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Credit authorship contribution statement

Valentina Wong-Benito: Methodology, Formal analysis, Writing – original draft. **Felipe Barraza**: Methodology, Formal analysis. **Agustín Trujillo-Imarai**: Methodology, Formal analysis. **Daniela Ruiz-Higgs**: Methodology, Formal analysis. **Ruth Montero**: Methodology, Formal analysis. **Ana María Sandino**: Conceptualization, Formal analysis. **Tiehui Wang**: Conceptualization, Methodology, Formal analysis. **Kevin Maisey**: Conceptualization, Methodology, Formal analysis. **Christopher J. Secombes**: Conceptualization, Formal research, Writing – original draft, Preparation, Supervision. **Mónica Imarai**: Conceptualization, Formal research, Writing – original draft, Preparation, Supervision.

Infectious pancreatic necrosis virus (IPNV) recombinant viral protein 1 (VP1) and VP2-Flagellin fusion protein elicit distinct expression profiles of cytokines involved in type 1, type *2*, and regulatory T cell response in rainbow trout (*Oncorhynchus mykiss*)

Valentina Wong-Benito[1*], Felipe Barraza[1*], Agustín Trujillo-Imarai[1*], Daniela Ruiz-Higgs[1*], Ruth Montero[2], Ana María Sandino[3], Tiehui Wang[4], Kevin Maisey[2], Christopher J. Secombes[4], and Mónica Imarai[1§]

*** Equally contributed**

1 Laboratorio de Inmunología. Centro de Biotecnología Acuícola, Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile, Alameda 3363, Santiago, Chile

2 Laboratorio de Inmunología Comparativa. Centro de Biotecnología Acuícola, Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile, Alameda 3363, Santiago, Chile

3 Laboratorio de Virología. Centro de Biotecnología Acuícola, Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile, Alameda 3363, Santiago, Chile

4 Scottish Fish Immunology Research Centre, School of Biological Sciences, University of Aberdeen, Aberdeen AB24 2TZ, United Kingdom

§ Corresponding author:
Mónica Imarai: monica.imarai@usach.cl

Authors e-mail address:
Valentina Wong: valentina.wong@usach.cl
Felipe Barraza: felipe.barraza@usach.cl
Agustín Trujillo: atrujillo@veterinaria.uchile.cl
Daniela Ruiz-Higgs: daniela.ruiz@usach.cl
Ruth Montero: ruth.montero.m@gmail.com
Ana María Sandino: ana.sandino@usach.cl
Tiehui Wang: t.h.wang@abdn.ac.uk
Kevin Maisey: kevin.maisey@usach.cl
Christopher J. Secombes: christopher.secombes@abdn.ac.uk

ABSTRACT

In this study, we examined the cytokine immune response against two proteins of infectious pancreatic necrosis virus (IPNV) in rainbow trout (*Oncorhynchus mykiss*), the virion-associated RNA polymerase VP1 and VP2-Flagellin (VP2-Flg) fusion protein. Since VP1 is not a structural protein, we hypothesize it can induce cellular immunity, an essential mechanism of the antiviral response. At the same time, the fusion construction VP2-Flg could be highly immunogenic due to the presence of the flagellin used as an adjuvant. Fish were immunized with the corresponding antigen in Montanide™, and the gene expression of a set of marker genes of Th1, Th2, and the immune regulatory response was quantified in the head kidney of immunized and control fish. Results indicate that VP1 induced upregulation of *ifn-γ, il-12p40c, il-4/13a, il-4/13b2, il-10a,* and *tgf-β1* in immunized fish. Expression of *il-2a* did not change in treated fish at the times tested. The antigen-dependent response was analysed by *in vitro* restimulation of head kidney leukocytes. In this assay, the group of cytokines upregulated after VP1-restimulation was consistent with those upregulated in the head kidney *in vivo*. Interestingly, VP1 induced *il-2a* expression after *in vitro* restimulation. The analysis of sorted lymphocytes showed that the increase of cytokines occurred in CD4-1^{+} T cells suggesting that Th differentiation happens in response to VP1. This is also consistent with the expression of *t-bet* and *gata3,* the master regulators for Th1/Th2 differentiation in the kidneys of immunized animals. A different cytokine expression profile was found after VP2-Flg administration, i.e., upregulation occurs for *ifn-γ, il-4/13a, il-10a,* and *tgf-β1,* while down-regulation was observed in *il-4/13b2* and *il-2a*. The cytokine response was due to flagellin; only the *il-2a* effect was dependent upon VP2 in the fusion protein. To the best of our knowledge this study reports for the first-time characteristics of the adaptive immune response induced in response to IPNV VP1 and the fusion protein VP2-Flg in fish. VP1 induces cytokines able to trigger the humoral and cell-mediated immune response in rainbow trout. The analysis of the fish response against VP2-Flg revealed the immunogenic properties of *Aeromonas salmonicida* flagellin, which can be further tested for adjuvanticity. The novel immunogenic effects of VP1 in rainbow trout open new opportunities for further IPNV vaccine development using this viral protein.

INTRODUCTION

Aquaculture is one of the most important sources of food, employment, and economic significance for millions of people worldwide FAO [1]. Unfortunately, aquaculture undergoes major problems regarding infectious diseases and outbreaks among reared fish, which can severely compromise fish welfare and cause significant economic losses. Vaccination has been routinely used for decades in aquaculture, being the most relevant prophylaxis method to control infectious diseases [2]. However, despite efforts in disease prevention, many fish species remain vulnerable to new and re-emerging diseases [3]. One of several viruses that infect farmed fish species is infectious pancreatic necrosis virus (IPNV), which causes infectious pancreatic necrosis (IPN), a highly contagious deadly disease [4]. IPNV affects salmonids [5] and many other fish species, including European barracuda (*Sphyraenas phyraena*), axillary seabream (*Pagellus acarne*), common two-banded seabream (*Diplodus vulgaris*), common pandora (*P. erythrinus*), Senegal seabream (*D. bellottii*), and surmullet (*Mullus surmuletus*) [6]. In salmonids, although the number of outbreaks has been reduced over the past decade due to the use of IPN-resistant broodfish genetically selected on the base of a major QTL marker [7, 8], the emergence of new variants and reports of massive outbreaks in farmed rainbow trout are evidence that IPNV will continue threatening the aquaculture industry [9].

The IPNV virus belongs to the genus Aquabirnavirus and the Birnaviridae family. The members of this family are characterized by having a double-stranded RNA (birnavirus) and a bisegmented genome (dsRNA), which is contained within a non-enveloped viral particle [10]. The first genomic segment, named A, contains two open reading frames. The first ORF, overlaps with the second ORF and codes for a non-structural protein called VP5 (17 kDa), which is detected only in infected cells [11]. The second ORF codes for a 106 kDa polyprotein (NH2-pV2-VP4-VP3-COOH) which, during the synthesis of viral proteins is co-translationally proteolyzed by the viral protease VP4 to produce the pVP2 precursor (62 kDa), VP4 (29 kDa) and VP3 (31 kDa). pVP2 is further processed, at its C-terminus, possibly by VP4 to produce VP2 (54 kDa). The VP2 protein assembles spontaneously to form the viral capsid [10, 12], whereas the VP3 protein corresponds to an internal protein of the viral particle [13]. The second genomic segment, named B, contains a single ORF that codes for the RNA-dependent RNA polymerase named VP1[14]. This protein is present in two forms: as a free polypeptide and as an associated protein to each genomic segment (VPg) at the 5' ends by a phosphodiester bond [4].

Several IPNV vaccines have been produced containing recombinant proteins [15], DNA [16] or based on viral-like particles (VLP) [17]. All these vaccines stimulate the immune system and induce the production of neutralizing antibodies [18]; however, IPNV vaccines still need important improvements as they are not protective enough to eliminate the virus [19] and none of them avoid outbreaks, which continue to appear even in QTL selected salmonids

[20]. The identification of highly immunogenic antigens within a given pathogen, such as IPNV, can be used to improve vaccine efficacy as they can induce protective immune responses. In this regard, and to the best of our knowledge, no previous studies have been done to evaluate immunogenicity or immune protection produced by VP1. Regarding the capsid protein VP2, using mouse monoclonal antibodies, it has been demonstrated that the central third of the protein contains several neutralization epitopes [21]. This is consistent with reports demonstrating that VP2 is immunogenic and can induce neutralizing antibodies [22, 23]. VP2 can confer different degrees of protection depending upon the system of antigen delivery and vaccine design [15, 23-28].

