



OPEN ACCESS

EDITED BY

María Del Mar Ortega-Villaizan,
Miguel Hernández University of Elche, Spain

REVIEWED BY

Gregory D. Wiens,
Agricultural Research Service (USDA),
United States
Johanna Hol Fosse,
Norwegian Veterinary Institute (NVI), Norway

*CORRESPONDENCE

Carolina Tafalla
✉ tafalla@inia.csic.es

RECEIVED 20 March 2024

ACCEPTED 21 June 2024

PUBLISHED 05 July 2024

CITATION

Perdiguero P, Jiménez-Barrios P, Morel E,
Abós B and Tafalla C (2024) Single-cell atlas
of rainbow trout peripheral blood leukocytes
and profiling of their early response to
infectious pancreatic necrosis virus.
Front. Immunol. 15:1404209.
doi: 10.3389/fimmu.2024.1404209

COPYRIGHT

© 2024 Perdiguero, Jiménez-Barrios, Morel,
Abós and Tafalla. This is an open-access article
distributed under the terms of the [Creative
Commons Attribution License \(CC BY\)](#). The
use, distribution or reproduction in other
forums is permitted, provided the original
author(s) and the copyright owner(s) are
credited and that the original publication in
this journal is cited, in accordance with
accepted academic practice. No use,
distribution or reproduction is permitted
which does not comply with these terms.

Single-cell atlas of rainbow trout peripheral blood leukocytes and profiling of their early response to infectious pancreatic necrosis virus

Pedro Perdiguero^{1,2}, Pablo Jiménez-Barrios¹, Esther Morel¹,
Beatriz Abós¹ and Carolina Tafalla^{1*}

¹Fish Immunology and Pathology Group, Animal Health Research Center (CISA-INIA), Valdeolmos, Madrid, Spain, ²Department of Genetics, Physiology and Microbiology, Faculty of Biological Sciences, Complutense University of Madrid (UCM), Madrid, Spain

The recent development of single cell sequencing technologies has revolutionized the state-of-art of cell biology, allowing the simultaneous measurement of thousands of genes in single cells. This technology has been applied to study the transcriptome of single cells in homeostasis and also in response to pathogenic exposure, greatly increasing our knowledge of the immune response to infectious agents. Yet the number of these studies performed in aquacultured fish species is still very limited. Thus, in the current study, we have used the 10x Genomics single cell RNA sequencing technology to study the response of rainbow trout (*Oncorhynchus mykiss*) peripheral blood leukocytes (PBLs) to infectious pancreatic necrosis virus (IPNV), an important trout pathogen. The study allowed us to obtain a transcriptomic profile of 12 transcriptionally distinct leukocyte cell subpopulations that included four different subsets of B cells, T cells, monocytes, two populations of dendritic-like cells (DCs), hematopoietic progenitor cells, non-specific cytotoxic cells (NCC), neutrophils and thrombocytes. The transcriptional pattern of these leukocyte subpopulations was compared in PBL cultures that had been exposed *in vitro* to IPNV for 24 h and mock-infected cultures. Our results revealed that monocytes and neutrophils showed the highest number of upregulated protein-coding genes in response to IPNV. Interestingly, IgM⁺IgD⁺ and IgT⁺ B cells also upregulated an important number of genes to the virus, but a much fainter response was observed in *cc14*⁺ or plasma-like cells (*irf4*⁺ cells). A substantial number of protein-coding genes and genes coding for ribosomal proteins were also transcriptionally upregulated in response to IPNV in T cells and thrombocytes. Interestingly, although genes coding for ribosomal proteins were regulated in all affected PBL subpopulations, the number of such genes transcriptionally regulated was higher in IgM⁺IgD⁺ and IgT⁺ B cells. A further analysis dissected which of the regulated genes were common and which were specific to the different cell clusters, identifying eight genes that were transcriptionally upregulated in all the affected groups. The data provided constitutes a comprehensive transcriptional perspective of how the different leukocyte populations present in blood respond to an early viral encounter in fish.

KEYWORDS

single cell transcriptomics, teleost, peripheral blood leukocytes (PBLs), infectious pancreatic necrosis virus (IPNV), B cells

1 Introduction

Teleost fish have a complex immune system with both innate and adaptive branches. For years, many of the advances made in fish immunology have been based on the identification of immune genes and analysis of their transcriptomic responses. These genes include homologues to multiple cytokines and their receptors, different classes of pattern recognition receptors (PPRs), immunoglobulins (Igs), T cell receptors (TCRs), major histocompatibility complexes (MHC I and MHC II), some specific markers of lymphocyte populations such as CD4 and CD8, and many transcriptional factors, among others, hence demonstrating an overall conservation of the fundamental components of the innate and adaptive immune system between teleost fish and other vertebrates (1). Despite this, the identification and characterization of leukocyte subsets has been a much harder task in most fish species, because in some cases the range of surface markers that define these subpopulations in mammals are not that well conserved in fish, and also due to difficulties in generating specific monoclonal antibodies against these markers when identified.

The explosion of the single-cell mRNA sequencing technology in recent years has drastically increased our capacity to analyze the transcriptome from thousands of individual cells, helping to the identification and characterization of specific immune populations at a transcriptional level in physiologic and pathogenic conditions without requiring their labeling with specific antibodies. These single-cell RNA sequencing technologies have been used in different species to dissect cellular heterogeneity in complex systems (2), including several fish species. For example, novel insights on T cell and natural killer (NK)-like cell functionality were revealed by single cell transcriptomics in zebrafish (*Danio rerio*) (3). Also in zebrafish, single-cell RNA sequencing was used to define cell heterogeneity within the kidney, revealing the existence of novel cell types including two types of NK-like cells and different hematopoietic progenitor subsets (4). Similarly, innate lymphoid cells (ILCs) have been identified and characterized in zebrafish following this methodology (5). In Atlantic cod (*Gadus morhua*), a species that lacks the MHC II system, several cell subpopulations were transcriptionally characterized among splenocytes and blood leukocytes, including cytotoxic T cells, B cells, erythrocytes, thrombocytes, neutrophils, and macrophages (6). Different subsets of non-specific cytotoxic cells (NCCs) were also identified in the Nile tilapia (*Oreochromis niloticus*) kidney using this technology (7). Single-nuclei RNA sequencing was similarly used to characterize different cell lineages from Atlantic salmon (*Salmo salar*) liver after challenge with *Aeromonas salmonicida*, identifying a subpopulation of defense-specialized hepatocytes and detecting the up-regulation of diverse immune genes (8). Regarding rainbow trout (*Oncorhynchus mykiss*), our group recently applied single cell transcriptomics to characterize peripheral blood B cells, recognizing several B cell clusters with specific gene profiles (9). Additionally, this technology was also used to establish that rainbow trout single B cells have the capacity to transcribe several immunoglobulin light chains (IgL) of different specificity (10).

