Early activation of teleost B cells in response to rhabdovirus infection

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To date, the response of B cells to specific pathogens has been only scarcely addressed in teleost. In the current work, we have demonstrated that viral hemorrhagic septicemia virus (VHSV), a fish rhabdovirus, has the capacity to infect rainbow trout spleen IgM⁺ cells although the infection is not productive. Consequently, we have studied the effects of VHSV on IgM⁺ cell functionality comparing these effects to those elicited by a TLR3 ligand, Poly I:C. We found that Poly I:C and VHSV significantly up-regulated TLR3 and type I interferon (IFN) transcription in spleen and blood IgM⁺ cells. Further effects included the up-regulated transcription of the CK5B chemokine. Significant inhibition of some of these effects in presence of baflomycin A1 (BAF), an inhibitor of endosomal acidification, suggests the involvement of an intracellular TLR in these responses. In the case of VHSV, these transcriptional effects were dependent on viral entry into B cells and initiation of viral transcription. VHSV also provoked the activation of NF-κB and the up-regulation of MHC-II cell surface expression on IgM⁺ cells, that along with an increased transcription of the co-stimulatory molecules CD80/86 and CD83 pointed to a VHSV-induced IgM⁺ cell activation towards an antigen-presenting profile. Finally, despite the moderate effects of VHSV on IgM⁺ cell proliferation, a consistent effect on IgM⁺ cell survival was detected.

Innate immune responses to pathogens established through their recognition by PRRs have been traditionally ascribed to innate cells. However, recent evidence in mammals has revealed that innate pathogen recognition by B lymphocytes is a crucial factor in shaping the type of immune response that is mounted. In teleost, these immediate effects of viral encounter on B lymphocytes have not been addressed to date. In our study, we have demonstrated that VHSV infection provoked immediate transcriptional effects in B cells, at least partially mediated by intracellular PRR signaling. VHSV also activated NF-κB and increased IgM⁺ cell survival. Interestingly, VHSV activated B lymphocytes towards an antigen-presenting profile, suggesting an important role of IgM⁺ cells in VHSV presentation. Our results provide a first description of the effects provoked by fish rhabdoviruses through their early interaction with teleost B cells.
Introduction

In mammals, toll like receptors (TLRs) recognize highly conserved structures of viral (TLR3, 7, 8 and 9) and bacterial (TLR1, 2, 4, 5, 6, 7, 8, and 9) origin. While TLRs 1, 2, 4, 5 and 6 - together with TLR11 and TLR12 in mice, and TLR10 in humans - are mostly expressed on the cell surface, a second group of TLRs including TLR 3, 7, 8 and 9 are localized within endosomal compartments and detect foreign nucleic acids (1). Recognition of pathogen associated molecular patterns (PAMPs) through TLRs and other pattern recognition receptors (PRRs) leads to the activation and maturation of innate immune cells such as macrophages or dendritic cells (DCs). Additionally, once the presence of several TLR receptors was verified on distinct populations of human and murine B cells, further investigations concluded that B cells have evolved to directly sense microbes and that this TLR-mediated activation of B cells contributes to the establishment of an adequate humoral response (2). However, controversy remains regarding as to what degree TLR signaling in B cells conditions the antibody response. On one hand, early studies showed that mice lacking B cell TLR signaling failed to mount an efficient antibody response (3). However, subsequent studies suggested a slightly different model in which these receptors play a role in the regulation of antibody class switch and in sustaining antibody secretion at late times after immunization in B cells (4), contributing to the amplification of the humoral response but not being completely responsible for it (5). In support of these observations, further studies demonstrated that the primary responses of some Ig subclasses (i.e. IgG2a or IgG2c) were absolutely dependent on the signaling through the adapter protein MyD88 used by most TLRs, whereas other Ig classes were not (IgG1 and IgG3) or much less (IgG2b, IgA) dependent on the MyD88 signaling cascade (6, 7). Interestingly, the
conditional deletion of MyD88 in either DCs or B cells revealed that the antibody
response to virus like particles required TLR signaling in B cells, while the response to
a soluble antigen was dependent on TLR signaling on DCs (8). This result reveals an
ability of B cells to discriminate among antigens based on their physical form.

Several studies have examined the expression of TLRs across B cell subsets in
mice and human tissues, revealing important species-specific differences in the range of
TLR expressed by each subset. In mice, evaluation of follicular B cells, marginal zone
B cells, B1 cells and Peyers` s patches B cells indicated broad (except TLR5 and TLR8)
yet differential TLR expression, and distinct responsiveness to TLR agonists (9). In
contrast, human naïve tonsil or blood B cells lack TLR3, TLR4 and TLR8 expression
(10, 11), even though the expression of these three TLRs can be detected in human
plasma cells (11). As a result, for example, human naïve B cells do not express TLR4
and therefore do not recognize the bacterial lipopolysaccharide (LPS), typically
recognized by this receptor in murine B cells (10). Despite these specificities, overall,
mammalian naïve B lymphocytes express relatively low levels of TLRs in comparison
to activated and memory cells (2). Furthermore, although the usual consequences of
TLR activation in responsive B cells are direct effects on cell proliferation and antibody
secretion, some additional effects have been reported in B1 cells. In this B cell subset,
TLR signaling directly induced nitric oxide (NO) via inducible NO synthase (iNOS)
activation (12), and increased the response to chemokines through a rapid specific
down-regulation of integrins and CD9 (13). In fact, the role of TLR signaling appears
prominent in innate B cell subsets; for example, B1 cells and marginal zone B cells are
known to generate natural antibodies through a T-independent mechanism that involves
the recognition of microbial products through TLRs and low affinity immunoglobulin
(Ig) receptors (14, 15). This rapid antibody response interferes with pathogen replication until more specific T-dependent antibody responses are mounted.

Given the propensity for delayed T-dependent responses in teleosts, TLR activation may play a more prominent role in B cell response to viral infections. Accordingly, rainbow trout (*Oncorhynchus mykiss*) IgM⁺ cells from different tissues including kidney, spleen, blood, intestine, gills and liver have relatively high constitutive transcription levels of TLR1, TLR2, TLR3, TLR5, TLR7, TLR8a, TLR9 and TLR22 (16). Furthermore, in sharp contrast to mammals where TLR3 (TLR responsible for sensing intracellular dsRNA) is not a predominant TLR in B cells, trout IgM⁺ cells appeared to be the main cell type expressing TLR3 in the spleen according to the ratios of TLR3 transcription observed between IgM⁺ and IgM⁻ populations (21 times higher in IgM⁺ cells than in IgM⁻ cells) (16). This suggests a high capacity of teleost B cells for sensing viral ligands. Unfortunately, to date, studies dealing with TLR responses in B cells are limited to examination of the proliferative and antibody-secreting responses observed after LPS stimulation (17, 18).

