seemed to be more pronounced than that provoked by IPNV, especially in the case of RTgutGC cells (Figure S1). Finally, although after 7 days of infection, a cytopathic effect was fully developed in both cell lines in response to both viruses, the cytopathic effect provoked by IPNV was more extensive in RTgill-W1 cells than that observed in RTgutGC cells (Figure S1).

Concerning the capacity of poly I:C to limit viral replication, we found that the VHSV titre reached on RTgutGC cultures after 7 days of infection was slightly reduced when cells were prestimulated with poly I:C at 20 and 100 μg/ml (Figure 4a). Noticeably, IPNV replication was reduced on the RTgutGC cell line approximately 6 logs when cells were prestimulated with any of the concentrations of poly I:C (Figure 4b). In the case of RTgill-W1 cells,

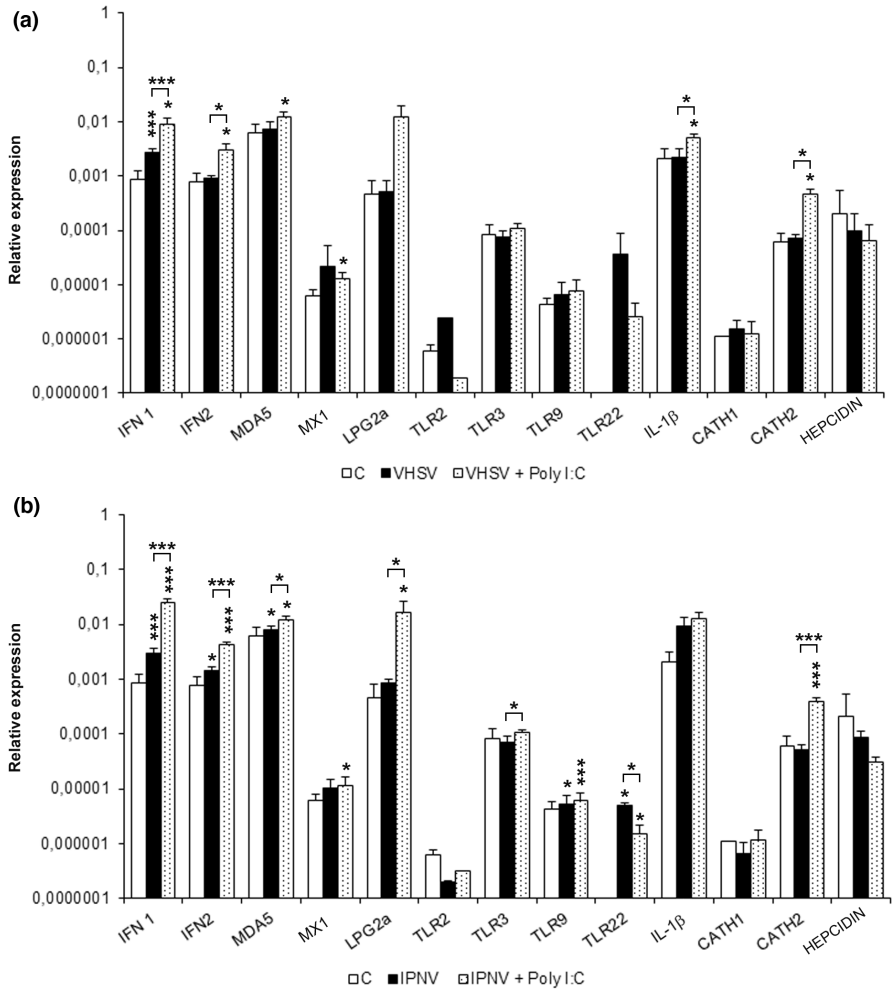

FIGURE 3 Transcriptional analysis of RTgill-W1 cells co-stimulated with VHSV or IPNV and poly I:C. Confluent cell cultures of RTgill-W1 cells (24-well plate format) were co-stimulated with VHSV (1×10^6 $TCID_{50}$/ml) and poly I:C (100 μg/ml) (a) or with IPNV (1×10^6 $TCID_{50}$/ml) and poly I:C (100 μg/ml) (b) for 24 h at 14°C. In both experiments, well plates with only virus infected cells and non-stimulated cells (control) were also included. After 24 h of incubation, RNA from treated or non-treated RTgill-W1 cells was extracted as described in 'Materials and Methods' section to study the transcription levels of different marker genes by real-time PCR. Results are shown as relative expression values to endogenous control EF-1α (mean ± SD; $n = 4$). Asterisks above bars denote significantly different values between treated and control cells, whereas asterisks above brackets indicate significant differences between groups, as indicated (*$p \leq 0.05$ and ***$p \leq 0.005$).

poly I:C prestimulation slightly reduced the VHSV replication capacity, although differences in viral titres were not significant (Figure 5a). By contrast, prestimulation of RTgill-W1 cells with the two highest poly I:C concentrations significantly limited the replication of the IPNV, although the differences in viral titres were of approximately 2 or 3 logs (Figure 5b). Thus, in both cell types, poly I:C was able to limit more efficiently the replication of IPNV than that of VHSV, although the antiviral effects were much more pronounced in RTgutGC than in RTgill-W1 cells.