Single-cell RNA sequencing technologies have also been widely applied to investigate the response to infectious diseases, increasing our knowledge on how different cell subsets specifically respond to pathogens. Thus, using this technology to investigate the response of complex cell populations to viruses has allowed the description of novel cell subpopulations involved in infectious responses; the determination of susceptible cell types and infection dynamics; the recognition of differentially expressed genes during the infection; or the discovery of biomarkers for infectious diseases. For example, knowing that CD4⁺ T cell permissiveness to Human Immunodeficiency Virus (HIV) infection is highly heterogeneous across individuals, single cell RNA-seq was applied to define markers of CD4⁺ T cell permissiveness (11). Similarly, a study performed with peripheral blood leukocytes (PBLs) from Dengue virus-infected patients identified Mx2 in naïve B cells and CD163 and IFIT1 in monocytes as predictive markers of disease onset (12). In another study, single-cell RNA sequencing was used to characterize the differentiation of plasmacytoid DCs during a cytomegalovirus infection in mice (13). Other studies have used this methodology to study virus variability such as that in which viral quasispecies were defined during hepatitis C virus (HCV) infection (14). Concerning fish, a single cell transcriptome of midbrain cells infected with Red spotted grouper nervous necrosis virus (RGNNV) performed in orange-spotted grouper (*Epinephelus coioides*) revealed that the brain was enriched in macrophages in RGNNV-infected fish, and described the transcriptional profile of these macrophages in response to the virus. Interestingly, further analysis revealed that, upon infection, the microglia transformed into M1-type activated macrophages that produced cytokines to help reduce the damage caused by the virus in this tissue (15). Different leukocyte subsets were also identified in the zebrafish spleen, which showed differential responses to spring viremia of carp virus (SVCV) infection (1). Single cell transcriptomics was also used to study the response of the SHK-1 cell line derived from Atlantic salmon head kidney to infectious salmon anemia virus (ISAV) (16).

Infectious pancreatic necrosis virus (IPNV) is a non-enveloped double stranded RNA (dsRNA) virus belonging to the genus *Aquabirnavirus* within the family *Birnaviridae*. IPNV can infect a wide range of salmonid species, including Atlantic salmon and rainbow trout, thus causing huge economic losses to the global salmonid industry every year (17). Although the percentage of blood leukocytes that supports viral replication in newly infected fish is low, the virus seems strongly associated with blood leukocytes in fish persistently infected with the virus, in which a carrier state is established (18). In this context, IPNV seemed like an adequate model to investigate the early immune responses of rainbow trout peripheral blood leukocytes (PBLs) to a viral encounter. To this aim, trout PBLs were exposed *in vitro* to the virus or mock-infected. After 24 h of incubation, a single cell transcriptomic analysis was undertaken using the 10x Genomics technology previously applied to fish cells (9, 10). Based on transcript heterogeneity and expression of known markers, PBLs were classified into 12 different clusters that included four different subsets of B cells, T cells, monocytes, two populations of dendritic-like cells (DCs), hematopoietic progenitor cells, NCCs, neutrophils and

thrombocytes. Further differences in the expression of protein-coding genes, long non-coding RNAs, ribosomal and mitochondrial genes were described for each subpopulation providing us with an atlas of blood leukocyte subsets in rainbow trout. When the transcriptional profile of these PBL subpopulations was compared between IPNV-infected and mock-infected cultures, we established that monocytes, neutrophils, IgM⁺IgD⁺ and IgT⁺ B cells, T cells and thrombocytes were the main responding cell types. An analysis of common and specific regulated genes proteins identified a set of viral-regulated genes involved in different cellular functions such as antigen presentation, effector processes, cell surface receptor signaling, phagocytosis or regulation of gene expression, that are thoroughly discussed throughout the paper. These results contribute to a better understanding of how different innate and adaptive immune populations respond to viral encounter in teleost fish, providing us with useful information for the development of prophylactic measures in the future.

2 Materials and methods

2.1 Isolation of PBLs from rainbow trout

Rainbow trout (*Oncorhynchus mykiss*) of approximately 70–100 g were obtained from *Piscifactoría Cifuentes* (Guadalajara, Spain). Fish were maintained at the Animal Health Research Centre (CISA-INIA) laboratory at 14°C in a re-circulating water system with 12:12 h light:dark photoperiod. Fish were fed twice a day with a commercial diet (Skretting, Spain). Prior to sampling, fish were acclimatized to laboratory conditions for 2 weeks and during this period no clinical signs were ever observed. At this point, two rainbow trout were killed by benzocaine (Sigma) overdose. Blood was extracted with a heparinized needle from the caudal vein and diluted 10 times with Leibovitz medium (L-15, Thermo Fisher Scientific) supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin (P/S, Thermo Fisher Scientific), 5% fetal calf serum (FCS, Thermo Fisher Scientific) and 10 IU/ml heparin (Sigma). PBLs were obtained by centrifugation (500 x g for 30 min at 4°C) of diluted blood on 51% continuous Percoll (GE Healthcare) density gradients. The interface cells were collected, washed twice in L-15 containing antibiotics and 5% FCS and adjusted to 1 x 10⁶ cells/ml. Dye exclusion test using trypan blue (Sigma) was used to determine the number of viable cells.