On the other hand, many viruses have a demonstrated capacity to infect mammalian B lymphocytes, affecting their functionality. For example, Epstein Barr virus establishes latency in B lymphocytes, which can be reactivated leading lymphocytes to shed virus (19). Porcine circovirus type 2 (PCV2) spliced capid mRNA (Cap mRNA) and viral DNA were detected in B lymphocytes from pigs, although no further studies were performed to determine if the presence of these nucleic acids corresponds to a productive infection, with release of viral progeny from these cells (20). The capacity of Simian foamy viruses (SFV) to transcribe genes in human B lymphocytes has also been recently described (21). Interestingly, live rabies vaccines have been shown to infect murine and human B lymphocytes (22).
In this context, we decided to undertake a functional study to examine the effects of viral hemorrhagic septicemia virus (VHSV) on the functionality of IgM$^+$ cells in fish. VHSV, a negative sense ssRNA enveloped rhabdovirus, is the etiological agent of a lethal disease for many cultivated fish species worldwide including rainbow trout. We have analyzed the capacity of VHSV to infect trout IgM$^+$ cells and also studied the effects that VHSV had on different aspects of B cell functionality, comparing these effects to those provoked by a TLR3 agonist such as Poly I:C and a TLR9 agonist (CpG). Our results demonstrate that VHSV infects B lymphocytes where it is able to transcribe viral genes and remain viable, even though active replication was not detected. Consequently, this early virus-B lymphocyte interaction provokes different effects in the cell, including transcriptional up-regulation of TLR3 and type I interferon (IFN), NF-κB activation, increased survival and increased major histocompatibility complex II (MHC-II) surface expression. These effects highlight the importance of B lymphocytes in the early immune response to VHSV and reveal an important role of innate immune signals in the viral response of teleost B cells, probably conditioning posterior acquired B lymphocyte responses.

**Materials and Methods**

*Fish*
Healthy specimens of female rainbow trout (*Oncorhynchus mykiss*) of approximately 50-70 g were obtained from Centro de Acuicultura El Molino (Madrid, Spain) located in a VHSV-free area. Fish were maintained at the Centro de Investigación en Sanidad Animal (CISA-INIA) laboratory at 16-18°C with a recirculating water system and 12:12 hours light:dark photoperiod. Fish were fed twice a day with a commercial diet (Skretting, Spain). Prior to any experimental procedure, fish were acclimatized to laboratory conditions for 2 weeks and during this period no clinical signs were ever observed. All experiments described comply with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals and were approved by the Ethics committee from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA).

Tissue collection

Rainbow trout were sacrificed via MS-222 (Sigma) overdose and blood was extracted with a heparinized needle from the caudal vein and diluted 10 times with Leibovitz medium (L-15, Life Technologies) supplemented with 100 I.U./ml penicillin, 100 μg/ml streptomycin (P/S), 10 units/ml heparin and 5% fetal calf serum (FCS) (all supplements also obtained from Life Technologies). Spleen was collected and single cell suspensions generated using 100 μm nylon cell strainers (BD Biosciences). Blood cell suspensions were placed onto 51% Percoll (GE Healthcare) cushions whereas spleen suspensions were placed onto 30/51% discontinuous density gradients. All suspensions were then centrifuged at 500 x g for 30 min at 4°C. The interface cells were collected and washed twice in L-15 containing 5% FCS.
**Cell stimulation**

Total leukocyte populations from individual fish were dispensed in 24-well plates at a density of $1 \times 10^6$ cells per ml and incubated with VHSV or the different TLR agonists. Non-stimulated controls were always included. Cells were then incubated at 20°C for different periods of time depending on the experiments. Poly I:C (Sigma) was used at a concentration of 50 µg/ml. For CpG stimulation, a plasmid containing four copies of a DNA sequence encoding different immunostimulatory CpG motifs which has been previously described (23) was used at a final concentration of 5 µg/ml.

VHSV propagated in the EPC cell line and titrated as previously described (24) was used at a final concentration $1 \times 10^6$ TCID$_{50}$/ml (tissue culture infective dose $50$ per ml).

For VHSV inactivation, the virus was incubated at 56°C for 30 min. Complete viral inactivation was confirmed by inoculation into EPC cells, followed by checking for cytopathic effect during 10 days. In some experiments, a 1 h pre-incubation of the cells with 50 nM of the endosomal TLR inhibitor bafilomycin A1 (BAF, Sigma) dissolved in DMSO was performed prior to the addition of other stimuli. LPS (Sigma) was used as a positive control for B cell proliferation at a final concentration of 100 µg/ml.

**Cell sorting**

After stimulation, leukocytes were collected from the wells by gentle scrapping, resuspended in PBS and incubated for 30 min on ice with a specific anti-trout IgM mAb (1.14) coupled to phycoerythrin (PE) (25). Following two washing steps, cells were
resuspended in PBS and IgM positive cells were isolated by FACS sorting in a BD FACSAria III (BD Biosciences), using first their FSC/SSC profiles (to exclude the granulocyte gate) and then on the basis of the fluorescence emitted by the anti-trout IgM antibody (16). IgM+ and IgM− cells were then collected in different tubes for RNA isolation. Expression of IgM and absence of T cell markers (CD3 and TCRα) was verified by PCR in some samples to assure an effective sorting.