Usually, antigens selected for use in subunit vaccines are chosen because antibodies are produced against them, neutralizing the viral infection by interfering with virion binding to receptors, or by preventing uncoating of the genomes. These antigens are typically proteins of the viral capsid. In this study, we have studied the immune response against two proteins of infectious pancreatic necrosis virus in rainbow trout, the virion-associated RNA polymerase VP1 and VP2-Flagellin (VP2-Flg) a fusion protein made using *Aeromonas salmonicida* flagellin. Since VP1 is not a structural protein, we hypothesize that VP1 can induce cellular immunity, which is an essential aspect of the antiviral response, while the fusion construct using VP2-Flg may be highly immunogenic due to the presence of the flagellin protein previously used as adjuvant in fish [29]. To assess the T cell immune response against these IPNV protein antigens, we performed expression profiling of marker genes in head kidney leukocytes of immunised fish, analysed the response to *in vitro* restimulation of head kidney leukocytes isolated from *in vivo* primed and control fish and assessed the effect of immunization in CD4-1^{+} sorted cells by cytokine expression profiling. The genes measured were: (i) for the Th1 type response, the cytokine genes encoding IFN-gamma and IL-12 (*ifn-γ* and *il-12p40*), and one gene encoding the master transcription factor T-bet (*t-bet*); (ii) for the Th2 type response, two genes encoding IL-4/13 cytokines (*il-4/13a*, and *il-4/13b2*) and the master transcription factor GATA-3 (*gata3*), (iii) for the T regulatory type response, two genes encoding immunosuppressive cytokines (*il-10a,* and *tgf-β1*) and (iv) for T cell proliferation, the gene encoding IL-2 which is a T cell growth factor (*il-2).*

METHODS

Production and purification of VP1 recombinant protein. *Escherichia coli* BL21 (DE3) were transformed with pET21a/VP1 plasmid, which contains the viral VP1 coding sequence (Graham et.al 2011). Transformed bacteria were grown in Luria-Bertani medium and the expression of VP1 was induced with 1 mM isopropyl 1-thio-b-D-galactopyranoside (Bioline) for 3 h at 30 °C, added during the exponential growth of the bacteria. Bacterial pellets were obtained and resuspended in solubilization buffer containing 50 mM NaH_2PO_4 pH 8.0, 10 mM imidazole, 0.3 M NaCl, 0.1% v/v Nonidet-P40 and EDTA-free protease inhibitors (Roche). Bacteria were disrupted with an ultrasonic homogenizer (Omni Sonic Ruptor) at 4 °C with 10 pulses of 20 s and 12 W. The insoluble fraction was recovered by centrifugation at 6,000 *g* for 1 h, at 4 °C and washed with a buffer containing 50 mM NaH_2PO_4 pH 8.0, 25 mM imidazole, 1 M NaCl, 0.1% v/v Nonidet-P40 and 20% glycerol. The insoluble fraction was washed two more times with a 50 mM Tris buffer pH 8 containing 20 mM β-mercaptoethanol, 0.1% triton x-100, 1.2 mM sodium deoxycholate, 5% glycerol and 500 µM EDTA and a 50 mM Tris buffer pH 8 containing 20 mM β-mercaptoethanol and 5% glycerol. The pellet was then suspended in 5 mL of denaturing buffer (50 mM Tris pH 8.0, 20 mM β-mercaptoethanol, 5% glycerol, 6 M guanidinium chloride) and incubated at 4 °C for 16 h. The soluble fraction was recovered and dialyzed sequentially at 4 °C against 50 mM Tris buffer pH 8 containing 3M guanidinium chloride for 2 h, then against 50 mM Tris pH 8 containing 2M guanidinium chloride for 1 h, against 50 mM Tris buffer pH 8 containing 1M guanidinium chloride for 2 h, and finally, against PBS (138 mM NaCl, 3 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) pH 8 for 16 h at 4 °C. The recombinant protein was further purified in a preparative SDS-PAGE and the band recovered with elution buffer. The protein was quantified, aliquoted and stored with 20% glycerol at −40 °C until use.

Western blot. After purification, rVP1 was analysed by 10% polyacrylamide-SDS gel electrophoresis. Later, the protein was transferred to a nitrocellulose membrane using eBlot Protein Transfer kit (GenScript, New Jersey, USA). The nitrocellulose membrane was incubated with blocking solution (0.05% Tween-PBS and 5% skim milk) overnight at 4 ° C with shaking and then incubated with anti-His Tag antibody (1:3,000) (Sigma Aldrich, Darmstadt, Germany) or with an affinity-purified polyclonal antibody (Rabbit) (1:5,000) produced against the CSFDPKARPQTPRSP peptide of VP1 (GenScript HK Limited, Hong Kong), recently used to detect VP1 from a lysate of IPNV infected CHSE-214 [30]. After 1 h incubation at room temperature with shaking, the membrane was washed three times with 0.05% Tween-PBS, and then incubated with the secondary antibodies: anti-mouse IgG peroxidase antibody (1:10,000) (Invitrogen, USA) for labelling anti-His Tag antibody or anti-rabbit IgG peroxidase (1: 10,000) (KPL) when the anti-VP1 antibody was used. Finally, after washing three times with 0.05% Tween-PBS, the blot was developed using the Pierce ECL Western Blotting Substrate kit (Thermo Scientific).

Production of VP2, Flagellin (Flg) and VP2-Flg recombinant proteins. The pTri-Ex6 (Novagen) was used for the expression of *vp2* from IPNV, *flg* and the *vp2-flg* encoding the fusion protein. The Flg gene was engineered from *flagellin* of the Gram-negative fish bacterial pathogen *Aeromonas salmonicida*. The amino acid sequences were detailed in **supplementary Figure 1**. For high-efficient expression, the nucleic acid sequences were codon optimised for the expression in *E. coli* using GENEius program (Eurofins Genomics), and cloned as pTRI-VP2, pTRI-Flg and pTRI-VP2-SF. After sequencing confirmation, the plasmids were used for transformation of BL21 Star (DE3) competent cells (Invitrogen). Induction of recombinant protein production, purification of VP2, Flg and VP2-Flg under denaturing conditions, refolding, re-purification under native conditions, SDS-PAGE analysis of proteins and quantification of protein concentration were as described previously [31].

Fish and experimental groups. Rainbow trout (*Oncorhynchus mykiss*) of approximate 70-80 g, were obtained from a local fish farm (Piscicultura Río Blanco, Los Andes, Chile) and maintained in tanks in the Fish Experimental Facility of the Centro de Biotecnología Acuícola. Procedures developed for this study were approved by the Ethics Committee at the Universidad de Santiago de Chile. Fish were kept in freshwater at a biomass of 5.7 kg/m^3, at 11-12 °C and with continuous aeration and fed with commercial pellets (Golden Optima, Biomar, Chile) at 1% body weight/day. Fish were separated into 4 tanks according to the experimental groups (described later) and were acclimated for 2 weeks prior to treatment. During acclimatization and the experimental phases, pH (7–7.5), dissolved oxygen (8.9–9.5 mg O_2/L) and ammonia (<0.1 mg/L) were recorded daily. The experimental groups consisted in four experimental groups of fish (n=7 per group) injected with 100 µL of an intraperitoneal (ip) injection containing: 50 µg of recombinant VP1 and Montanide™ adjuvant solution ISA 763A VG (Montanide™, Seppic) (group 1), Montanide™ adjuvant alone (group 2), 50 µg of VP2-Flg and Montanide™ (group 3) and 50 µg of Flg and Montanide™ (group 4). Injections were repeated for two additional times at two-week intervals, as within 15 days post challenge, a significant decrease in specific antibodies occurs in stimulated fish [32]. On day 33, when immune cells are expected to be activated, fish were anaesthetised using benzocaine (Veterquímica, Santiago, Chile), killed and head kidneys removed for further analysis (**Figure 1**). A further independent experiment was performed with groups 1 and 2 to analyse additional genes (n=5 per group). Leukocytes isolated from the head kidney of these fish were used for the transcriptional expression analysis and for antigen-specific stimulation assay performed *in vitro*.