2.2 *In vitro* infection of PBLs with IPNV

IPNV (Sp strain, ATCC VR 1318) was propagated in the RTG-2 (Rainbow Trout Gonad-2) cell line, an established line of fibroblasts from rainbow trout gonads (19). RTG-2 cells were routinely grown in 75 cm² culture flasks (ThermoFisher Scientific) at 19°C, and split 1:2 when confluent, after detaching the cells with 0.25% trypsin-EDTA in PBS (Phosphate Buffer Saline) (Gibco). To produce viral stocks, complete culture media was removed from 80% confluent cell cultures in 75 cm² culture flasks and IPNV (200 µl of 1x10⁷ TCID₅₀/ml) inoculated. Immediately after, L-15 medium with

antibiotics and 2% FCS was added and the cells cultured at 14°C for approximately 5–7 days. When cytopathic effect was extensive, the supernatant was harvested and centrifuged to eliminate cell debris (2000 x g for 15 min at 4°C). Clarified supernatants were used for the experiments. All virus stocks were titrated in 96-well plates according to the procedure described by Reed and Muench (20).

For the *in vitro* infection of rainbow trout PBLs, cells resuspended in L-15 containing antibiotics and 5% FCS were disposed in 24-well plates (500 µl per well). Immediately after, 10 µl of IPNV (5 x 10⁷ TCID₅₀/ml) were added to each well, to achieve a multiplicity of infection (MOI) of 1. At this point, the cells were incubated at 18°C for 24 h, before they were harvested to perform the single-cell transcriptomic analysis. Non-infected control cells from each fish were included and treated in the same conditions.

2.3 Construction of the 5' single cell library and sequencing

After the 24 h of culture, cell viability was checked on a FACS CelestaTM flow cytometer (BD Biosciences) after staining the cells with DAPI (0.2 µg/mL; Sigma-Aldrich). After confirming cell viabilities of approximately 85%, cells gently pipetted and diluted in L-15 medium supplemented with antibiotics and 5% FCS to a concentration of 1,000 cells/µl were used for cell isolation on a 10x Genomics Chromium Controller instrument. Cell suspensions from each sample were loaded into the chips of the ChromiumTM Single Cell 5' Gel Beads Kit (10x Genomics) and subjected to the Chromium Controller instrument to generate single cell Gel Bead-In Emulsions (GEMs) following manufacturer's instructions. Next, GEMs were subjected to library construction using the ChromiumTM Single Cell 5' Library Kit v1 (10x Genomics). As a first step, reverse transcription was performed, resulting in cDNA tagged with a cell-specific barcode and unique molecular index (UMI) per transcript. Fragments were then size selected using SPRIselect magnetic beads (Beckman Coulter). Next, Illumina sequencing adapters were ligated to the size-selected fragments and cleaned up using SPRIselect magnetic beads (Beckman Coulter). Finally, sample indices were selected and amplified, followed by a double sided size selection using SPRIselect magnetic beads (Beckman Coulter). The quality of the libraries was assessed using an Agilent 2100 Bioanalyzer (Agilent technologies) and samples were then sequenced using a NextSeq instrument (Illumina) with 150PE chemistry.

2.4 Alignment and initial processing of sequencing data

The Cell Ranger software (10x Genomics, v3.1) was used to process the sequenced libraries. The complementary DNA reads from each sample were mapped against a previously adapted *Oncorhynchus mykiss* reference transcriptome (9) using the "Cell Ranger count" tool. Through this system, filtered UMI expression matrices from each sample were generated. As a result, raw expression data was obtained containing transcriptomes for

individual cells, both for control non-infected leukocytes and for leukocytes exposed to IPNV during 24 h, from each of the two fish included in analysis.

2.5 Quality filtration of cells

A quality control of datasets was performed in order to filter out contaminants such as abnormal cells in all datasets. For this purpose, filtered UMI expression matrices were conducted to Seurat package (v3.1) for successive analysis. Cells with at least 200 detected genes, and only those genes that appeared in at least three cells were included in an initial matrix for each fish. A cell was considered to be abnormal if any of the following criteria were met: (i) detected gene number $>3,500$; (ii) detected count number $>15,000$ or (iii) $>25\%$ of reads in a cell mapped to mitochondrial genes. Additionally, cells containing $>1\%$ of reads mapped to hemoglobin genes were considered contaminant red blood cells or doublets involving these undesired cells, thus cells achieving this threshold were also filtered out at this step.

2.6 Sample integration and data reduction analysis

The SCTransform method from the Seurat software was applied in order to normalize the four filtered single-cell datasets that were then integrated using the PrepSCTIntegration tool to avoid a batch effect between samples. The merged data was subjected to dimensionality reduction using the principal component analysis (PCA) followed by uniform manifold approximation and projection (UMAP) using 35 dimensions.

2.7 Marker identification and functional analysis

The identification of genes showing differential expression associated to a specific cluster was performed using the FindCluster tool from the Seurat software, considering a significant association for those genes showing an adjusted $p < 0.001$ and $\log_2FC \geq 0.25$. The information relative to the gene description contained in the *O. mykiss* genome v1.0 Omyk_1.0 (GCF_002163495.1) was taken into account for gene name association. In order to obtain an actualized functional annotation, the nucleotide sequences from the genes identified as markers were compared with proteins from a set of model species (*Homo sapiens*, *Mus musculus*, *Danio rerio*, *Macaca mulata*, *Drosophila melanogaster*, and *Xenopus tropicalis*) using the Blastx software applying as threshold a minimum E value of 10^{-5} . Blast results were subjected to the Blast2GO software for GO term mapping. Sequences were also compared against domain databases using the InterProScan tool implemented in Blast2GO. GO term annotations were inferred for rainbow trout transcripts. Single enrichment analysis was performed by comparing the

functions associated to genes from each cluster taking into account differences with an adjusted $p < 0.05$.