4 | DISCUSSION

Fish mucosal surfaces continually interact with the water environment that surrounds them and thereby constitute the first line of defence against pathogens. Nevertheless, the role of epithelial cells in mucosal antiviral immunity is often neglected. In this context, the use of epithelial cell lines derived from mucosal tissues such as the ones used in our study constitutes an interesting tool to further decipher how these cells behave and in this sense, numerous studies in the recent years have used RTgutGC and RTgill-W1 cells to investigate their response to different types of stimuli or infectious agents (Holen et al., 2021; Kawano et al., 2011; Lisser et al., 2017; Mandal et al., 2020; Misk et al., 2022). However, only a few of these studies focussed on establishing the response to viral agents.

Recently, Mandal and collaborators established that RTgill-W1 cells increased their cellular integrity in response to poly I:C (Mandal et al., 2020). In that work, the induced transcription of some IFN-related genes was also confirmed in response to poly I:C (Mandal et al., 2020). In the current study, we have compared the transcriptional response to poly I:C of RTgill-W1 and RtgutGC cells, including a wider range of immune genes, such as genes involved in the IFN response, AMPs, innate receptors and a pro-inflammatory gene. As representatives of the type I IFN response, we chose two type I IFN genes (IFN1 and IFN2) and Mx1, an IFN-induced protein with direct antiviral effects, given that all of these genes are well-known to be up-regulated in response to poly I:C (Leong et al., 1998; Tafalla et al., 2007; Zou et al., 2007). As epithelial cells are known to be major producers of AMPs, we also studied the transcriptional response of some of these genes, namely hepcidin and cathelicidin 1 and 2. These AMPs have been shown to possess a significant antiviral activity against a variety of viruses, including VHSV and IPNV (Brunner et al., 2020). Additionally, IPNV infection in Atlantic salmon leads to an upregulation of hepcidin transcription in liver and red blood cells and of cathelicidin in head kidney and red blood cells (Tarifeno-Saldivia et al., 2018). In rainbow trout, both hepcidin and cathelicidin were upregulated by