**Real time PCR analysis of sorted cells**

Total cellular RNA was isolated from IgM+ sorted populations from different tissues using the Power SYBR Green Cells-to-Ct Kit (Life Technologies) following manufacturer’s instructions. RNAs were treated with DNase during the process to remove genomic DNA that might interfere with the PCR reactions. Reverse transcription was also performed using the Power SYBR Green Cells-to-Ct Kit (Invitrogen) following manufacturer’s instructions. To evaluate the levels of transcription of the different genes, real-time PCR was performed with a LightCycler® 96 System instrument (Roche) using SYBR Green PCR core Reagents (Applied Biosystems) and specific primers previously described (16, 26-28) and summarized in Table 1. The efficiency of the amplification was determined for each primer pair using serial 10 fold dilutions of pooled cDNA, and only primer pairs with efficiencies between 1.95 and 2 were used. Each sample was measured in duplicate under the following conditions: 10 min at 95°C, followed by 45 amplification cycles (15 s at 95°C and 1 min at 60°C) plus a dissociation cycle (30 s at 95°C, 1 min 60°C and 30 s at 95°C). The expression of individual genes was normalized to the relative expression of trout
EF-1α and the expression levels were calculated using the $2^{\Delta Ct}$ method, where \( \Delta Ct \) is determined by subtracting the EF-1α value from the target Ct. Negative controls with no template were included in all the experiments. A melting curve for each PCR was determined by reading fluorescence every degree between 60ºC and 95ºC to ensure only a single product had been amplified.

**Western blot**

To determine if VHSV was actively replicating inside spleen IgM$^+$ cells, splenocytes were incubated with VHSV during 24 h and IgM$^+$ and IgM$^-$ cells sorted and lysed independently using RIPA buffer containing protease inhibitors (Roche). Proteins were fractionated onto a denaturing 12% SDS-PAGE gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore Merck). A VHSV extract was included as positive control. After blocking in PBS with 5% skim milk for 1 h, the membrane was incubated with a mAb against the VHSV N protein kindly donated by Dr. Niels Lorenzen (University of Aarhus, Denmark) (IP5B11, 1:1000) in blocking solution at 4ºC overnight. After three washing steps, the membrane was incubated for 1 h with the secondary antibody, a goat anti-mouse IgG-HRP conjugate (GE Healthcare Life Sciences). The reactive bands were visualized with the ECL system (GE Healthcare Life Sciences). Beta-actin staining was used as a loading control, visualized using a mAb raised against zebrafish β-actin (29) and a donkey anti-rabbit IgG-HRP conjugate (GE Healthcare Life Sciences).

**Analysis of VHSV replication in IgM$^+$ cells**
To further determine whether VHSV replication was taking place inside IgM+ cells, splenocyte cultures were infected as described before. At 24 h post-infection, IgM+ and IgM− cells from control and infected cultures were sorted, washed twice with fresh medium and resuspended in 50 μl of medium. The cells were lysed by repeated freezing-thawing cycles and used to inoculate EPC cells, highly permissive to VHSV infection. Confluent EPC cultures in 24-well plates were infected with the cell lysates at 1:10, 1:100 and 1:1000 dilution in L-15 medium supplemented with 2.5% FBS and maintained at 14°C for 7 days. At this point cytopathic effects were visualized under the microscope.

NF-κB translocation to the nucleus

Trout splenocytes were isolated and seeded in complete MGFL-15 medium (MGFL-15 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, 10% newborn calf serum (Gibco) and 5% carp serum). Cells were treated for 24 h in the presence of Poly I:C (50 μg/ml). Following stimulation, cells were fixed in 1% formaldehyde, and washed twice in PBS with 2% calf serum and 0.1% saponin (permeabilization buffer). To determine nuclear translocation in IgM+ cells, splenocytes were stained with anti-p65 (Santa Cruz Biotechnology) and anti-trout IgM (1.14) for 30 min at 4 °C followed by 20 min at room temperature. Following the primary staining, cells were washed and stained with goat anti-rabbit APC (Jackson ImmunoResearch) and rabbit anti-mouse FITC (Jackson ImmunoResearch). Prior to acquisition, Hoechst33342 nuclear stain (Molecular Probes) was added as per manufacturer’s
recommendations. Data was collected on an ImageStream MKII and analyzed using IDEAS software (Amnis), as previously described (30).

MHC-II expression

The levels of MHC-II expression on the surface of IgM+ cells were measured via flow cytometry using a mAb against trout MHC-II (31, 32). Stimulated or unstimulated splenocytes were washed in Staining Buffer (PBS with 1% BSA and 0.02% sodium azide) and co-incubated with PE-labelled anti-trout IgM and the Alexa 647-labelled anti-MHC-II antibody for 30 min at 4°C protected from light. Finally cells were washed twice with the same buffer and analysed by flow cytometry (BD FACSCalibur, BD Biosciences). To check viability, the cells were then stained with 4 μg/ml 7-aminoactinomycin D (BD) at 4°C for 15 min and analyzed in a FACSCalibur with excitation wavelength at 488 nm and emission at 650 nm. The dye is excluded from live cells whereas dead cells are stained. Cell viability in our cultures was always higher than 95%. Dead cells were excluded from the analysis.

B cell proliferation

The BrdU Flow Kit (Becton Dickinson) was used to measure the specific proliferation of IgM+ cells following manufacturer’s instructions. Splenocytes at a concentration of 2 x 10^6 cells per ml were incubated for 3 days at 20 °C with different stimuli (Poly I:C, CpG, a combination of Poly I:C and CpG, VHSV or LPS) as described above. Bromodeoxyuridine (BrdU, 10 μM) was then added to the cultures and
cells were incubated for an additional 24 h. After that time, trout cells were collected and stained with anti-IgM-PE (1.14) antibody and then fixed and permeabilized with Cytofix/Cytoperm Buffer for 15 min on ice. Afterwards, cells were incubated with Cytoperm Permeabilization Buffer Plus for 10 min on ice and re-fixed with Cytofix/Cytoperm Buffer during 5 min at room temperature. Cells were then incubated with DNase (30 µg/10⁶ cells) for 1 h at 37 ºC to expose the incorporated BrdU. Finally, they were stained with FITC anti-BrdU antibody for 20 min at room temperature and analysed by flow cytometry (BD FACSCalibur, BD Biosciences).

Statistics

Statistical analyses were performed using a two-tailed paired Student's t test with Welch's correction when the F test indicated that the variances of both groups differed significantly using the GraphPad Prism 4 software and the GraphPad Prism 6 software in the case of NF-κB translocation experiments. The differences between the mean values were considered significant when P<0.05.