2.8 Differential gene expression analysis of cell subsets in response to IPNV interaction

The differential expression analysis between leukocytes exposed to IPNV and non-infected cells was conducted globally and within each cell subset using the MAST package and the FindMarkers function from the Seurat package (version 3.1.5). Multiple tests corrections were applied to the p values to control the FDR using the procedure described by Benjamini and Hochberg (21), as implemented in MAST.

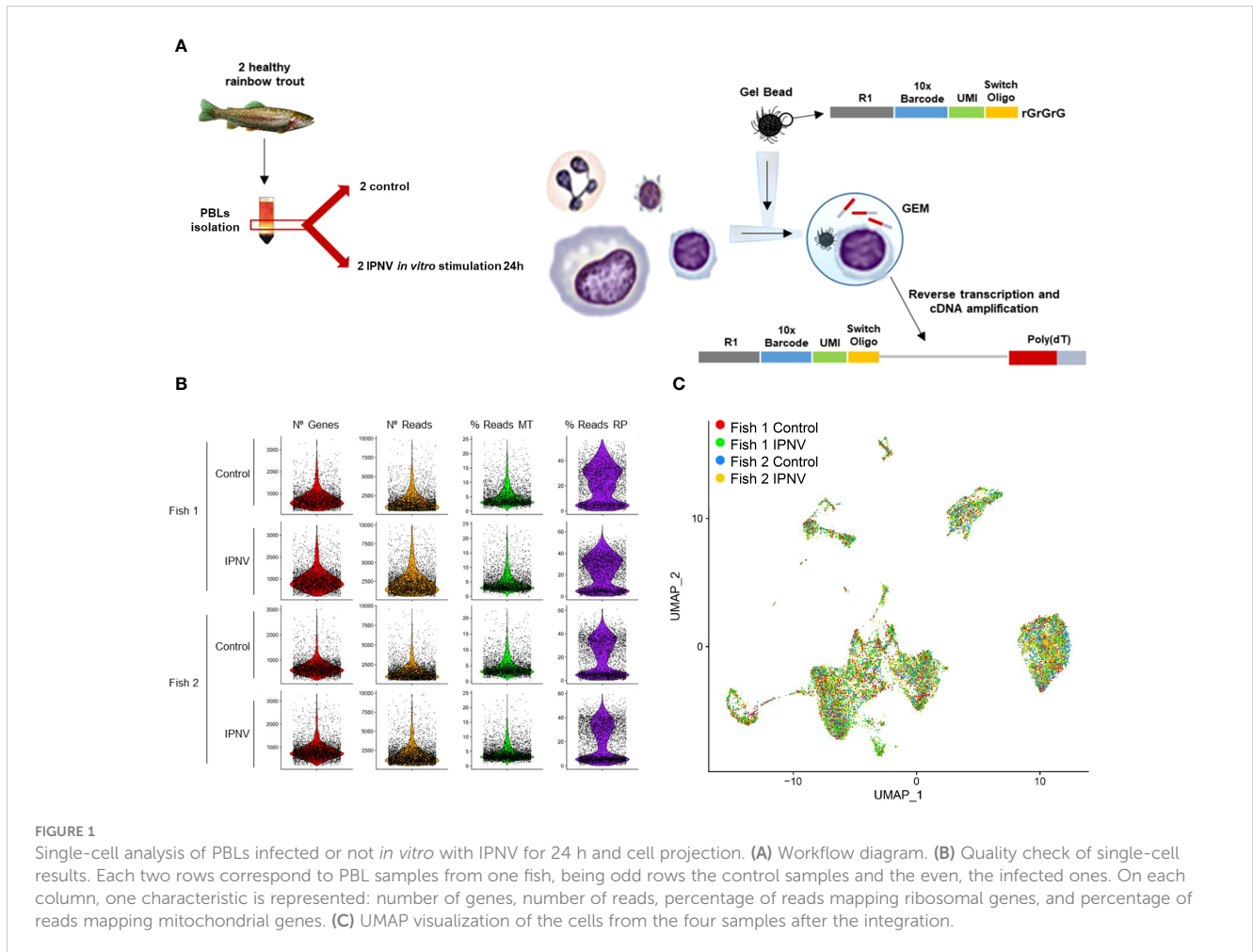
3 Results

3.1 Single-cell transcriptome sequencing of rainbow trout PBLs

To explore the heterogeneity of rainbow trout PBLs and their responses to a virus infection, a transcriptomic profile at single-cell resolution was constructed for all isolated cells, both control and IPNV-infected cells from each fish (Figure 1A). Single cell cDNA libraries were sequenced using Illumina HiSeq 150PE obtaining a total number of 145,325,945 and 166,384,599 raw reads for control and IPNV-infected PBLs from fish 1, respectively, as well as 287,908,169 and 284,601,127 for control and IPNV-infected PBLs from fish 2, respectively. Using the Cell Ranger software around 75–85% of raw reads per sample were maintained after the identification of valid cell barcodes, that when mapped to the transcriptome resulted in approximately 65–75% of mapped reads. Filtered data from Cell Ranger was loaded to the Seurat software (v3.0) for successive analysis. Finally, cells showing more than 1% of reads mapping to hemoglobin genes were excluded as potential contaminant erythrocytes. After filtering, all datasets showed a similar distribution (Figure 1B). All datasets were integrated together using the SCTransform function, and the resulting matrix was dimensionally reduced by applying PCA, followed by UMAP. The cell projection reflected a homogeneous separation of cells in the four samples included in the analysis (Figure 1C).

3.2 Clustering and assignment of cell identities

The application of the FindCluster algorithm from Seurat software resulted in the detection of 9 different clusters of cells showing clear differences in gene expression patterns. Meanwhile, analyzing UMAP projection, a set of 88 cells were catalogued as doublets and filtered out at this step due to discordances between cluster assignment and location in cell projection. After this last filtering step, a total of 3,108, 3,724, 3,584 and 4,167 cells were retained for successive analysis in the samples from fish 1 (control and IPNV-infected) and fish 2 (control and IPNV-infected),



respectively. Three small groups of cells, which contained cells from the four samples analyzed, were found to clearly separate in cell projection. Thus, these three groups, which included *irf4*⁺ B cells, dendritic cells (DCs), and DC-like cells, were manually defined as new clusters achieving a total of 12 final clusters (Figure 2A). These clusters were visualized both in control and infected samples (Supplementary Figure S1).