Leukocyte extraction from head kidney. Isolated head kidneys were disaggregated in L-15 medium using a syringe plunger. The cell suspension was filtered through a 70 µm strainer (BD Falcon) and then washed with L-15 medium by centrifugation for 5 min at 400 x*g*. To obtain the lymphoid population, red cell lysis was performed as described by Hu et al. [33].

Briefly, the supernatant was removed, and the pellet was resuspended in 1 mL PBS containing 2% Fetal Calf Serum (FCS) (IF media). Then, 8 mL of ice-cold MiliQ water wase added and gently mixed; after 15 s, 1 mL of 10X PBS was added and the cells resuspended to stop the lysis process. Cells were once again washed by centrifugation for 5 min at 400 x *g* and resuspended in IF media. All procedures were performed at 4°C. Cells were counted and cell viability determined using trypan blue.

RNA extraction and cDNA synthesis. Cell pellets were resuspended in 1 mL of TRIzol® Reagent (Ambion®, Life Technologies) and homogenized by passing the cells several times through the pipette tip. Total RNA was extracted according to the manufacturer´s protocol. The extracted RNA was resuspended in diethyl pyrocarbonate–treated water (Invitrogen) and quantified. RNA (2 µg) samples were treated with DNase I (AMPD1-1KT, Sigma) and cDNA synthesis was performed using reverse transcriptase Moloney murine leukemia virus (Sigma), oligo (dT) (Promega), and dNTPs (Promega) according to the manufacturer's instructions. RNA samples were kept at -80 °C and cDNA at -20 °C until use.

qPCR analysis. The real-time PCR reactions were performed in 96-well plates (Axygen) covered with optical caps (Axygen) in a Stratagene Mx3000P (Stratagene). PCR reaction efficiencies were determined by generating cDNA standard curves using serial dilutions (1:10) of a mix of total RNA isolated from head kidney and spleen of Atlantic salmon. Table I shows the target gene, accession number, primer sequencies and the calculated efficiencies. Elongation factor-1 alpha (*ef1α*) was used as an internal reference because it was stably expressed in the head kidney of the rainbow trout under the experimental conditions used here. Each reaction was carried out in 25 µL final volume containing 12.5 µL SensiMix SYBR Low-ROX master mix (23) (Bioline), 5 nM forward primers, 5 nM reverse primers, 8 µL ultrapure distilled water (Invitrogen), and 2 µL cDNA (diluted 1:10 for housekeeping gene). The cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 58-62 °C for 15-30 s, and 72 °C for 30 s (depending on the primer set). Data were analysed using MxPro quantitative PCR software (Agilent Technologies). Data were expressed as normalized expression, known as 2^-ddCt [34]. PCR product quality was monitored using post-PCR melt curve analysis.

Antigen stimulation assay. Head kidney cells were obtained from VP1-immunized and VP2-Flg-immunized rainbow trout. First, red cells were lysed as described above, and then 1 mL of kidney cells (1 x 10^6/mL in supplemented L-15 medium) of each fish were seeded into 6-well plates. Next, the corresponding antigens were added for lymphoid stimulation. Thus, 100 µL of L-15 RPMI containing 100 µg of rVP1 or rVP2 were added to cells of the VP1-immunized fish and of VP2-Flg-immunized fish, respectively. As a control, cells of both groups were incubated with supplemented RPMI alone (control). The plates were incubated at 15 ºC for 72 h. After incubation, cells were collected and centrifuged at 400 *g* for 8 min at 4 ºC. Total RNA was prepared for the analysis of gene expression by RT-PCR as described

above. CD4-1$^+$ T cells were also quantified after antigen stimulation using flow cytometry, as outlined below.

Flow cytometry. Isolated cells (5 x 10^5 cells per sample) were incubated in 400 µL IF for 30 min at 4 °C to block potential nonspecific binding sites. Then, 1 mL of IF was added, centrifuged at 400 *g* for 5 min at 4°C and the supernatant removed. For single staining, cells were resuspended in 200 µL IF containing rabbit polyclonal anti-trout CD4-1 antibody (1:1,000) [35] or mouse monoclonal anti-trout IgM (1.14 supernatant) [36], and were incubated for 30 min at 4°C. After washing with IF, cells were incubated with the corresponding secondary antibodies, i.e., Alexa Fluor® 647 Goat anti-rabbit IgG (1:800) and Alexa Fluor® 488 Donkey anti-mouse IgG (1:600), respectively (Thermo Scientific). For double staining, cells were first incubated with anti-trout CD4-1 for 30 min at 4°C, then washed and incubated with the secondary antibody Alexa Fluor® 647 Goat anti-rabbit IgG. After washing again, cells were incubated with anti-trout IgM (1.14) as above. Cells were then washed and incubated with Alexa Fluor® 488 Donkey anti-mouse IgG. Single and double staining samples were washed after the last incubation with the corresponding antibody and resuspended in 500 µL IF for fluorescence measurement in a FACSCanto II cytometer (Becton Dickinson). At least 10,000 events were recorded for each sample. Flow cytometry analyses routinely included cell viability staining for exclusion of dead cells using 1 µg/mL propidium iodide. The staining was detected in the PerCP channel. Leukocytes exhibited a characteristic distribution in forward (FSC-A) and side scatter (SSC-A) allowing the distinction between the lymphoid ($FSC^{low}SSC^{low}$) and the myeloid cell population ($FSC^{hi}SSC^{hi}$). Cells were analysed on a gate set for lymphocyte-sized cells, since some myeloid cells are also CD4-1$^+$ in rainbow trout [37].

Fluorescence-activated cell sorting (FACS). To obtain sorted CD4-1$^+$ T cells, four fish were i.p. injected with VP1 (50 µg) and Montanide™ and four more were injected with Montanide™ alone, as described above. For cell sorting, cells were isolated from head kidneys and resuspended with IF and quantified. Then 1x10^6 cells were incubated with the rabbit affinity purified anti-trout CD4-1 antibodies (1:100) [35], and mouse monoclonal anti-trout IgM supernatant (1.14) [36] to ensure the separated CD4-1$^+$ cells were IgM$^-$. Secondary antibodies used were Alexa Fluor® 647 Donkey anti-rabbit IgG and Alexa Fluor® 488 Goat anti-mouse IgG (Thermo Fisher Scientific), respectively. Stained cells were washed and resuspended in 500 µL IF. The CD4-1$^+$ T cells were separated using the BD FACSMelody™ Cell Sorter and harvested into tubes containing heat-inactivated FCS. Post-sort analysis was done to verify that the purity of the sorted cells was ≥95%, prior to use for gene expression profiling. The populations obtained by cell sorting were resuspended in TRIzol® and stored at -80°C until RNA extraction.

Statistical analysis. GraphPad Prism 9.0.2 for MacOSX was used for statistical procedures and graph drawing. Statistical analyses of gene expression were performed using the Mann-Whitney U test or one-way ANOVA followed by Tukey's post hoc test. $p < 0.05$ was considered statistically significant.

RESULTS

Recombinant VP1 and VP2 proteins Recombinant VP1 was prepared from *E. coli* BL21 (DE3) transformed with the plasmid pET21a/VP1. Cells were induced using IPTG, and as expected, a protein band of approximately 94 kDa was detected in the whole protein extract. The protein purification was performed under denaturing conditions with extensive washing of the insoluble fractions, using detergent-containing buffers to remove LPS. The purified band was analysed by SDS-PAGE (**Figure 2A)** and then examined by western blot using an anti His-tag antibody (not shown) and an anti VP1 polyclonal antibody raised against a 15 amino acid synthetic peptide of the protein (**Figure 2B**). The results showed that the 94 kDa band is immunoreactive with both antibodies, verifying that this protein band corresponds to recombinant VP1 (rVP1). IPTG-induced expression of *E. coli* transformed with pTRI-VP2 was analysed by SDS-PAGE, and as expected for VP2, an overexpressed protein of approximately 54 kDa was detected (**Figure 2C**). In *E. coli* transformed with pTRI-VP2-SF, the SDS-PAGE showed overexpression of a protein band at the VP2-Flg expected size (approximately 80 kDa) (**Figure 2D**). Purified VP2 and VP2-Flg proteins used in this study are shown in Figures 2C and 2D.