Results

VHSV infects rainbow trout spleen IgM⁺ cells
Firstly, we studied if VHSV was capable of replicating in trout splenic IgM+ cells. For this, spleen leukocytes were infected in vitro with VHSV and after 24 h, IgM+ cells were FACS sorted and the transcription of viral genes and the viral protein expression detected. We could detect transcription of VHSV genes in sorted IgM+ cells from samples incubated with active VHSV, but not on control or VHSV inactivated treated samples (Fig. 1A), indicating that VHSV can actively enter inside IgM+ cells and start viral gene transcription. On the other hand, viral N protein was not detected by Western blot in splenic IgM+ cells after 24 h of infection (Fig. 1B) or later times post-infection (data not shown). Despite these results, when lysates from IgM+ cells obtained from infected cultures were inoculated into the permissive EPC cell line, a clear cytopathic effect was visible at day 7 post-inoculation, demonstrating that the virus remained viable inside IgM+ cells, even though the viral titer inside the cells did not increase with time (data not shown). Nevertheless, our results demonstrate that even if VHSV has the capacity to infect trout B lymphocytes, an active replication is not taking place within IgM+ cells.

VHSV activates TLR3 transcription in spleen IgM+ cells

Having established that spleen IgM+ cells could be infected by VHSV, we wanted to assess if these cells were responsive to VHSV. Cells stimulated with the TLR3 agonist, Poly I:C or with CpG were included for comparative reasons, together with non-stimulated controls. After 1 h, 24 h and 48 h, IgM+ cells were FACS-sorted and used for RNA extraction and evaluation of gene transcription through real time PCR.
First, we analyzed the response of trout TLRs which had been previously suggested to be implicated in nucleic acid recognition, namely TLR3, 7, 8, 9 and 22 (33, 34). We observed that TLR3 was significantly up-regulated in response to Poly I:C and VHSV after 24 and 48 h of incubation (Fig. 2), whereas no significant changes were observed in the transcription of the other TLRs analyzed. Surprisingly, CpG stimulation also resulted in significant up-regulation of TLR3 transcription in IgM+ cells. Similar results were observed when IgM+ cells were sorted prior to stimulation, however, cell viability was largely decreased when the experiment was conducted following this protocol due to the activation of cells throughout the sorting procedure by antibody engagement of the BCR (data not shown).

Finally, we also studied the effects of Poly I:C and VHSV on the levels of transcription of two retinoic acid-inducible gene I (RIG-I)-like receptors known to sense viral RNA in the cytoplasm, namely MDA5 (Melanoma Differentiation Associated protein 5) and LPG2A (Laboratory of Genetics and Physiology 2A). Only after 48 h of stimulation with Poly I:C, we could detect a significant increase in the levels of transcription of MDA5 in IgM+ cells (data not shown). VHSV, on the other hand, had no effect on the transcription levels of these cytoplasmic sensors in IgM+ cells. Although TLRs and other PRRs may be implicated in viral sensing in teleost B cells without a requirement for this transcriptional modulation, our results placed particular emphasis on TLR3 as a potential regulator of viral recognition by teleost B cells.

\textit{VHSV activates IFN1 and Mx transcription in spleen IgM+ cells}
We also studied the effect of VHSV, Poly I:C and CpG on genes known to be regulated through the activation of TLR3 (35). We focused on IFN1 and IFN2, type I IFN molecules because of their antiviral activity (36), as well as Mx, an IFN-responsive gene (36) and the CK5B chemokine, a likely trout homologue of mammalian CCL5 (37). We focused on this chemokine because a previous proteomic analysis performed by our group showed that CK5B was one of the main proteins produced by IgM+ cells (data not shown). After 1 h, 24 h and 48 h of incubation with the different stimuli, splenic IgM+ cells were FACS-sorted and the transcription of these genes studied through real-time PCR. A significant up-regulation of IFN1 transcription was observed in IgM+ cells from cultures stimulated with Poly I:C after 1 or 24 h of incubation or with VHSV at all the time points studied (Fig. 3). A significantly higher Mx transcription was also observed in spleen IgM+ cells from cultures incubated with VHSV after 24 h in comparison with IgM+ cells from unstimulated cultures (Fig. 3). Additionally, a 48 h incubation with either Poly I:C or VHSV resulted in significant up-regulation of CK5B transcription levels in IgM+ B cells (Fig. 3). Finally, CpG stimulation induced the up-regulation of IFN1 at 48 h post-stimulation and of CK5B transcription at both 24 h and 48 h post-stimulation. These results reveal that VHSV and TLR agonists such as Poly I:C or CpG stimulate IgM+ cells to produce type I IFN and the CK5B chemokine.

*TLR3 and IFN1 transcription in response to VHSV on IgM+ cells is mediated by an endosomal TLR*
To study whether the induction of TLR3, IFN1 and CK5B observed in IgM+ cells in response to VHSV or Poly I:C was in fact mediated by the activation of an intracellular TLR we used bafilomycin A1 (BAF), a highly specific inhibitor of vacuolar type H+-ATPase. Inhibitors of endosomal acidification abolish signaling of all those TLRs located in endosomes, including TLR3-dependent responses (38). We found that pre-incubation of splenocytes with BAF significantly reduced the up-regulation of both TLR3 and IFN1 transcription observed in IgM+ cells in response to either Poly I:C or VHSV, when compared to the expression levels in cells stimulated in the absence of the inhibitor BAF (Fig. 4). These results strongly suggest that an intracellular TLR, possibly TLR3, mediates the IFN1 response in trout splenic IgM+ cells. Nevertheless, no changes were found in CK5B mRNA levels when cells were pre-incubated with BAF and the appropriate stimuli, suggesting that the up-regulation of CK5B observed after VHSV and Poly I:C incubation is not a direct consequence of intracellular TLR activation.

Blood IgM+ cells are also responsive to VHSV and Poly I:C

Having shown that spleen IgM+ cells transcribe TLR3, IFN1 and CK5B in response to VHSV and knowing that blood IgM+ cells also express TLR3 constitutively (16), we wondered whether blood B cells were similarly responsive. We first examined TLR3, IFN1, Mx and CK5B transcription on IgM+ cells from Poly I:C or VHSV stimulated and control blood cultures after 24 h of incubation. We observed that blood IgM+ cells significantly increased TLR3, IFN1 and CK5B mRNA levels after
stimulation with either Poly I:C or VHSV (Fig. 5A). There was also a significant up-regulation of Mx in the case of Poly I:C-treated cells.