Once the main cell clusters were defined, a global identification of differentially expressed genes for each cell cluster was performed. Differentially expressed genes were analyzed to identify known markers commonly associated to different cell types which allowed the assignation of putative identities (Figure 2B, Supplementary Table S1). Four clusters were clearly associated with B cells according to the expression of the constant regions of Ig heavy (H) and light (L) chains as well as different genes encoding CD79 (Figure 2B). The first cluster of B cells is preferentially represented by IgM⁺IgD⁺ B cells (known to be the main subset of B cells in systemic compartments), whereas the second one is mainly represented by IgT⁺ B cells. The third group of B cells identified also preferentially contained IgM⁺IgD⁺ B cells, but with a differential expression pattern defined mainly by the expression of *ccl4*, a chemokine previously defined as a marker for a specific subset of B cell in salmonids (9). Finally, a reduced group of 30 cells was identified as plasma-like cells based on the reduced expression

of IgD, the increased expression of IgM and the higher expression of *irf4*, a common marker of plasma cells (22). Only one cluster was clearly identified as a T cell population according to the expression of the constant region for TCR α and other common markers such as CD3d, CD3e, CD4 or ZAP-70 (Figure 2B). Three clusters seemed to correspond to myeloid cells according to the expression of different genes annotated as CD209. The biggest of these groups, represented by ~ 5% of total cells, was identified as monocytes attending to the expression of CD209 and lysozyme II (*lyz2*) genes, common markers for this subset in several species (23) (Figure 2B). The other two clusters were represented by only 26 and 22 cells. The first one was annotated as a DC cluster, based on the higher expression of different genes encoding components of the MHC II complex (*LOC11049409*, *oncmk-dab*, *oncmk-dbb*) and an homologue of the zinc finger protein 366 (*LOC110536334*), a DC-specific marker in mammals (DC-SCRIPT) (24) (Figure 2B). The last one of these small myeloid groups represents a rare population of cells similar to the DC population, which also transcribes other genes encoding CD209 antigen-like protein C (*LOC110533211*) and E (*LOC110534781*) (Figure 2B). A small cell population of approximately 2% of total cells was defined as hematopoietic progenitor cells (HPCs) attending to the specific expression of an FLT3-like gene, also known as CD135, a common marker of this cell type in mice and human (25). Interestingly,

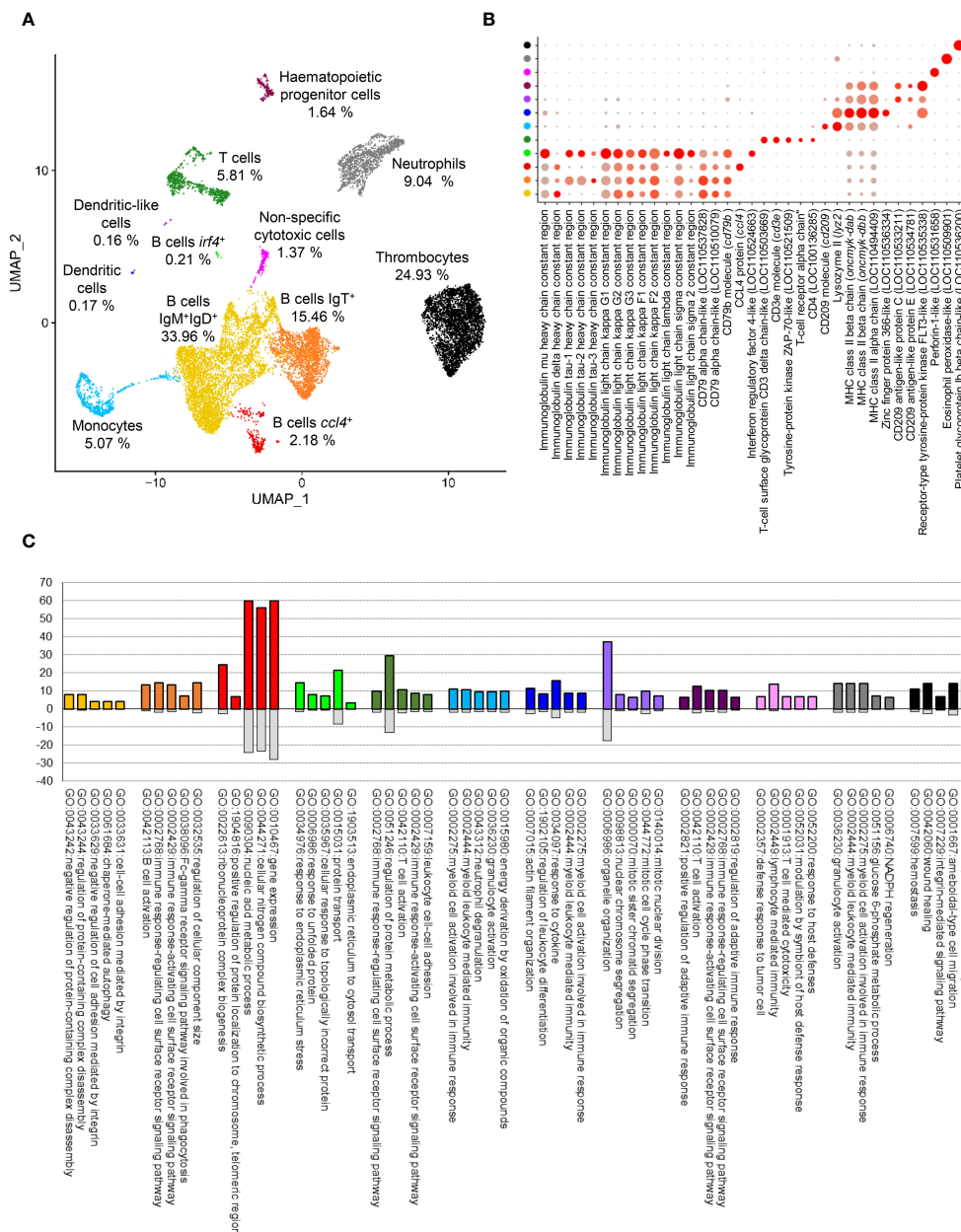


FIGURE 2 Clustering of integrated data and group identities. **(A)** UMAP visualization showing the different clusters obtained at a resolution of 0.15 and the percentage of cells in each one. **(B)** Dotplot representation of known markers for each cell type. **(C)** Top 5 GO-term showing significant enrichment at level 6 of 'Biological process' category in each cell cluster identified.