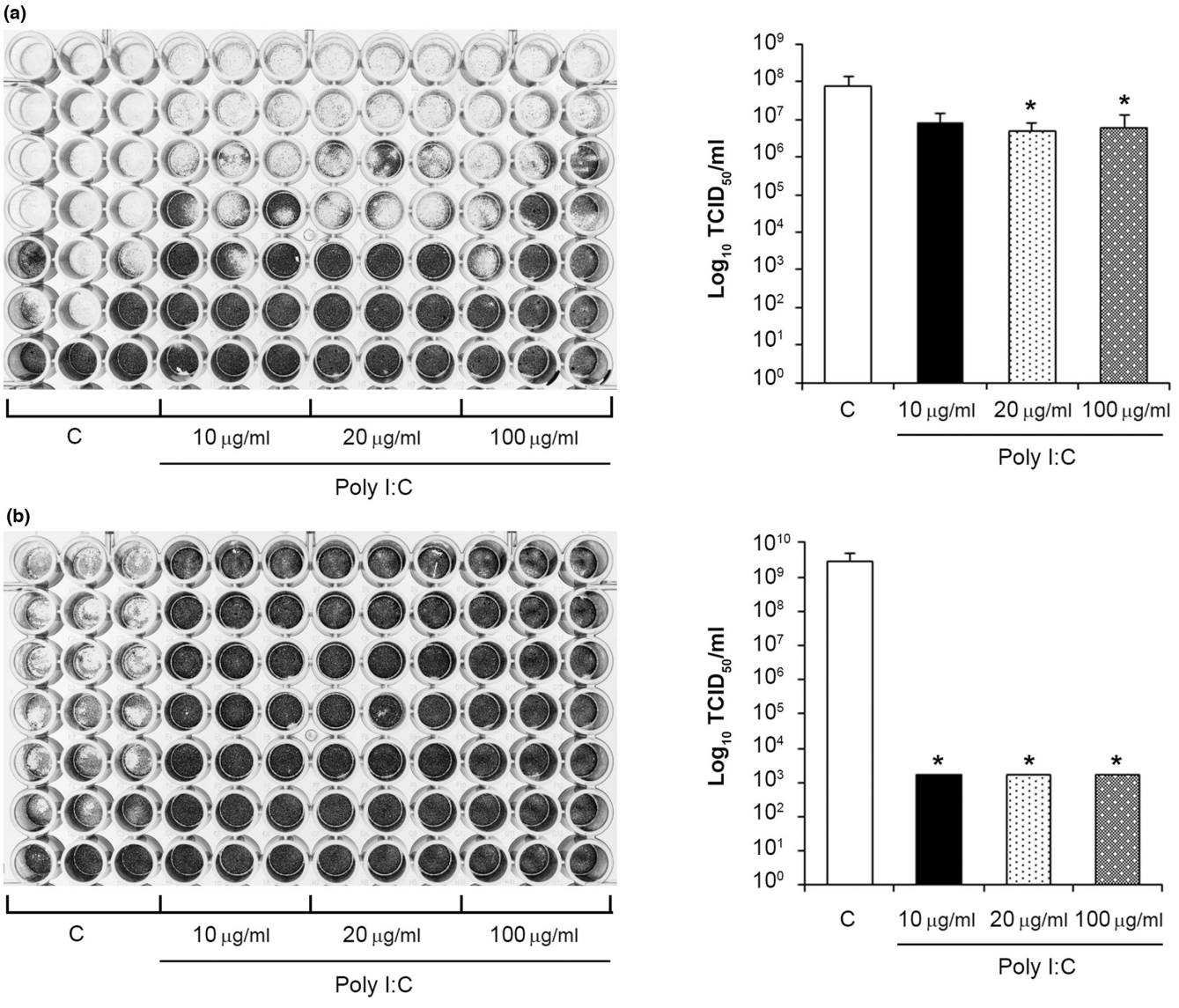

FIGURE 4 Analysis of the effect of poly I:C cellular prestimulation on the cytopathic action of VHSV and IPNV in the RTgutGC cell line. Cell cultures of RTgutGC cells (96-well plate format) were stimulated for 24 h at 19°C with different concentrations of poly I:C (10, 20 and 100 μg/ml) or non-stimulated (control). After that time, prestimulated and control cells, in confluent conditions, were infected with virus serial dilutions (ranging from 10^{-3} to 10^{-9}; triplicates) and incubated for a week at 14°C. Cells not infected but stimulated with poly I:C at 10, 20 or 100 μg/ml, and uninfected and unstimulated cells, were also included and were used as internal controls in each of the plates. (a,b) Representative images of a 96-well plate showing RTgutGC cells infected with serial dilutions of VHSV (a) or IPNV (b) and stained with crystal violet to better visualize the cytophatic effect of the virus in the different stimulation conditions, along with the corresponding graphs showing the viral titre expressed by the dose infective cytotoxic 50% ($TCID_{50}$/ml) are shown (mean ± SD; $n = 6$) (C = virus control). Asterisks denote significantly different values between treated and virus control cells (*$p \leq 0.05$).