Subsequently we assessed whether the induction of TLR3, IFN1 and CK5B observed in blood IgM+ populations was mediated by intracellular TLR activation using BAF. The results showed that, in this case, BAF was only capable of significantly down-regulating the TLR3 transcription induced by VHSV and not that induced by Poly I:C (Fig. 5B). Consistent with our observations in splenic IgM+ cells, BAF significantly reduced the IFN1 transcription elicited in blood IgM+ cells by both Poly I:C and VHSV (Fig. 5B). On the other hand, unlike spleen IgM+ cells, blood IgM+ cells, up-regulated CK5B transcription in response to Poly I:C in a BAF-sensitive fashion (Fig. 5B). These data indicate that although both spleen and blood IgM+ cells are highly responsive to VHSV and Poly I:C, there are some differences in Poly I:C signaling between the two populations.

The effect of VHSV on IgM+ cells is dependent on viral transcription

Because we have demonstrated that VHSV has the capacity to infect spleen IgM+ cells, starting viral transcription within the cells, we next studied whether this viral transcription was necessary for the activation of the TLR3, IFN1 and CK5B transcription detected. We demonstrated that the induction of all three genes observed in response to VHSV in sorted IgM+ cells was dependent on virus viability, because when heat-inactivated VHSV (iVHSV) was used, TLR3, IFN1 and CK5B transcription levels were significantly lower than those obtained with live virus (Fig. 6A). These results seem to indicate that VHSV does not exert its transcriptional effects on IgM+ cells
through cross-linking of the B cell receptor (BCR). Furthermore, additional studies revealed that cross-linking of the BCR through incubation with anti-IgM antibody provoked a down-regulation of TLR3 transcription in IgM+ cells and no significant effects on IFN1 and CK5B transcription (Fig. 6B). Altogether our results strongly suggest that the effects on the IFN and CK5B transcriptional responses elicited by VHSV in IgM+ B cells are dependent on viral transcription since, as described above, we verified that no viral transcription was detected inside IgM+ cells treated with the inactivated virus (Fig. 1A).

**VHSV activates NF-κB in spleen IgM+ cells**

To study whether viral TLR agonists activate the transcription factor NF-κB in IgM+ B cells, we undertook two different approaches. First, we analyzed the transcription of IκBα and IL-1β in response to the different stimuli. IκBα is a member of the family of NF-κB inhibitors, previously shown to be up-regulated in fish when NF-κB activation occurs (39), whereas IL-1β is a pro-inflammatory cytokine highly responsive to NF-κB activation (40). We observed a significant up-regulation of IκBα in sorted IgM+ cells from VHSV-treated cultures after 24 and 48 h of stimulation and in Poly I:C-treated cells at 24 h (Fig. 7A). Concerning IL-1β, only VHSV was capable of significantly up-regulating its transcription levels after 48 h of incubation (Fig. 7A). Additionally, we analyzed whether a dsRNA such as Poly I:C promoted NF-κB translocation to the nucleus of IgM+ cells using an anti-NF-κB antibody and a quantitative imaging flow cytometry approach. Through this methodology, translocation of NF-κB to the nucleus of B cells in response to dsRNA was confirmed (Fig. 7B).
Altogether, our results strongly support the hypothesis that VHSV infection in the spleen leads to NF-κB activation in IgM+ B cells, being this possibility the mechanism through which IFN1 production is elicited.

VHSV activates IgM+ cells to an antigen presenting cell (APC) profile

In relation to their role as antigen presenting cells (APCs), it has been previously shown that different TLR agonists including Poly I:C were capable of up-regulating MHC-II expression in murine B cells (41). Thus, we examined whether the same was true for teleost B cells infected with VHSV. We evaluated MHC-II surface expression through flow cytometry using an anti-trout MHC-II. For this, splenocyte cultures treated for 48 h with Poly I:C, VHSV or untreated controls were co-stained with anti-trout MHC-II and anti-trout IgM mAbs. We observed a significant increase on MHC-II cell surface expression on VHSV-treated IgM+ cells when compared with control cells but not in Poly I:C-treated cultures (Fig. 8A). This up-regulation was not detected in IgM cells. Additionally, we evaluated the effect of VHSV on the levels of transcription of MHC-II together with that of CD80/86, CD83 and CD40, co-stimulatory molecules involved in antigen presentation. After 24 h of infection, VHSV provoked a significant up-regulation of CD80/86 and CD83 mRNA levels in IgM+ B lymphocytes that was not visible in the IgM- cell fraction (Fig. 8B). These results strongly suggest that spleen IgM+ cells act as APCs during the course of a VHSV infection.

Effect of VHSV on IgM+ cell proliferation
It is well known that LPS stimulation can induce B cell proliferation in fish (17), thus we assessed whether VHSV or the different TLR agonists could also induce IgM⁺ proliferation on their own. For this, we incubated splenocytes with Poly I:C, CpG, Poly I:C in combination with CpG, VHSV or LPS (as a positive control) for 4 days, having added Bromodeoxyuridine (BrdU) during the last 24 h of culture. We then measured the proliferative response of IgM⁺ cells, co-staining with anti-IgM and anti-BrdU mAbs in order to detect cell proliferation specifically in IgM⁺ populations. Results showed that IgM⁺ cells significantly proliferated in response to LPS and also in response to the combination of Poly I:C and CpG, but not when the stimuli were added independently (Fig. 9A, B), suggesting that in some cases two independent signals are necessary for direct effect on proliferation of unprimed trout IgM⁺ cells. On the other hand, some degree of IgM⁺ proliferation was observed in some individuals in response to VHSV, with overall differences not statistically significant (p=0.08). Despite these moderate results in the number of proliferating cells, we consistently detected an increase in the total number of IgM⁺ cells in VHSV-infected cultures after 24 h of incubation compared to control cultures, indicating that the virus provoked an increased survival of IgM⁺ cells (Fig. 9C, D).

Discussion
Despite recent evidence in mammals for important contribution of B cell TLR signaling to antibody production and antigen presentation (2, 41), there is limited information available on TLR signaling in teleost B lymphocytes. Our data indicate that VHSV, a natural pathogen for rainbow trout, can enter B lymphocytes where it provokes multiple immediate effects on these cells, conditioning B cell functionality. VHSV could effectively transcribe viral genes inside IgM+ B cells, but viral translation seemed interrupted as we could not detect viral proteins inside the cells through Western blot, despite the fact that the presence of infective viral particles inside IgM+ B cells was confirmed. Live rabies vaccines have also been shown to infect murine and human B lymphocytes (22). In that case, even though viral proteins could be detected inside B lymphocytes, the infection also turned out as not productive and no viral particles were released into the supernatants. Similarly, murine dendritic cells are also infected by rabies virus, but the infection is suppressed inside these cells, rendering it non-productive (42). Therefore, it seems that rhabdoviruses have the capacity of infecting several immune cells types, affecting for sure their functionality.