Gene expression profiling in head kidney after* in vivo *stimulation with rVP1 and VP2-Flg The gene expression of master transcription factors and cytokines involved in T helper and regulatory responses was analysed after rVP1, and VP2-Flg immunisation by RT-qPCR to determine the immunogenicity of rVP1 and VP2-Flg. Total head kidney leukocytes from juvenile rainbow trout injected with rVP1 as outlined in the Methods were isolated for the analysis. Results show that rVP1 increased expression of the Th1 type cytokine *ifn-γ,* relative to adjuvant controls (**Figure 3**). In addition, rVP1 induced gene expression of *il-4/13a* and *il-4/13b2*, both encoding cytokines related to the Th2 type response (**Figure 3**). Regarding immunosuppressive cytokines, rVP1 triggered up-regulation of the immunosuppressive cytokine *il-10a* (**Figure 3**) and there was a trend to upregulation for *tgf-β1* (data not shown). Only *il-2* was not induced by the antigen stimulation (**Figure 3**). The results from an independent experiment confirmed that rVP1 induces a type 1 type response because up-regulation of *tbet*, the master transcription factor of Th1 development, and the *il-12p40c* cytokine gene was also observed (**Figure 3**). Similarly, induction of a type 2 response was also confirmed as *gata3*, the gene encoding the master regulator of Th2 cell development, was also up-regulated by antigen stimulation (**Figure 3**). In this case, up-regulation of *tgf-β1* gene expression reached statistical significance (**Figure 3**) while three genes tested for reproducibility *ifn-γ, il-4/13a* and *il-4/13b2* were also upregulated relative to adjuvant controls (not shown). The cytokine expression profile in response to VP2-Flg inoculation was also examined (**Figure 4**). VP2-Flg induced expression of *ifn-γ, il-4/13a, il-10a* and *tgf-β1*, relative to adjuvant controls (**Figure 4**) but in contrast, the expression of *il-4/13b2* and *il-2* decreased (**Figure 4**). Except for *il-4/13a* and *il-2a*, the changes in expression levels of

the immune marker genes relative to adjuvant controls were also observed in the fish injected with Flagellin alone (**Supplementary figure 2**).

Gene expression profiling of* in vitro *antigen-stimulated CD4-1$^+$ T cells

Immune cells isolated from VP1-injected fish were *in vitro* stimulated with rVP1 or were kept without stimulation for 72 h to analyse the antigen dependent T cell response. Similarly, fish immunized with VP2-Flg were *in vitro* stimulated with rVP2. CD4-1$^+$ T cells were quantified using flow cytometry, as after antigen-specific stimulation responding cells are expected to proliferate. **Figure 5A** shows that the CD4-1$^+$ T cells (CD4-1$^+$ IgM$^-$ cells) did not change after 72 h stimulation with VP1, while CD4-1$^+$ T cells from fish stimulated with VP2-Flg showed a small decline after *in vitro*-stimulation with VP2 (**Figure 5B**). Since antigen stimulation should also induce differentiation, the transcriptional expression of five cytokine markers of T cell differentiation (*ifn-γ, il-4/13a, il-10a, tgf-β1 and il-2a*) were also analysed in the *in vitro*-restimulated and non-stimulated control cells. The expression profile is shown for each fish tested as a relative expression against paired control cells (**Figure 5**). The results show that each fish reacts to antigen stimulation with a unique expression profile of the cytokine genes (**Figure 5C-D**). For cells from VP1 immunized fish, restimulation of fish 1 cells showed a moderate increase in expression of the five cytokines tested, i.e., *il-2a, ifn-γ, il-4/13a, il-10a,* and *tgf-β1*; fish 2 cells responded with a high increase in expression of *il-2a* only, whilst fish 3 cells responded with very high induction of *ifn-γ and il-4/13a* and moderate increase of *il-2a, il-10a,* and *tgf-β1* (**Figure 5C**). The other fish showed decreased expression of all tested cytokines (not shown), suggesting that other cytokines that were not analysed may have been stimulated. For VP2 stimulated fish, only cells from one of the five fish studied, fish 1, showed a modest increase of *ifn-γ, il-4/13a,* and *il-10a* (**Figure 5D**). Cells from fish 2 showed sustained expression of *il-4/13a, il-10a,* and *tgf-β1,* the latter also seen with cells from fish-3 (**Figure 5D**). Cells from the other two fish showed no responses against VP2 after *in vitro* restimulation (not shown).

Trout CD4-1$^+$ T cells response in VP1 immunized fish.

The next aim was to determine whether the cells that produce the cytokines in response to VP1 immunization are trout T helper cells. Head kidney CD4-1$^+$ T cells were isolated by sorting from four VP1-immunized fish and three control fish receiving adjuvant only. The cells were collected and cytokine expression of *ifn-γ, il-4/13a, il-4/13b2, il-10a, tgf-β1* and *il-2a* were analysed. **Figure 6A** shows the gating of isolated CD4-1$^+$ T cells. The cytokine expression profile for each fish is shown as a fold change relative to the transcriptional level in cells of the control group. The CD4-1$^+$ T cells produced transcripts for *ifn-γ, il-4/13a, il-4/13b2, il-10a, and tgf-β1,* but not *il-2a* (**Figure 6B**). Furthermore, the expression profile in four analysed fish showed that immunization triggers a unique T cell response in each fish (**Figure 6B**). Thus, T cells from one fish expressed most of the cytokines analysed, i.e., Th2 type cytokines (*il-4/13a* and *il-4/13b2*), the Th1 type cytokine *ifn-γ*, and *il-10a*; T cells from

386 a second fish expressed *ifn-γ* and *il-10a* only, whilst T cells from the other two only produced
387 transcripts for Th2 type cytokines and one regulatory cytokine (*il-10a* or *tgf-β1*) (**Figure 6B**).

DISCUSSION

This study investigated the rainbow trout immune response against two infectious pancreatic necrosis virus proteins, VP1 and VP2-flagelin, which can be used as immunogens in IPNV vaccine design. VP1 is the RNA-dependent RNA polymerase located internally in the virion [4, 14], whilst VP2 forms the viral capsid [10, 12].

Recombinant VP1, administered using Montanide™ as an adjuvant, induced the expression of *ifn-γ*, *tbet*, and *il-12p40c* in the head kidney of rainbow trout. In higher vertebrates, IFN-γ, Tbet, and IL-12 are directly involved in Th1 differentiation [38]. IL-12 (p35/p40), produced by antigen-presenting cells, induces IFN-γ production by T and natural killer cells and, in turn, IFN-γ and IL-12 induce *t-bet* in $CD4^+$ T cells, the master transcription factor that controls Th1 differentiation and the expression of *ifn-γ* [39, 40]. In teleost fish, *ifn-γ*, *tbet*, and *il-12* are conserved and although functional studies are scarce, reports have shown conserved interrelationships depicted between IFN-γ, Tbet, and IL-12. For example, it has been demonstrated that recombinant IFN-γ induces increased expression IL-12-p35 and IL-12-p40 in goldfish macrophages [41], and in flounder, IFN-γ and IL-2 upregulate the expression of *tbet* in sorted CD4-1^+ and CD4-2^+ T lymphocytes [42]. In rainbow trout, IL-12 isoforms p35/p40c and p35/p40b increase transcript levels of IFN-γ1 and IFN-γ2 in head kidney cells [43]. Accordingly, gene expression results here showed that rVP1 from IPNV induced a type 1 immune response in rainbow trout which will trigger cell-mediated mechanisms of immunity. Interestingly, the type 1 response occurs even though the VP1 is a soluble antigen. This is probably due to the adjuvant present (Montanide™) since rVP1 administered in saline did not induce expression of *ifn-γ* and *tbet* (data not shown). These findings are consistent with a recent study reporting that a bacterin administered to rainbow trout upregulates expression of genes driving a type 1 response due to the presence of Montanide™ ISA 763A VG, which was not observed when another adjuvant (Montanide™ ISA 761 VG) was used [44].