several cells within this population also express CD209 antigen-like protein C and E, which may indicate they preferentially correspond to myeloid progenitor cells. In fact, it has been shown that in mammals most lymphoid tissue DCs derive from Flt3⁺ progenitors whereas a few derive from Flt3⁻ monocytes and these DCs seem to maintain Flt3 in the steady-state (25). Therefore, it is difficult to unequivocally establish whether these cells correspond to a progenitor cell type or to yet another DC subset, but because they are clustered independently from other DC subtypes and given they lack some other DC markers, in this paper, we will refer to them as HPCs. The identity of the last three clusters identified during the analysis was easily assigned according to differentially expressed

genes. The first one was identified as NCCs, considered as evolutionary precursors of natural killer cells (NK) in teleost (7), defined by the expression of perforin-1-like gene (LOC110531658). The second one was clearly identified as a neutrophil population, attending to the expression of an eosinophil peroxidase-like gene (LOC110509901), previously associated to fish neutrophils (6). The final of these clusters is a highly represented one, corresponding to approximately 25% of total cells. The cells in this cluster corresponded to thrombocytes according to the expression of platelet glycoprotein Ib beta chain-like gene (LOC110536200) (Figure 2B). Once the different clusters were defined, an enrichment analysis was performed to identify functional

annotations associated with each PBL population (Figure 2C, Supplementary Figure S2, Supplementary Table S2).

3.3 Identification of novel markers and functions associated to rainbow trout PBL populations

In addition to the well-known markers previously mentioned, clear differences among rainbow trout PBL populations were detected during the analysis, both in the expression of coding genes and long non-coding RNAs (Supplementary Table S1) and in the enrichment of specific features or functions (Figure 2C, Supplementary Figure S2, Supplementary Table S2). For instance, monocytes showed higher values in total number of reads and in total number of expressed genes than other populations (Supplementary Figures S3A, B). On the other hand, both T and B cells show a clear enrichment in the percentage of reads mapping to ribosomal proteins (Supplementary Figure S3C), which also results in a higher expression of these genes, identifying several of them as markers in these populations (Supplementary Table S1). The expression of mitochondrial genes also showed an irregular distribution, with a higher percentage of reads mapping these genes in monocytes, DC-like cells, HPCs and neutrophils (Supplementary Figure S3D). The differential expression analysis also highlighted a set of coding genes and long non-coding RNAs which effectively differentiate at a transcriptional level among PBL populations (Tables 1, 2). These genes were differential taking into account transcripts from control and infected samples altogether, and also in control samples exclusively.

Analyzing B cells globally (the 4 clusters), the expression of a gene encoding a phospholipase A and acyltransferase 1-like (LOC110505920) was identified in approximately 85% of these cells and was only identified in 0.04% of other leukocyte subsets. Also, transcriptional activity of zinc finger proteins 34-like (LOC110500012) and 35-like (LOC110490446) was identified in 53 and 66% of B cells, respectively, whereas it was only expressed in 0.05-0.06% of other PBL populations (Table 1). In addition, an uncharacterized gene (LOC110533868), that codes for a protein that shows similarities with a domain from the ectropic viral integration site 2A protein (EVI2A), was also identified as preferentially expressed in B cells, both in IgM⁺IgD⁺ and IgT⁺ B cells. Specific markers differentiating B cell subpopulations were also identified. For instance, ~ 83% of cells included in the IgT⁺ B cell cluster express a prosaposin-like coding gene (LOC110509903). Finally, the *irf4*⁺ subpopulation identified as a plasma-like population showed differential expression of several enzymes, such as dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit-like (LOC110492237), protein disulfide-isomerase A6-like (LOC110529378) and A4-like (LOC110489602), expressed by approximately 90% of the cells from this group but only expressed in a 0.07-0.09% of cells from all other PBL subsets, including other B cell populations (Table 1). Different long non-coding RNAs also seemed strongly associated with this plasma-like cell subpopulation, such as LOC110499933

and LOC110526128, which are expressed in ~ 87% and 60% of cells, respectively (Table 2).

Concerning T cells, the expression of a gene encoding the protein S100-A5-like (LOC110520885) was observed in 82% of these cells at very high transcript levels. Other genes with expression highly associated with T cells encode an SH2 domain-containing protein 1A-like (LOC110530635), a PLAC8-like protein 1 (LOC110489105) or a granzyme K-like (LOC110523798) identified in at least 62% of T cells but transcribed at very low levels by other cells subsets (Table 1). Surprisingly, the percentage of cells expressing some common markers used for identity assignment were only identified in 30-50% of cells. Hence, for example, the transcription for the TCR α constant region was observed only in ~ 31% of cells and ZAP70 in ~ 47% of cells (Supplementary Table S1). The transcription of other known T cell markers such as CD8 or TCR β was practically residual (Supplementary Table S1).

Cells from the HPC population defined by the differential expression of an FLT3-like gene as previously mentioned, widely transcribed other interesting genes such as two genes encoding beta-galactoside-binding lectins (*leg* and LOC110512958), three genes encoding tetraspanin-8-like proteins (LOC110499719, LOC110499718 and LOC110499717), a low affinity immunoglobulin gamma Fc region receptor II-like protein (LOC110515346) and an osteoclast stimulatory transmembrane protein-like (LOC110505982) (Table 1, Supplementary Table S1). Additionally, cells included in this leukocyte population were also characterized by the transcription of one long non-coding RNA, LOC110524566, whose transcription was observed in 100% of cells while residually expressed in other PBL populations (0.3% of cells) (Table 2).