To date, eight TLRs have been identified in rainbow trout, namely TLR1, 2, 3, 5, 7, 8, 9 and 22 (33), among which, TLR3, 7, 8 and 22 have been suggested as viral sensing TLRs (34). TLR3 recognizes dsRNA and does not signal through the adaptor molecule MyD88 commonly used by other TLRs, but through the alternative adaptor molecule TRIF (Toll/IL-1R domain-containing adaptor inducing IFN-β) (43). While TLR3 is mostly intracellular, TLR22, a fish-specific TLR identified in several species (33, 34, 44), is expressed on the cell surface where it also senses dsRNA. As for TLR3, TLR22 viral sensing capacities were indirectly demonstrated through transcriptional studies (44-46), and direct evidence of its capacity to recognize long dsRNAs from the cell surface was also obtained in fugu (47). In mammals, TLR7 and TLR8 are activated...
by synthetic antiviral imidazoquinoline compounds and are implicated in the recognition of ssRNA, but in fish no direct evidence for ligand specificity has been provided to date and some contradictory results have been obtained regarding their transcriptional regulation in response to viruses and imidazoquinoline compounds (34).

In our studies, only TLR3 transcription was affected by VHSV infection in teleost IgM+ cells, as it was in response to Poly I:C, a well-known TLR3 agonist. Despite the fact that none of these other TLRs were transcriptionally regulated in IgM+ B cells in response to neither VHSV nor any of the other TLR agonist used, we cannot rule out their implication in viral sensing in teleost B lymphocytes, exclusively on the basis of undetected transcriptional regulation. However, our results do suggest that, on the contrary of what occurs in mammalian B cells, TLR3 is an important mediator of viral sensing in teleost B cells. In mammals, two independent studies determined that whereas murine follicular B cells, B1 cells and Peyer’s patches B cells showed very low levels of TLR3, marginal zone B cells transcribed TLR3 at very high levels (9, 48). In humans, TLR3 is present in CD138+ plasma cells (11), but not in naïve nor on memory B cells (10 4587, 11, 49). Thus, either human B cells sense dsRNA through an alternative mechanism or the engagement of TLR3 in DCs is enough to compensate for the lack of this receptor in B cells. Interestingly, a specific subset of mucosal B cells in the upper respiratory tract expressing TLR3 and responding to viral dsRNA was identified in humans (50). It was speculated that this B cell population contributed to the innate resistance against certain respiratory viruses.

In the current work, we have also demonstrated that trout spleen and blood IgM+ cells respond to Poly I:C and VHSV increasing the transcription of type I IFN. In mammals, there are only a few reports of IFN production by B cells, since previous studies had pointed to DCs as the main IFN-producer leukocytes (51, 52). In both
spleen and blood IgM+ cells, this up-regulation of IFN1 transcription was significantly reduced by BAF, revealing the implication of a receptor located in the endosomal compartment. The fact that mammalian endosomal TLR3 is known to induce the production of type I interferons (IFNs) (53), and that it was transcriptionally regulated in our studies, strongly suggest that IFN induction in trout IgM+ cells is a consequence of TLR3 signaling. However, we cannot rule out the implication of other endosomal TLRs, such as TLR7 and TLR8 in VHSV detection (54), because studies performed with mammalian rhabdovirus such as vesicular stomatitis virus (VSV) have pointed to either TLR7 (55) or TLR13 (56) as the receptors responsible for sensing the virus. Interestingly, how BAF affected the transcriptional regulation of TLR3 and CK5B in response to Poly I:C differed in blood and spleen IgM+ populations, revealing slight differences in how these two populations sense dsRNA. These differences might be a consequence of different stages of activation / differentiation between IgM+ cells in blood and spleen.

Once this type I IFN has been produced by spleen IgM+ cells, in addition to its demonstrated direct antiviral activity (28), it will surely have important consequences on both the innate and acquired regulation of other cell types and on IgM+ cells themselves, such as those previously reported in mammalian models (57). For example, in mammals, IFN drives isotype switching to the IgG2 isotype (58), the antibody isotype predominantly elicited by viral infections (59), demonstrating that PAMPs polarize the antibody response to that best suited to deal with a specific pathogen (60). Additionally, IFN-α/β has shown to directly up-regulate CD69, CD86 and CD25 molecules in B cells (61, 62), increase their survival, and lower their threshold to further activation through the BCR with effects on calcium fluxes, IgM internalization, induction of activation markers and proliferation (61). Therefore, it could be possible
that some of the effects that VHSV provokes on B cells, such as an increased survival and the activation of co-stimulatory molecules are a consequence of the type I IFN elicited. Furthermore, type I IFN has shown to precipitate autoantibody production through a polyclonal activation of auto-reactive B cells (63), such as that observed in patients with systemic lupus erythematosus (64). On the other hand, it seems that the IFN1 secreted by trout IgM+ cells does not trigger a strong antiviral state in IgM+ cells, because Mx was poorly induced in comparison to IFN1, thus it might be possible that the effects that IFN1 has on a non-immune cell are directed towards an antiviral activation, whereas the effects on B cells are directed towards activation of specific B cell functions.

Interestingly, TLR3 and IFN1 up-regulation was also detected when trout IgM+ cells were treated with CpG, even though CpG is thought to be an agonist for TLR9 and its detection by TLR3 has not been reported in any species. Since TLR3 transcription is enhanced by LPS-induced autocrine IFN-β in murine macrophages (65), it could be possible that it is the IFN produced after CpG stimulation (significantly increased at 48 h) by IgM+ cells and/or by other immune cells from the spleen the responsible for the up-regulation of TLR3. Type I IFN production in response to CpG was already demonstrated in murine B cells through an IRF3-dependent mechanism (52).