Interestingly, rVP1 injection also increased the expression of *il-4/13a*, *il-4/13b2* and *gata-3* genes. In higher vertebrates, IL-4, IL-13 and GATA3 are the central factors of type 2 immunity. The transcription factor GATA-3 is induced by IL-4 and triggers the differentiation of naive T cells to Th2 cells [45]; IL-4 helps class switching to IgG1 and IgE; IL-4 and IL-13 induce alternative macrophage activation, and all of them have a role in tissue repair [46]. In rainbow trout, *il-4/13a* and *il-4/13b* encode cytokines evolutionary related to IL-4 and IL-13 [47]. Rainbow trout IL-4/13A and B cytokines induce the gene expression of antimicrobial peptides, acute phase proteins and IL-10 but down-regulate IL-1β and IFN-γ in isolated head kidney cells. They also modulate the expression of the receptors of IFN-γ, and their own potential receptors in head kidney cells stimulated *in vitro* [47]. Similar effects have been observed when IL-4/13A is injected into Atlantic salmon [48]. *Gata3* orthologues have also been identified in several fish species, including rainbow trout [49], which indicates

that the type-2 mechanisms of immunity are present in teleost fish. Thus, the increased expression of *il-4/13a, il-4/13b2* and *gata-3* in VP1 immunized trout in this study provides evidence for the induction of type-2 immunity in these fish which may be related to humoral immunity or tissue repair, both effector functions that must be further investigated. Another important effect of rVP1 immunization was the upregulation of *il-10a* in the head kidney of rainbow trout, which suggests that a well-regulated and limited immune response has been elicited. This is supported by several functional studies of IL-10 in goldfish, common carp, grass carp, tongue sole (*Cynoglossus semilaevis*) and spotted knifejaw (*Oplegnathus punctatus*), showing that fish IL-10 inhibits pro-inflammatory gene expression, and reduces respiratory burst, nitrogen radical production and phagocytic activity [50-53] as occurs in higher vertebrates [52, 54]. Up-regulation of *tgf-β1* gene expression was also observed in response to rVP1 immunization. The results of two independent experiments showed a slight discrepancy, in one the increase was a trend, while the difference reached statistical significance in the other. Although we did not identify the source of the difference, we reported this observation for further analysis, since parameters such as environmental factors may explain the differences. The functional role of TGF-β1 has been tested in some fish species, i.e., TGF-β1 inhibits IL-1β in grass carp and down-regulates TNFα in goldfish [55, 56] therefore, induction by rVP1 immunization in rainbow trout may also contribute to the homeostasis and regulation of the immune response.

Further analyses performed *ex-vivo* and *in vitro* to identify the cells responsible for the type of immune response observed and to verify antigen-dependent responsiveness showed high inter-individual variations. Since reporting averaged data would have led us to disregard the existence of distinct and relevant responses, as noted before [57], in this study we reported the individual response profiles of analysed fish as each fish response can give clues to help obtain the desirable population level responses. Furthermore, studying the origin of inter-individual variations [58] can help develop ways and therapies to induce an immune response in a higher number of individuals of a given population, for example, after vaccination.

In this context, the expression analysis in sorted CD4-1$^+$ T cells revealed that most of the upregulated cytokines are produced by T helper cells indicating that T helper differentiation occurred in response to VP1 immunization as in mammals. One fish had T cells expressing cytokines indicating that T cell differentiation towards Th1 and Th2 lymphoid cells has occurred, although the presence of separate lineages of CD4$^+$ T cells was not demonstrated. The presence of *il-10* expression in the CD4-1$^+$ T cells of this fish, suggests that the antigen response also includes lymphoid differentiation of T cells with a regulatory role. In mammals, several lineages of CD4$^+$ T cells can produce IL-10, for example, Th2 type cells, Treg cells and Tr1 cells [59]. Treg cells also exist in fish and play a role in immunotolerance [60-63], therefore, it may be such T cells that are expressing *il-10* in this study. Indeed, the cytokine expression profile of the CD4-1$^+$ T cells was highly distinct in the studied fish. Thus, in

another fish, the expression of *ifn-γ* and *il-10a* indicated that only Th1 differentiation occurred but also in the presence of regulatory T cells, while in two others Th2 differentiation was apparent again accompanied by regulatory T cells expressing regulatory cytokines (either *il-10a* or *tgf-β1*). As in mammals, a Th1 type response may induce cellular immune responses in fish to provide effective mechanisms of defence against viral infections and other intracellular pathogens [43]. Our data suggests that Th1 response occurred in only 50% of the immunized animals, which might be related to genetic differences of animals, different intrinsic factors such as sex and diverse immune history including response to vaccination.

rVP1 *in vitro* stimulation of leukocytes isolated from immunized fish also revealed a very different immune profiling in each fish. Consistent with the results observed *in vivo*, the antigen stimulation assay showed an increase of *ifn-γ, il-4/13a,* and *il-10* in 2 of 3 responders. Interestingly, the three responders also showed a rise in *il-2* expression after 4-h antigen stimulation even though increased expression of *il-2a* was not observed in leukocytes isolated from the head kidney, nor in the CD4-1^{+} T cells obtained 3 days after the last rVP1 immunization. Upregulation of *il-2a* after *in vitro* restimulation is likely linked to the fact that IL-2 is a T cell growth factor produced and secreted mainly by T cells that have been activated by stimulation with mitogens or by interaction of the T cell receptor with the antigen/MHC complex on the surface of APC [64]. In rainbow trout, the role of IL-2 in promoting lymphoid cell proliferation has been demonstrated [65]. Moreover, it is known that IL-2 secretion in murine CD4^{+} T cells is rapid and transient *in vivo*, lasting less than 20 h [66], which can explain why this study did not detect upregulated expression in leukocytes isolated after three days (72 h) stimulation.

The immune response against recombinant VP2 produced as a fusion protein with flagellin of *Aeromonas salmonicida* was also examined. VP2 has been previously used as a target antigen in vaccines because it is the main protein of the IPNV capsid and immunization is expected to produce antibodies against neutralizing epitopes [21]. Since flagellin activates pro-inflammatory responses in rainbow trout [29, 67], the rationale was that this bacterial protein could be used as a vaccine adjuvant to help stimulation of the type 1 response and cellular immunity against VP2. After immunization with VP2-Flg, the expression profiling of leukocytes isolated from trout head kidneys showed an increase of *ifn-γ, il-4/13a, il-10,* and *tgf-β1.* The expression profile was different from that observed with rVP1 and two cytokines showed a decrease of expression (*il-4/13b2* and *il-2a*). The effects produced by the administration of VP2-Flg were mostly due to the presence of flagellin within the chimera and only the reduction of *il-2* was a VP2-dependent effect. *In vitro* restimulation of immune leukocytes, which seeks to examine the presence of effector lymphoid cells responding specifically to VP2, consistently showed that responding leukocytes did not upregulate IL-2 after 4 h stimulation in three of the four examined fish. Interestingly, one fish showed a small increase of *ifn-γ, il-4/13a* and *il-10* in leukocytes after VP-2 *in vitro* restimulation, indicating