Monocytes singularly expressed genes coding for the protein Olfactomedin-4-like (LOC110513914 and LOC110494071) or the lysozyme C II (LOC110530635) or keratine-related proteins (LOC110528492 and *krt79*), among others (Table 1). Also, the long non-coding RNA, LOC110516627, which mapped the small inducible cytokine A13 (*ccl13*), was found to be present in ~ 56% of monocytes, while only in ~ 6% of the other leukocyte subsets (Table 2).

The gene encoding for the macrophage expressed 1 (*mpeg1*) was the most predominantly transcribed in DCs (Table 1). Other characteristic gene products from this PBL population were the myristoylated alanine-rich C-kinase substrate-like (LOC110529520), two ictacalcin-like (LOC110536275 and LOC110520890), and one uncharacterized gene (LOC110523838). In the other population of DC-like cells, genes encoding reductase subunits (LOC110529451 and *rrm1*) stood up, together with genes mapping topoisomerase DNA II alpha (*top2a*) or tubulin beta-1 chain (LOC110535859) (Table 1).

The genes that most distinguished neutrophils from the rest were those encoding choriolytic enzymes (*hce23* and LOC110526217) and complement factor D (*cfD*), while the most enriched gene products in thrombocytes were integrins (LOC110491676 and LOC110538030) and the coagulation factor XIII A chain-like (LOC110500457) (Table 1).

TABLE 1 Top 5 protein coding genes showing the highest differences in percentage of cells expressing these genes between PBL populations.

PBL population	Gene	avg_logFC	pct.1	pct.2	p_val_adj
All B cells	Zinc finger protein 34-like (LOC110500012), transcript variant X1	260,940	0.529	0.047	0
	Uncharacterized LOC110490088 (LOC110490088)	1.102	0.638	0.038	0
	Zinc finger protein 35-like (LOC110490446)	11.885	0.655	0.055	0
	C-C chemokine receptor type 9-like (<i>ccr9</i>)	4.208	0.829	0.252	0
	Phospholipase A and acyltransferase 1-like (LOC110505920)	24.286	0.841	0.044	0
a) B cells IgM ⁺ IgD ⁺	Uncharacterized LOC110533868 (LOC110533868)	0.681	0.639	0.2	0
	C-C chemokine receptor type 9-like (<i>ccr9</i>)	1.609	0.811	0.416	0
	B-cell linker protein-like (LOC110526728)	0.264	0.54	0.175	0
	Glycerol-3-phosphate acyltransferase 3-like (LOC110520337)	0.793	0.874	0.534	0
	Elongation factor 2 (LOC110524284)	0.348	0.848	0.517	0
b) B cells IgT ⁺	Prosaposin-like (LOC110509903)	3.608	0.824	0.146	0
	Spectrin beta chain, non-erythrocytic 1-like (LOC110530138)	0.957	0.656	0.235	0
	Myocyte enhancer factor 2C (<i>mef2c</i>)	1.632	0.659	0.267	0
	Uncharacterized LOC110533868 (LOC110533868)	0.452	0.664	0.289	8.8E-243
	Gamma-adducin-like (LOC110485619)	1.613	0.596	0.237	4.7E-273
c) B cells <i>ccl4</i> ⁺	C-C motif chemokine 4-like (LOC110494096)	220.790	0.547	0.009	0
	Serine/threonine-protein kinase pim-2-like (LOC110492581)	26.737	0.649	0.128	2.8E-178
	Jun proto-oncogene (<i>jun</i>)	17.095	0.525	0.047	5.6E-294
	NHP2-like protein 1 (LOC110499221)	11.654	0.599	0.15	3.2E-122
	NOP56 ribonucleoprotein (<i>nop56</i>)	6.328	0.509	0.081	2.0E-164
d) B cells <i>ifr4</i> ⁺	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit-like (LOC110492237)	7.086	0.9	0.068	1.6E-75
	Protein disulfide-isomerase A6-like (LOC110529378)	11.801	0.9	0.091	4.6E-57
	Cysteine-rich with EGF-like domain protein 2 (LOC110490158)	3.928	0.9	0.097	2.1E-50
	Thioredoxin domain-containing protein 5-like (LOC110535240)	19.607	0.8	0.024	9.6E-165
	Protein disulfide-isomerase A4-like (LOC110489602)	4.168	0.833	0.064	6.9E-67
T cells	Protein S100-A5-like (LOC110520885)	8.960	0.82	0.018	0
	SH2 domain-containing protein 1A-like (LOC110530635)	4.583	0.673	0.011	0
	PLAC8-like protein 1 (LOC110489105)	4.801	0.762	0.101	0
	Granzyme K-like (LOC110523798)	9.800	0.624	0.002	0
	Death-associated protein-like 1-A (LOC110520057)	10.734	0.802	0.2	0
Monocytes	Olfactomedin-4-like (LOC110513914)	34.517	0.802	0.003	0
	Lysozyme C II (LOC110523157)	20.949	0.827	0.032	0
	Keratin, type I cytoskeletal 18-like (LOC110528492)	16.927	0.812	0.02	0
	Olfactomedin-4-like (LOC110494071)	22.396	0.786	0.001	0
	Simple type II keratin K8b (S2) (<i>krt79</i>)	25.830	0.802	0.023	0
Dendritic cells	Macrophage expressed 1 (<i>mpeg1</i>)	5.248	1	0.039	5.5E-136

(Continued)