VHSV in the RTG-2 cells (Cano et al., 2021). Regarding innate receptors, MDA5 and LGP2a are two intracellular type RIG-I-like receptors involved in virus detection (Yoneyama & Fujita, 2009), similarly to TLR-3 that has been functionally identified as an intracellular sensor of virus-derived dsRNA (Alexopoulou et al., 2001). In mammals, TLR-2 is a major TLR that can recognize lipoproteins derived from bacteria,

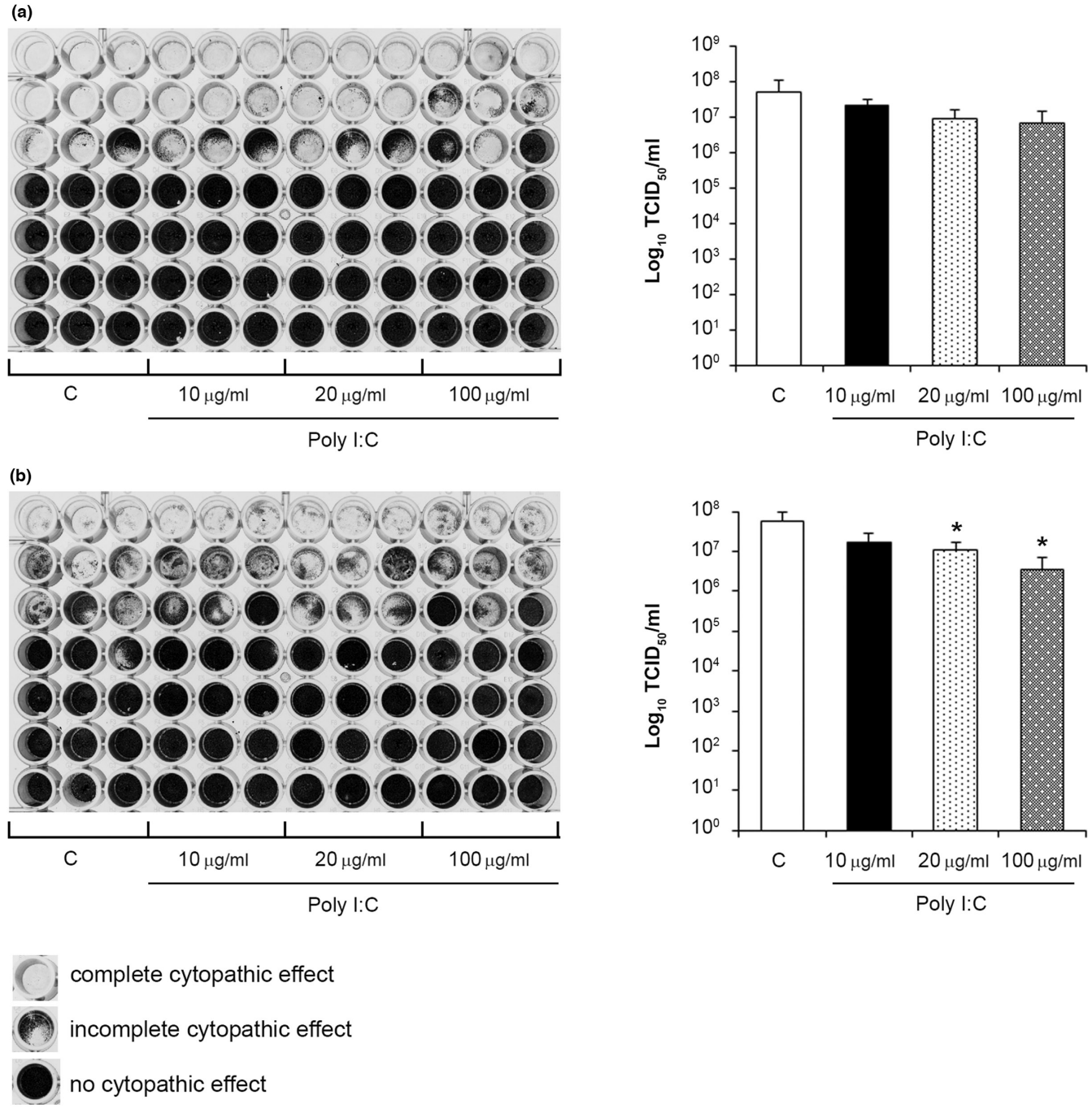

FIGURE 5 Analysis of the effect of poly I:C cellular prestimulation on the cytopathic action of VHSV and IPNV in the RTgill-W1 cell line. Cell cultures of RTgill-W1 cells (96-well plate format) were stimulated for 24 h at 19°C with different concentrations of poly I:C (10, 20 and 100 μg/ml) or non-stimulated (control). After that time, prestimulated and control cells, in confluent conditions, were infected with virus serial dilutions (ranging from 10^{-3} to 10^{-9}; triplicates) and incubated for a week at 14°C. Cells not infected but stimulated with poly I:C at 10, 20 or 100 μg/ml, and uninfected and unstimulated cells, were also included and were used as internal controls in each of the plates. (a,b) Representative images of a 96-well plate showing RTgill-W1 cells infected with serial dilutions of VHSV (a) or IPNV (b) and stained with crystal violet to better visualize the cytophatic effect of the virus in the different stimulation conditions, along with the corresponding graphs showing the viral titre expressed by the dose infective cytotoxic 50% ($TCID_{50}$/ml) are shown (mean ± SD; $n = 6$) (C = virus control). Asterisks denote significantly different values between treated and virus control cells ($^*p \leq 0.05$).