that although not frequent a type-1 response against VP2 is possible. Upregulation of *ifn-γ, il-4/13a, il-10,* and *tgf-β1* in head kidney after *in vivo* stimulation with flagellin of *A. salmonicida,* suggests that this bacterial protein could be used as an adjuvant to trigger *i1* and *i2* type immune responses in rainbow trout. In fact, mostly in mammals, flagellin from several bacteria exhibits adjuvant activity when used as recombinant proteins consisting of flagellin fused to heterologous antigens [68]. Several of the target antigens are viral proteins such as influenza hemagglutinin and produce protective immune responses [69, 70]. To the best of our knowledge, this type of analysis to test the potential adjuvanticity of flagellin with heterologous antigens has not been performed in fish species, although, it has been demonstrated that flagellin is a good immunostimulant [29] and improves the performance of several vaccines in fish, including bacterins and fusion proteins of flagellin and antigenic proteins of particular bacteria [71-74]. This strategy of adjuvanticity using fusion proteins containing flagellin and other heterologous protein antigens in vaccines for fish will need analysis on a case-by-case basis since flagellins from different microorganisms and even different flagellins from a single species can differ in their immunological properties and therefore in their adjuvant activity [72]. Moreover, as we have seen in this study, when flagellin induces an increased immune response, it may be mounted against flagellin and not to the heterologous antigen, which is the target for protection. To understand whether this type of cytokine response in rainbow trout is beneficial to counteract IPNV infection, the effects on experimental infection must be studied further, because protective immunity will depend on the magnitude and kinetics of the response, on the mechanisms of virulence and on immune evasion displayed by IPNV. For example, in Atlantic salmon, it is known that up-regulation of *il-10* in spleen and head kidney cells occurs after IPNV infection in Atlantic salmon [75-77]. Although this may be beneficial to ensure a controlled inflammatory response during infection, a high and early induction of *il-10* might counteract protective immunity and help the development of persistent infection [76, 77]. Indeed, early and high upregulation of *il-10* that drops with time has been observed in IPNV-susceptible families of infected Atlantic salmon while in resistant families IL-10 shows a discrete increase which remains unchanged with time [78].

Altogether, this study reports for the first time a characterization of the adaptive immune response induced by the IPNV VP1 protein and a fusion protein of VP2 with flagellin, as new approach to induce immunity in fish against IPNV infection. The results indicate that recombinant VP1 is a very good immunogen for rainbow trout, that induces upregulation of type 1, type 2 and regulatory response cytokines which is evidence for a well-regulated humoral and cellular response. In addition, the immunogenic properties of flagellin from *Aeromonas salmonicida* are reported, which can be further tested for adjuvanticity. Although additional research needs to be done to determine potential protective effects of VP1 for IPNV infection in rainbow trout, these novel findings open new opportunities for further IPNV vaccine design and development.

ACKNOWLEDGMENTS

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555 **FIGURES**

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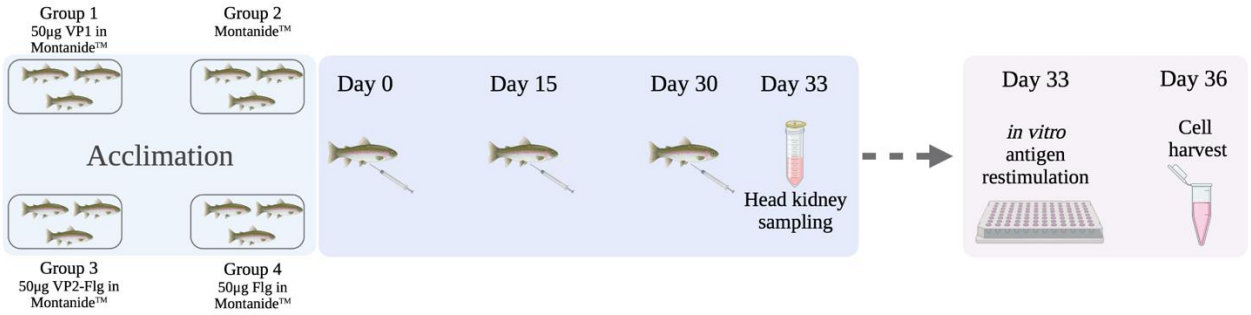

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559 **Figure 1.** Graphical summary of the experimental set up (created in BioRender.com)

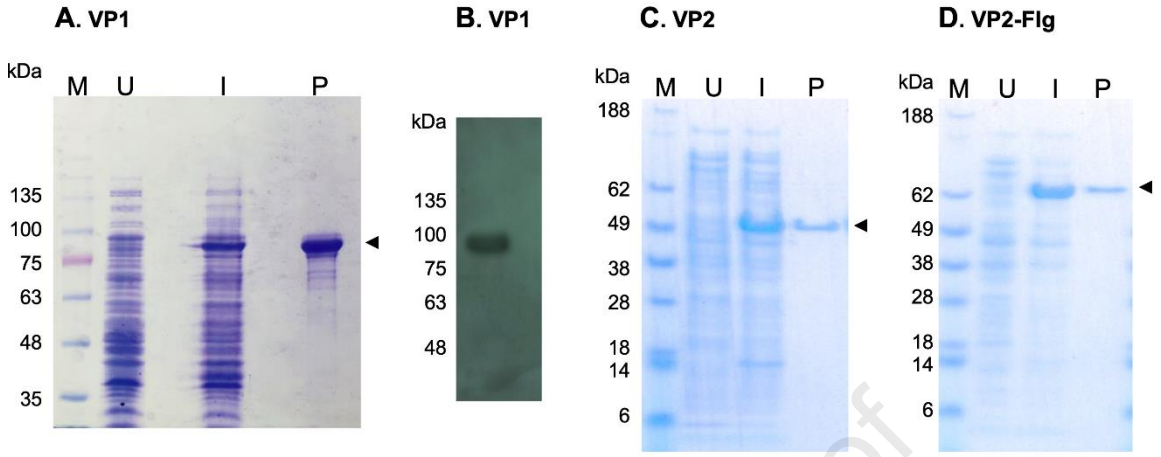


Figure 2. Expression of VP1 and VP2 proteins in *E. coli*. **(A)** Recombinant VP1: M, RGB pre-stained protein ladder (Maestrogen); U, whole protein extract from uninduced *E. coli* transformed with pET21a/VP1; I, whole protein extract from *E. coli* transformed with pET21a/VP1 and induced by 1 mM IPTG; and P, purified recombinant VP1 analysed by SDS–PAGE (12 %) and stained using Coomassie Brilliant Blue. **(B)** Western blot analysis of the recombinant protein with an antibody against VP1 synthetic peptide. **(C)** Recombinant VP2: M, SeeBlue pre-stained protein standard; U, cell lysate from uninduced *E. coli* transformed with pTRI-VP2; I, cell lysate from *E. coli* transformed with pTRI-VP2 induced by IPTG; P, VP2 purified recombinant protein. Analysis by SDS–PAGE and stained using Coomassie Brilliant Blue. **(D)** Recombinant VP2-Flg: M, SeeBlue pre-stained protein standard; U, cell lysate from uninduced *E. coli* transformed with pTRI-VP2-SF; I, cell lysate from *E. coli* transformed with pTRI-VP2-SF induced by IPTG; P, VP2-Flg purified recombinant protein. Analysis was by SDS–PAGE and stained using Coomassie Brilliant Blue.