The CK5B chemokine was also induced in spleen and blood IgM+ cells in response to Poly I:C or VHSV infection. Despite the difficulty of ascribing true orthologues among fish CC chemokines and their mammalian counterparts, a recent CC chemokine classification grouped rainbow trout CK5B with mammalian CCL5/RANTES genes (66). Although the specific cell targets for CK5B have not been yet described, interestingly, mammalian CCL5 is associated with the recruitment of T helper cells (67). Therefore it seems probable that trout IgM+ cells produce this
chemokine in response to viral infections to recruit T helper cells and present the virus to the recruited cells. This hypothesis also correlates with the activation of B cells towards an APC profile observed in response to VHSV infection. We demonstrated that VHSV up-regulated MHC-II surface expression in IgM+ cells, even though transcriptional regulation was not detected. Even though these results seemed surprising, in mammals it has been shown that B cells regulate MHC-II at a translational level (68). Additionally, VHSV up-regulated the levels of transcription of two co-stimulatory molecules, CD83 and CD80/86. This last molecule with similar homologies to mammalian CD80 and CD86 was consequently designated as CD80/86 and has shown to increase interleukin 2 (IL-2) expression (69). All these results point to an important role of trout B cells in VHSV presentation. The recent discovery of the phagocytic capacity of B cells led to the identification of an important role in antigen presentation of particulate antigens by teleost B lymphocytes (70, 71). Recently, an additional role of teleost B lymphocytes in the presentation of soluble antigens was revealed in zebrafish (71) and is now supported by our results.

On the other hand, as occurs in mammalian TLR3-bearing B cell populations (9, 48), the viral TLR agonists were not capable of significantly inducing a B-specific cell proliferation on their own. In the human population of TLR3-expressing B cells found in the upper respiratory tract, Poly I:C alone induced very little proliferation, whereas it was in combination with BAFF that significant proliferation was achieved (50). Similarly, Poly I:C had a synergistic effect on the CpG-induced proliferation, suggesting that TLR3 signaling renders the cells more sensitive to a further stimulation. In our experiments, VHSV up-regulated IgM+ cell proliferation in some animals, but this was not consistent and the differences were only marginally significant. However, a
consistent effect on IgM$^+$ cell survival was observed, maybe as an indirect consequence of IFN production.

In conclusion, we have demonstrated that trout splenic IgM$^+$ cells can be infected by VHSV, even though the infection is not productive. This early interaction of the virus with B lymphocytes activates TLR3 and IFN1 transcription, the later through a mechanism residing on an endosomal receptor, probably TLR3. Additionally, VHSV activated NF-κB and up-regulated the transcription of CK5B, a potential homologue of CCL5, a mammalian chemokine associated with the recruitment of T helper cells. Accordingly, VHSV activated IgM$^+$ cells towards an APC profile increasing MHC-II expression on the cell surface and the transcription of co-stimulatory molecules. Our findings provide new evidence that B cells are highly responsive to viral stimuli, suggesting an important role of teleost B cell antigen presentation in rhabdovirus infections.
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**FIG. 1.** VHSV replication in trout spleen IgM+ cells. A. Transcription of VHSV N gene was assessed by real-time PCR in sorted IgM+ cells after 24 h of viral inoculation. Mean from 6 independent experiments is shown. B. VHSV N protein expression in IgM+ and IgM- sorted cells previously incubated with the virus during 24 h was evaluated by Western blot. β-actin was included as loading control.

**FIG 2.** Effect of TLR agonists on the transcription of TLR genes in sorted spleen IgM+ cells. Total spleen leukocyte populations were stimulated with Poly I:C (50 µg/ml), CpG (5 µg/ml) or VHSV (1 x 10^6 TCID₅₀ ml⁻¹) and incubated 1 h, 24 h or 48 h at 20°C. Non-stimulated controls were also included. After the different incubation periods, IgM+ B cells were sorted using an anti-trout IgM mAb. RNA was extracted from sorted IgM+ cells and the levels of transcription of TLR3, TLR7, TLR8a2, TLR9 and TLR22 evaluated through real time PCR in duplicate. Data from 7 independent fish are shown as the mean gene expression relative to the expression of an endogenous control (EF-1α) ± SD. Asterisks denote significant differences between cells treated with TLR agonist and their corresponding control. * p < 0.05; ** p < 0.01; *** p < 0.001.

**FIG 3.** Effect of TLR agonists on the transcription of immune genes related to TLR3 activation in sorted spleen IgM+ cells. Total spleen leukocytes were treated as described in the legend of Fig. 1. RNA was extracted from sorted IgM+ cells and the levels of transcription of IFN1, IFN2, Mx and CK5B evaluated through real time PCR in
duplicate. Data from 7 independent fish are shown as the mean gene expression relative to the expression of an endogenous control (EF-1α) ± SD. Asterisks denote significant differences between cells treated with TLR agonists and their corresponding control. *p < 0.05; **p < 0.01.

**FIG 4.** BAF down-regulates Poly I:C and VHSV-induced TLR3 and IFN1 transcription on IgM⁺ cells. Spleen leukocyte suspensions were treated with BAF (50 nM) 1 h prior to the addition of Poly I:C or VHSV. Control wells without BAF were included for all conditions. After the TLR agonists were added, the cells were incubated for 24 h (for TLR3 and IFN1) or 48 h (for CK5B) at 20ºC and IgM⁺ cells were sorted. The transcription of TLR3, IFN1 and CK5B was evaluated in sorted cells through real time PCR. Data from 5 independent experiments are shown as the mean gene expression relative to the expression of an endogenous control (EF-1α) ± SD. *p < 0.05.

**FIG 5.** Blood IgM⁺ cells are also responsive to VHSV and Poly I:C. A. Leukocyte cultures from blood were incubated with Poly I:C (50 µg/ml) or VHSV (1 x 10⁶ TCID₅₀/ml) for 24 h at 20ºC. IgM⁺ cells were then sorted and the levels of expression of TLR3, IFN1, Mx and CK5B evaluated in sorted IgM⁺ cells through real time PCR. Data are shown as the mean gene expression relative to the expression of an endogenous control (EF-1α) ± SD (n=4-8). B. In some experiments, the TLR3 inhibitor BAF was added 1 h prior to the addition of the stimuli. *p < 0.05; **p < 0.01; ***p < 0.001.

**FIG 6.** The effect of VHSV on IgM⁺ cells is dependent on viral transcription. A. Spleen leukocytes were treated with VHSV or heat-inactivated VHSV (VHSVᵉ) or with media
alone in the case of controls. After 24 h of incubation at 20ºC, IgM+ cells were sorted and the transcription of TLR3, IFN1 and CK5B evaluated through real time PCR. Data from 4-6 independent experiments are shown as the fold change relative to unstimulated controls ± SD. * p < 0.05. B. Effect of anti-IgM on the transcription of TLR3, IFN1 and CK5B in sorted spleen IgM+ after 24 h of stimulation. Data are shown as the mean gene expression relative to the expression of an endogenous control (EF-1α) ± SD in 7 independent experiments. * p < 0.05.