viruses, fungi and parasites (Oliveira-Nascimento et al., 2012), while TLR-9 preferentially binds DNA present in bacteria and viruses (Hemmi et al., 2000). In fish, although transcriptional studies point to a similar response of these homologues, whether they detect the same ligands as those recognized by their mammalian counterparts has not been clearly established yet (Langevin et al., 2013; Pietretti & Wiegertjes, 2014). On the contrary, TLR22 is a fish-specific TLR, identified in a variety of fish species, which plays a significant role

in systemic and mucosal defence after viral or bacterial infection (Li et al., 2017). In some species, TLR22 has been identified as also capable of sensing dsRNA similarly to TLR3 (Li et al., 2017; Xiao et al., 2011). Although some authors suggested that TLR22 is located in the cell surface sensing the presence of dsRNA outside the cell, recent studies revealed it is also localized in the endosomal membrane sensing dsRNA in endosomes (Ding et al., 2018).

Our results demonstrated that the transcriptional response to poly I:C was much higher in RTgutGC cells than that observed in RTgill-W1 cells. Hence, while all genes studied, with the exception of TLR2, TLR9 and IL-1β, were upregulated in response to poly I:C in RTgutGC cells, only IFN1, IFN2, TLR2, TLR3, cathelicidin 2 and hepcidin were transcriptionally regulated by poly I:C in RTgill-W1 cells and to a much lesser extent. Interestingly, the basal levels of transcription of TLR3 were much higher in RTgutGC cells than in RTgill-W1 cells and in the case of TLR22, although none of the cell lines constitutively transcribed this gene, it was only induced by poly I:C in the case of RTgutGC cells. Thus, the lower responsiveness of RTgill-W1 cells to dsRNA could be explained by a much lower constitutive expression of TLR receptors with a known capacity to sense dsRNA. However, a previous study reported that these differences could be due to the fact that dsRNA binding in RTgutGC cells is dependent on the expression of class A scavenger receptors, while that is not the case in RTgill-W1 cells (Poynter et al., 2015).