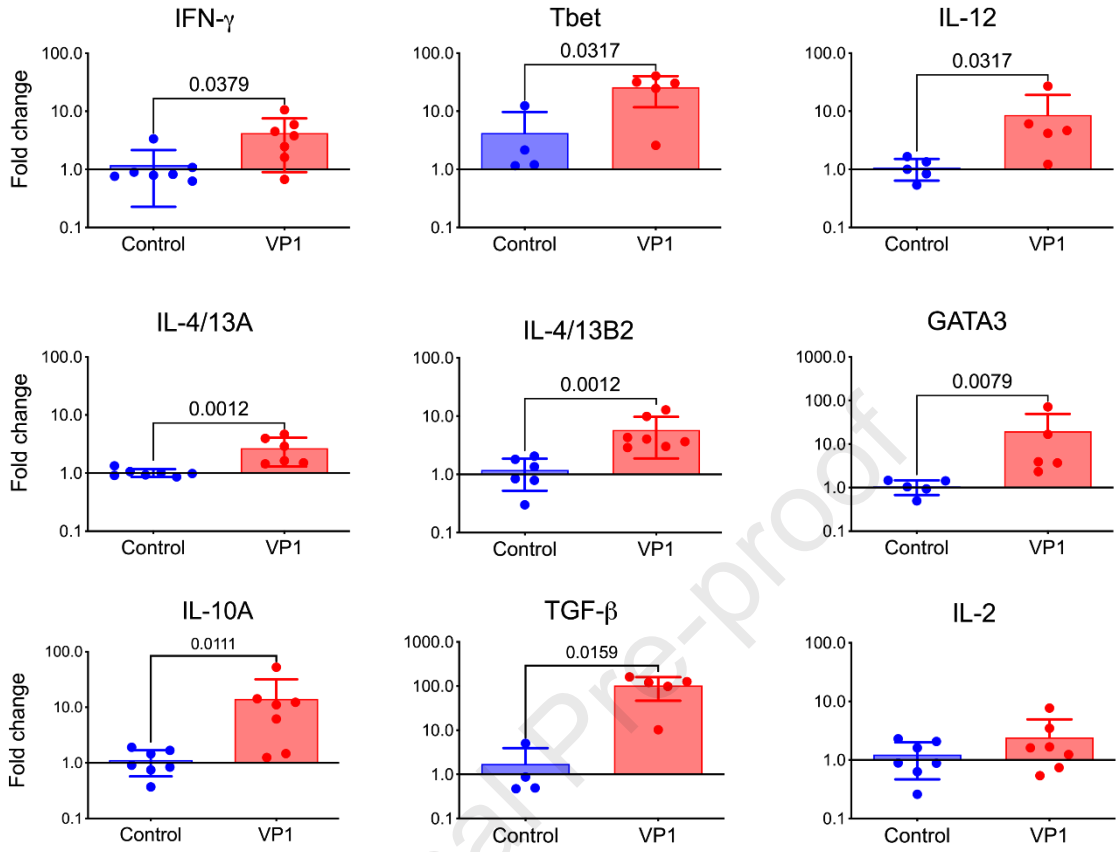
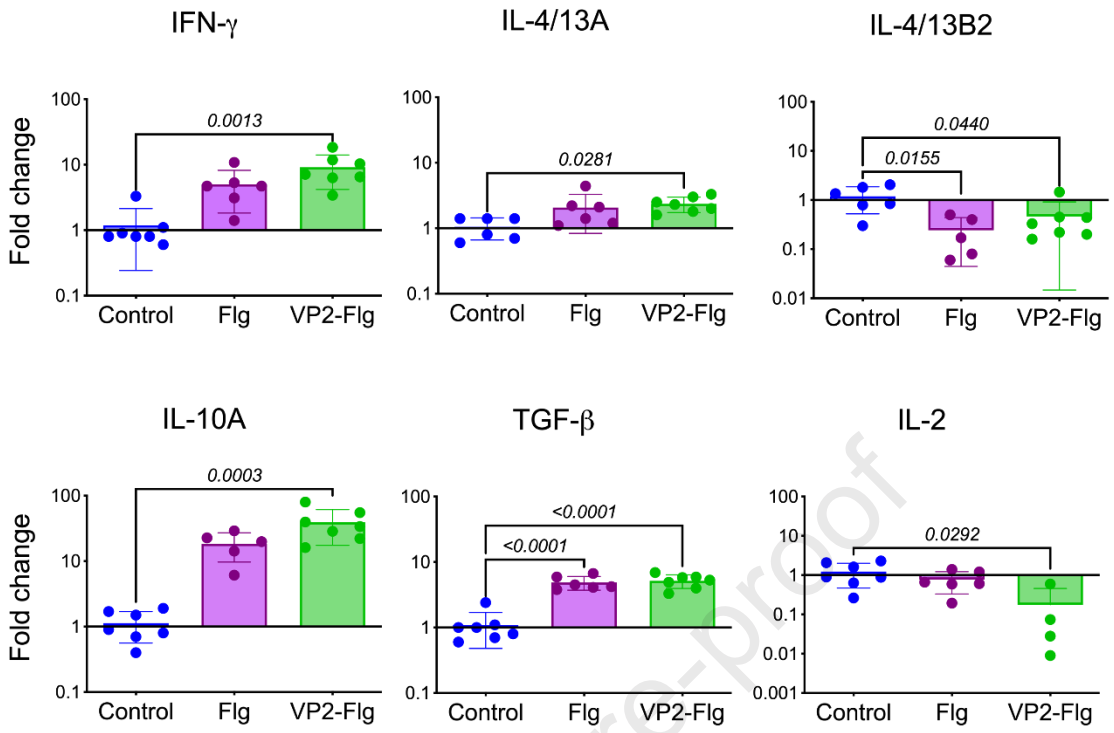


Figure 3. Transcriptional expression profiling in the head kidney of rainbow trout immunized *in vivo* with rVP1. The expression levels analysed by real-time PCR were normalized against *ef1a* expression. The fold-change was calculated as $2^{-\Delta\Delta Cq}$, relative to control group (fish injected with adjuvant alone). The data is expressed as mean ± SD, and each dot represents one fish. Data were analysed using a Mann-Whitney U test, with differences considered statistically significant when $p < 0.05$. p values are shown above the brackets.



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589

590 **Figure 4**. Transcriptional expression profiling in the head kidney of rainbow trout immunized
591 *in vivo* with VP2-Flg. The expression levels analysed by real-time PCR were normalized
592 against *ef1a* expression. The fold-change was calculated as $2^{-\Delta\Delta Cq}$, relative to control group
593 (fish injected with adjuvant alone). Flagellin (Flg) (with adjuvant) injected fish are also
594 shown. The data are expressed as mean ± SD, and each dot represents one animal. The data
595 were analysed using a one-way ANOVA and Tukey *post hoc* test, with differences
596 considered statistically significant when $p < 0.05$. *p* values are shown above the brackets, in
597 paired comparisons.