FIG 7. VHSV and Poly I:C activate NF-κB in IgM+ B cells. A. Splenocytes were stimulated as described in the legend of Fig. 1. IgM+ B cells were then sorted and RNA extracted for the evaluation of IκBα and IL-1β transcription levels by real time PCR (n=7). Data are shown as the mean gene expression relative to the expression of an endogenous control (EF-1α) ± SD. * p < 0.05. B. Translocation of NF-κB to the nucleus in trout IgM+ splenocytes following Poly I:C stimulation. Trout splenocytes were stimulated with Poly I:C or with media alone for 24 h at 20ºC. NF-κB translocation was determined in IgM+ cells following staining with anti-p65 and anti-trout IgM mAbs. Hoechst stain was used to visualize cell nuclei. The percentages of NF-κB translocation in trout IgM+ cells are represented together with representative images of positive nuclear translocation (Translocated) and negative translocation (Non-translocated) based on an ImageStream MkII imaging flow cytometer (Amnis). Data was collected from five different trout (n=5) over three independent experiments. ** p < 0.05 are based on comparisons with media alone controls.
**FIG 8.** VHSV activates IgM$^+$ cells to an antigen-presenting phenotype. A. MHC-II cell surface expression on IgM$^+$ and IgM$^-$ cells after 48 h of incubation with Poly I:C or VHSV. Data are shown as the mean fluorescence intensity (MFI) ± SD in 5 independent experiments. * P < 0.05. Representative histograms are shown below for both IgM$^+$ and IgM$^-$ cells. B. Splenocytes were stimulated as described in the legend of Fig. 1. IgM$^+$ and IgM$^-$ cells were sorted after 24 h of incubation and RNA extracted for the evaluation of MHC-II, CD80/86, CD83 and CD40 transcription levels by real time PCR (n=5-7). Data are shown as the mean gene expression relative to the expression of an endogenous control (EF-1α) ± SD. * P < 0.05.

**FIG 9.** Effect of VHSV on IgM$^+$ cell proliferation and survival. Spleen leukocytes were incubated with Poly I:C (50 µg/ml), CpG (5 µg/ml), Poly I:C combined with CpG, VHSV (1 x 10$^6$ TCID$_{50}$/ml) or LPS (100 µg/ml) for 3 days at 20°C. After this time, cells were labeled with BrdU and incubated for a further 24 h. The percentage of proliferating (BrdU positive) IgM$^+$ cells was then determined as described in the Materials and Methods section. A. Data are shown as the percentage of proliferating IgM$^+$ cells out of all IgM$^+$ cells. Circles indicate percentages observed in individual fish, while black bars represent mean values in each group. Significant differences between cells treated with TLR agonist and control cells. * p < 0.05; ** p < 0.01. B. Dot plots from a representative fish are shown for each condition.

C. D. Spleen leukocytes were infected *in vitro* with VHSV (1 x 10$^6$ TCID$_{50}$/ml) and after 48 h the number of IgM$^+$ cells in the cultures determined through flow cytometry. Data are shown as the percentage of IgM$^+$ cells in control and infected cultures from 5 individual trout, along with dot plots from a representative trout.
Table 1. Primers used in this study

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Fig. 1

A  

B  

Relative N gene transcription

Control  VHSV  VHSV

0.0001

0.001

0.0001

VHSV N

β-actin

50 KDa

42 KDa

on July 14, 2017 by guest http://jvi.asm.org/ Downloaded from
Fig. 2

Graphs showing relative expression of TLR3, TLR7, TLR8, TLR9, and TLR22 at 1 h, 24 h, and 48 h post-infection. TLR3 expression is significantly increased at 48 h post-infection compared to other time points. TLR7 and TLR8 expression remains relatively stable across time points. TLR9 expression is slightly increased at 24 h post-infection. TLR22 expression shows significant increase at 24 h and 48 h post-infection.

Significance levels: *** p < 0.0001, ** p < 0.01, * p < 0.05.
Fig. 3

![Graph showing relative expression of IFN1, IFN2, Mx, and CK5B. The x-axis represents time (1 h, 24 h, 48 h), and the y-axis represents relative expression levels. The legend indicates different treatments and time points.](image-url)
Fig. 4
Fig. 5

A

B
Fig. 6

A

B

TLR3 IFN1 CK5B

Fold change

Control VHSV VHSVi

*  *

0.001 0.01 0.1

Relative expression

Control Anti-IgM

*
Fig. 7

A

Relative expression

IL-1β

Lc-β

Translocated
Non-translocated

IgM-FITC Hoechst NF-κB-APC Brightfield Overlay

B

% NF-κB Translocation

Translocated
Non-translocated

Brightfield
Hoechst NF-κB-APC Overlay
Fig. 8

A

B

IgM+ cells

IgM- cells

MHC-II medians

MHC-II medians

Counts

Counts

Relative expression

Relative expression

Control Poly I:C VHSV

Control Poly I:C VHSV

IgM+ cells

IgM- cells

MHC-II CD86/CD80 CD83 CD40

MHC-II CD86/CD80 CD83 CD40

0.001 0.01 0.1 1

0.001 0.01 0.1 1

Control VHSV

Control VHSV
Fig. 9

A

\[ \% \text{BrdU}^+\text{IgM}^+ \text{Cells} \]

\* \( p=0.04 \)

\** \( p=0.002 \)

\( p=0.07 \)

Control       Poly I:C    Poly I:C/CpG    VHSV    LPS

B

\[ \text{FSC} \quad \text{SSC} \quad \text{IgM} \]

\[ \alpha\text{-IgM} \quad \alpha\text{-BrdU} \]

\( 0.57 \quad 2.09 \quad 0.83 \)

\( 0.80 \quad 0.48 \quad 1.26 \)

C

\[ \% \text{IgM}^+ \text{Cells} \]

\[ \text{Control} \quad \text{VHSV} \]

\[ \text{trout 1} \quad \text{trout 2} \quad \text{trout 3} \quad \text{trout 4} \quad \text{trout 5} \]

D

\[ \text{SSC} \quad \text{FSC} \quad \text{IgM} \]

\[ \text{Control} \quad \text{VHSV} \]

\[ 30.2 \quad 30.4 \quad 30.8 \]

\[ 40.4 \quad 40.6 \]