The transcriptional response of both cell lines to VHSV and IPNV was also markedly different. Thus, while VHSV by itself upregulated the transcription of IFN1, IFN2, MDA5, Mx1, TLR3, IL-1β and cathelicidin 2 in RTgutGC cells, in similar conditions, it was only capable of significantly upregulating IFN1 transcription in RTgill-W1 cells. Although the transcriptional response to IPNV alone was also higher in RTgutGC cells than in RTgill-W1 cells, in this case, the virus upregulated IFN1, IFN2, MDA5, TLR9 and TLR22 in the later cell line. Hence, the antiviral response induced by IPNV in RTgill-W1 cell line is higher than that induced by VHSV in the same cell line. When these viruses were combined with poly I:C to stimulate the cells, many of these genes were further upregulated to significantly higher levels. This occurred in response to both viruses and in both cell lines, although again the transcription responses of RTgill-W1 cells were lower than those of RTgutGC cells.

Multiple studies have demonstrated that poly I:C induces an antiviral state in fish cells that subsequently limits viral replication (Jensen et al., 2002; Skjesol et al., 2009; Zou et al., 2007). To test whether this was the case also in these mucosal epithelial cells, we preincubated RTgutGC and RTgill-W1 for 24 h with poly I:C and then infected them with either of the two viruses. Prior to conducting this experiment, we visually confirmed that both cell lines were susceptible to both VHSV and IPNV. The capacity of VHSV to replicate in RTgill cells had already been described (Pham et al., 2013), whereas the susceptibility of this cell line to IPNV had never been established, nor that of the RTgutGC cells to both viruses. When we studied how poly I:C preincubation affected the replication capacity, variable results were observed depending on the virus and cell line. In the case of RTgutGC cells, while IPNV replication was greatly reduced in cells preincubated with poly I:C, only a slight reduction in titres was observed when the cells were infected with VHSV. This stronger capacity of poly I:C to reduce IPNV replication than that of VHSV was also observed in RTgill-W1 cells, suggesting that IPNV is much more sensitive to dsRNA antiviral mechanisms triggered than VHSV. Similarly, Jensen and Robertsen in 2002 demonstrated that IPNV and ISAV (infectious salmon anaemia virus) showed very different sensitivities to the antiviral mechanisms induced by poly I:C in Atlantic salmon cells (Jensen et al., 2002). Given that most viruses have varied strategies to counteract the action of cellular antiviral mechanisms (Lucas et al., 2001; Skjesol et al., 2009), it would be interesting to study in the future the mechanism through which VHSV is capable of escaping the antiviral mechanisms elicited by poly I:C in these cell lines. Interestingly, recent studies have shown that pretreatment of RTgill-W1 cells with inactivated VHSV resulted in reduced subsequent VHSV replication that did not imply the upregulation of key antiviral genes such as Mx1, IFN1 or IFN2 (Al-Hussinee et al., 2016; Misk et al., 2022). Furthermore, Liu and colleagues demonstrated that both viral gene copy number and VHSV N protein significantly decreased when RTgill-W1 cells were treated with autophagy-blocking (chloroquine) and autophagy-inhibiting reagents (deoxynivalenol and 3-methyladenine) (Liu et al., 2020). Altogether, this could be suggesting that, at least in RTgill-W1 cells, other mechanisms different that those elicited by dsRNA would have a greater capacity to block VHSV infection. Additionally, we observed that the capacity of poly I:C to reduce viral replication was much higher in RTgutGC cells than in RTgill-W1 cells, as would be expected from the limited capacity of RTgill-W1 cells to respond to dsRNA.

5 | CONCLUSIONS

In summary, taking advantage of the established rainbow trout RTgutGC and RTgill-W1 mucosal epithelial cell lines, we have been able to investigate the response of these cell types to viral infections and to a viral stimuli such as poly I:C. Our results demonstrated that the transcriptional response of RTgill-W1 cells to dsRNA or to viral encounter was much lower than that of RTgutGC cells, possibly as a consequence of a less effective IFN system in RTgill-W1 cells. This was also implied given that poly I:C almost completely blocked IPNV replication in RTgutGC cells but had only minor effects in RTgill-W1 cells. Our results highlight the usefulness of these established cell lines to investigate mucosal immune responses in fish and contribute to a further understanding of how they respond to viruses.

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DATA AVAILABILITY STATEMENT

The data sets generated during this study are available from the corresponding author upon reasonable request.

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