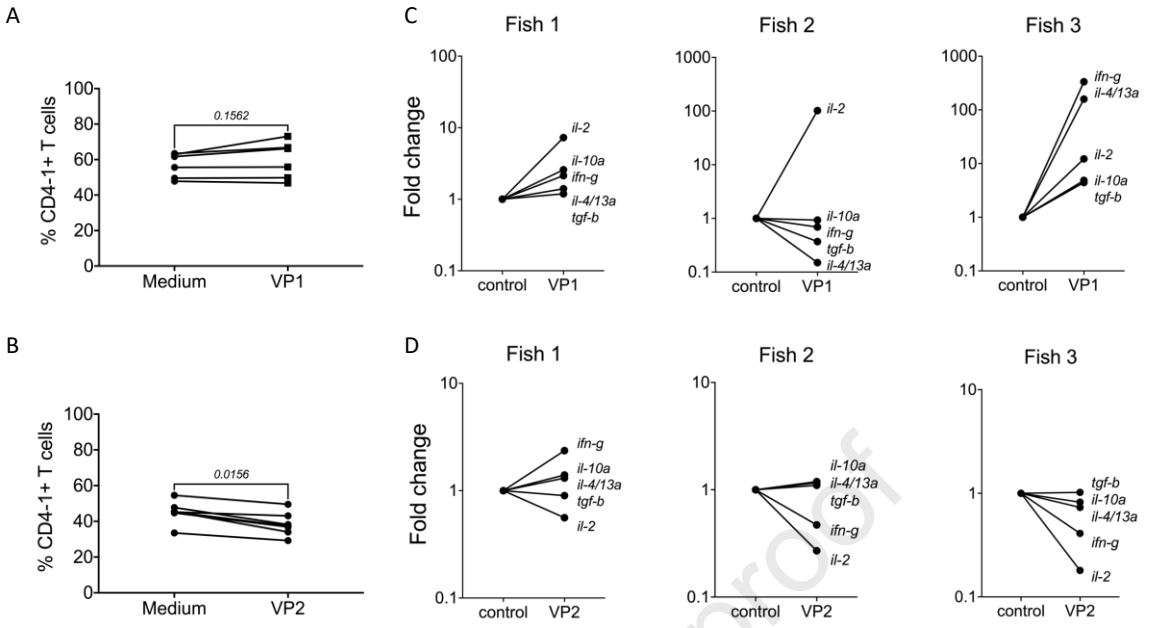
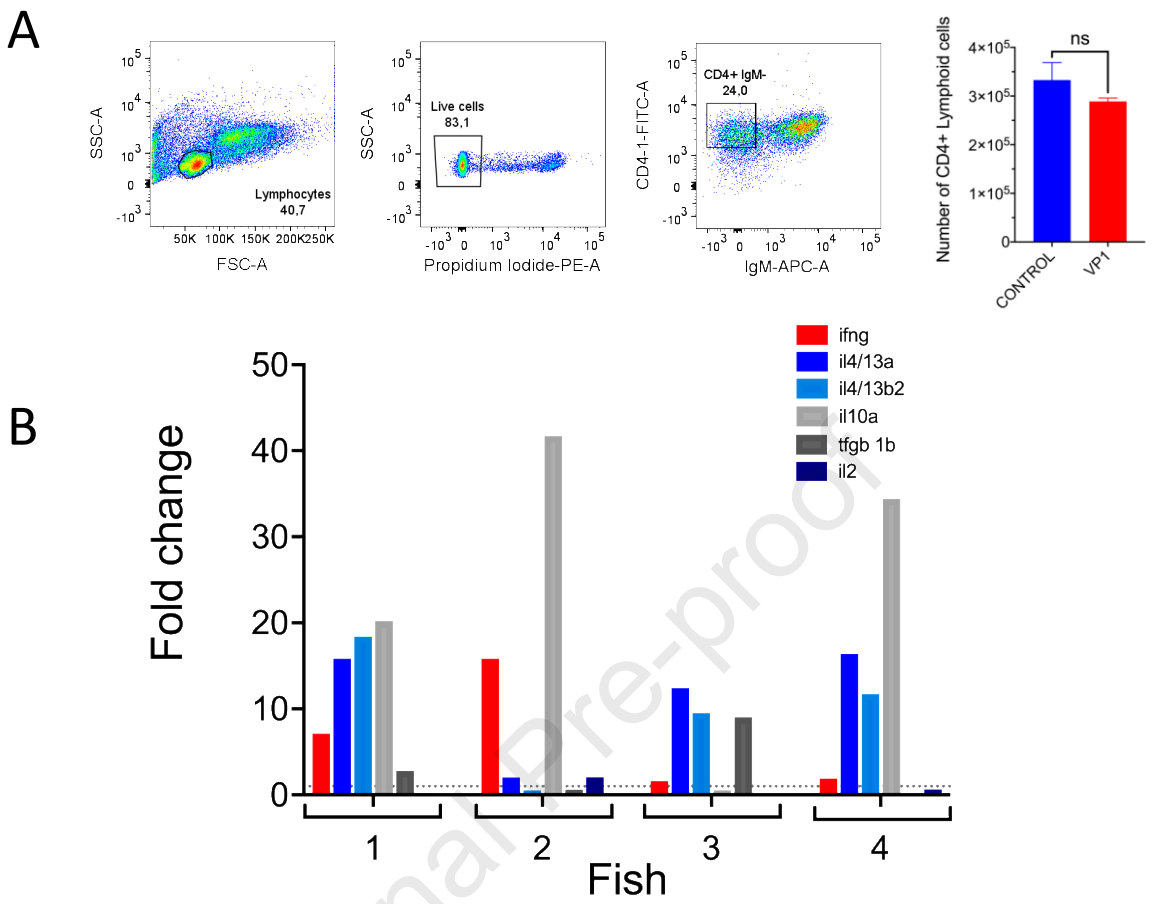


Figure 5. Transcriptional expression profiling in CD4-1+ T cells *in vitro* restimulated with rVP1. **(A)** CD4-1^{+} T cells obtained from head kidneys of VP1-immunised fish were stimulated for 72 h with medium or 1 mL medium containing 100 μg of rVP1. Each dot represents the percentage of CD4-1 T cells with respect to the lymphoid cells, while each line represents a paired comparison between kidney cells stimulated with medium and those stimulated with VP1 for each fish. **(B)** CD4-1^{+} T cells obtained from head kidneys of VP2-Flg immunised fish were stimulated for 72 h with medium or medium containing 100 μg of rVP2. Each dot represents the percentage of CD4-1 T cells with respect to the lymphoid cells, while each line represents a paired comparison between kidney cells stimulated with medium and those stimulated with VP2 for each fish. Paired t-tests were used to determine statistical significance. **(C)** Transcriptional expression of cytokine indicators of T cell differentiation in head kidney cells of VP1-treated fish *in vitro*-restimulated with rVP1. Each line represents a paired comparison between cytokine transcript levels analysed in cells stimulated with medium and those stimulated with VP1 for each fish. The data were normalized against expression values obtained in the control cells. **(D)** Transcriptional expression of cytokine indicators of T cell differentiation in head kidney cells of VP2-Flg treated fish *in vitro*-restimulated with VP2. Each line represents a paired comparison between cytokine transcript levels analysed in cells stimulated with medium and those stimulated with VP2 for each fish. The data were normalized against expression values obtained in the control cells.



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623 **Figure 6.** Transcriptional expression of cytokines in head kidney CD4-1^{+} T cells isolated by
624 sorting cells from VP1-immunized fish. **(A)** Flow cytometry and gating of the sorted cell
625 CD4-1^{+} IgM^{-} cells. The bar graph shows the average number of CD4-1+ T cells obtained
626 from VP1-treated (n=4) and control fish (n=3). Data are means ± SD. **(B)** Cytokine
627 expression profile for individual fish. Data are shown as fold change relative to the average
628 transcriptional level in cells of the control group.
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TABLE 1. SEQUENCE OF PRIMERS

Gene	Accession Number	Sequence (5'→ 3')
ifn-γ	NM_001124620.1	F 5´ CCGTACACCGATTGAGGACT 3´
		R 5´ GCGGCATTACTCCATCCTAA 3´
tbet	NM_001195793.1	F 5´GGTAACATGCCAGGGAACAGGA 3´
		R 5´TGGTCTATTTTTAGCTGGGTGATGTCTG 3´
il-12 p40c	NM_001124392.1	F 5´GAGCCAAGTCTTATGGCTGC 3´
		R 5´GTTCAAACTCCAACCCTCCA 3´
il-4/13a	NM_001246341.1	F 5' GTCAGAGGAACTTCTGGAAACA 3'
		R 5' GTTGTAAACCCTCAGATGTCG 3'
il-4/13b2	HG794525.1	F 5' CTCCTCTTCTCCTTTGCATTTGTG 3'
		R 5' TACAGCTTCAGCACTCTACTGATTT 3'
gata3	NM_001195792.1	F 5´CCAAAAACAAGGTCATGTTCAGAAGG 3´
		R 5´TGGTGAGAGGTCGGTTGATATTGTG 3´
il-10a	NM_001124339.1	F 5' GGATTCTACACCACTTGAAGAGCCC 3'
		R 5' GTCGTTGTTGTTCTGTGTTCTGTTGT 3'
tgf-β1	X99303.1	F 5' AGCTCTCGGAAGAAACGACA 3'
		R 5' AGTAGCCAGTGGGTTCATGG 3'
il-2a	NM_001164065.2	F 5´GAAACCCAATTCCCAGACTCCT 3´
		R 5´GTCCGTTGTGCTGTTCTCCT 3´
ef1α	NM_001124339.1	F 5' CAA GGA TAT CCG TCG TGG CA 3'
		R 5' ACA GCG AAA CGA CCA AGA GG 3'

HIGHLIGHTS

- The rainbow trout adaptive immune response against IPNV VP1 and VP2-Flagellin was studied.
- VP1 induces Th1 and Th2 type cytokines able to trigger the humoral and cell-mediated immunity in rainbow trout.
- CD4-1^{+} T cells produced most of the cytokines indicating that Th differentiation occurred in response to VP1.
- rVP1 *in vitro* stimulation of leukocytes revealed a different cytokine profiling in each fish.
- Trout response against VP2-Flg revealed the immunogenic properties of *Aeromonas salmonicida* flagellin