TABLE 1 Continued

PBL population	Gene	avg_logFC	pct.1	pct.2	p_val_adj
	Uncharacterized LOC110523838 (LOC110523838)	10.442	0.962	0.054	2.5E-92
	Ictacalcin-like (LOC110536275)	9.209	0.923	0.077	4.2E-59
	Myristoylated alanine-rich C-kinase substrate-like (LOC110529520)	5.509	0.846	0.034	8.4E-112
	Ictacalcin-like (LOC110520890)	8.539	0.923	0.122	3.4E-34
Dendritic-like cells	Ribonucleoside-diphosphate reductase subunit M2 (LOC110529451)	1.240	1	0.082	9.6E-58
	Topoisomerase (DNA) II alpha (<i>top2a</i>)	1.467	0.955	0.046	1.5E-91
	Ribonucleotide reductase catalytic subunit M1 (<i>rrm1</i>)	2.934	0.955	0.066	1.7E-63
	Tubulin beta-1 chain (LOC110535859)	11.400	0.955	0.087	3.9E-48
	PCNA-associated factor-like (LOC110522455)	6.271	0.864	0.019	5.9E-169
Haematopoietic progenitor cells	Beta-galactoside-binding lectin (<i>leg</i>)	58.116	0.863	0.087	0
	Tetraspanin-8-like (LOC110499719)	1.460	0.765	0.003	0
	Low affinity immunoglobulin gamma Fc region receptor II-like (LOC110515346)	6.177	0.697	0.047	0
	Osteoclast stimulatory transmembrane protein-like (LOC110505982)	5.555	0.637	0.025	0
	Beta-galactoside-binding lectin-like (LOC110512958)	4.691	0.564	0.019	0
Non-specific cytotoxic cells	Eotaxin-like (LOC110490824)	2.014	0.486	0.005	0
	Membrane-spanning 4-domains subfamily A member 4D-like (LOC110505995)	1.737	0.471	0.003	0
	Glycine-rich RNA-binding protein 1-like (LOC110526019)	0.250	0.678	0.252	7.4E-54
	Perforin-1-like (LOC110500520)	1.641	0.399	0.016	0
	FYN-binding protein-like (LOC110536305)	0.728	0.351	0.01	0
Neutrophils	Complement factor D (adipsin) (<i>cfid</i>)	79.281	0.817	0.005	0
	Leukotriene A-4 hydrolase-like (LOC110500254)	12.585	0.814	0.035	0
	High choriolytic enzyme 1 (<i>hce23</i>)	69.794	0.767	0.004	0
	Low choriolytic enzyme-like (LOC110526217)	54.283	0.759	0.003	0
	Uncharacterized LOC110528322 (LOC110528322)	10.787	0.816	0.089	0
Thrombocytes	Integrin beta-3-like (LOC110491676)	17.068	0.989	0.008	0
	Integrin alpha-IIb-like (LOC110538030)	8.725	0.988	0.01	0
	Coagulation factor XIII A chain-like (LOC110500457)	30.007	0.999	0.022	0
	Hyaluronidase-3-like (LOC110492323)	8.496	0.978	0.012	0
	Connective tissue growth factor-like (LOC110522165)	30.0752	0.965	0.01	0

3.4 Differential gene expression of rainbow trout PBL subpopulations during IPNV *in vitro* exposure

After having defined the transcriptional profile of the different leukocyte populations in rainbow trout blood, we proceeded to analyze the differential expression between control and IPNV infected cells, which allowed the identification of several genes that significantly altered their transcript levels in response to viral

exposure. A first analysis was conducted treating the expression data like a bulk RNA-seq (without differentiating among leukocyte subsets) with an adjusted p value < 0.001 and an average $\log_2FC > 0.25$. This initial analysis identified a total of 497 transcripts that were significantly modified their transcript levels in response to IPNV (Figure 3A, Supplementary Table S3). Among them, approximately 67% corresponded with protein-coding transcripts whereas approximately 29%, 3% and 1.6% represent ribosomal proteins, long non-coding RNAs and mitochondrial genes,

TABLE 2 Long non-coding RNAs showing significant differences between populations.

PBL population	Gene	avg_logFC	pct.1	pct.2	p_val_adj
B cells IgM⁺IgD⁺	Uncharacterized LOC110490117	0.354	0.278	0.12	2.735E-121
	Uncharacterized LOC110488454	0.277	0.354	0.223	3.847E-61
B cells IgT⁺	Uncharacterized LOC110520552	4.233	0.837	0.478	4.456E-303
	Uncharacterized LOC110490117	0.394	0.34	0.142	2.556E-113
B cells <i>ccl4</i>⁺	Uncharacterized LOC110496411	4.027	0.429	0.116	2.8435E-65
B cells <i>ifr4</i>⁺	Uncharacterized LOC110499933	45.533	0.867	0.197	4.1436E-20
	Uncharacterized LOC110526128	7.316	0.6	0.097	3.2592E-17
	Uncharacterized LOC110487501	8.868	0.467	0.015	5.8973E-85
T cells	Uncharacterized LOC110496534	11.546	0.56	0.061	0
	Uncharacterized LOC110495953	4.265	0.479	0.001	0
	Uncharacterized LOC110526706	0.897	0.369	0.061	7.984E-231
Monocytes	Uncharacterized LOC110516627	8.011	0.559	0.004	0
	Uncharacterized LOC110504793	2.135	0.508	0.008	0
	Uncharacterized LOC110505986	21.418	0.485	0.008	0
Dendritic cells	Uncharacterized LOC110518168	18.865	1	0.198	1.7221E-30
	Uncharacterized LOC110521442	165.327	1	0.24	1.2234E-22
	Uncharacterized LOC110507655	6.9117	0.692	0.016	2.59E-149
Rare dendritic-like cells	Uncharacterized LOC110489840	16.495	0.955	0.422	1.8421E-06
	Uncharacterized LOC110519496	1.633	0.591	0.062	4.4261E-21
	Uncharacterized LOC110515983	1.235	0.864	0.413	0.00040676
Haematopoietic progenitor cells	Uncharacterized LOC110524566	57.116	1	0.003	0
	Uncharacterized LOC110519670	2.854	0.449	0.013	0
	Uncharacterized LOC110511519	1.553	0.483	0.066	2.346E-133
Non cytotoxic cells	Uncharacterized LOC110515983	2.502	0.678	0.41	5.1177E-17
		0.390	0.26	0.104	3.7576E-09

(Continued)

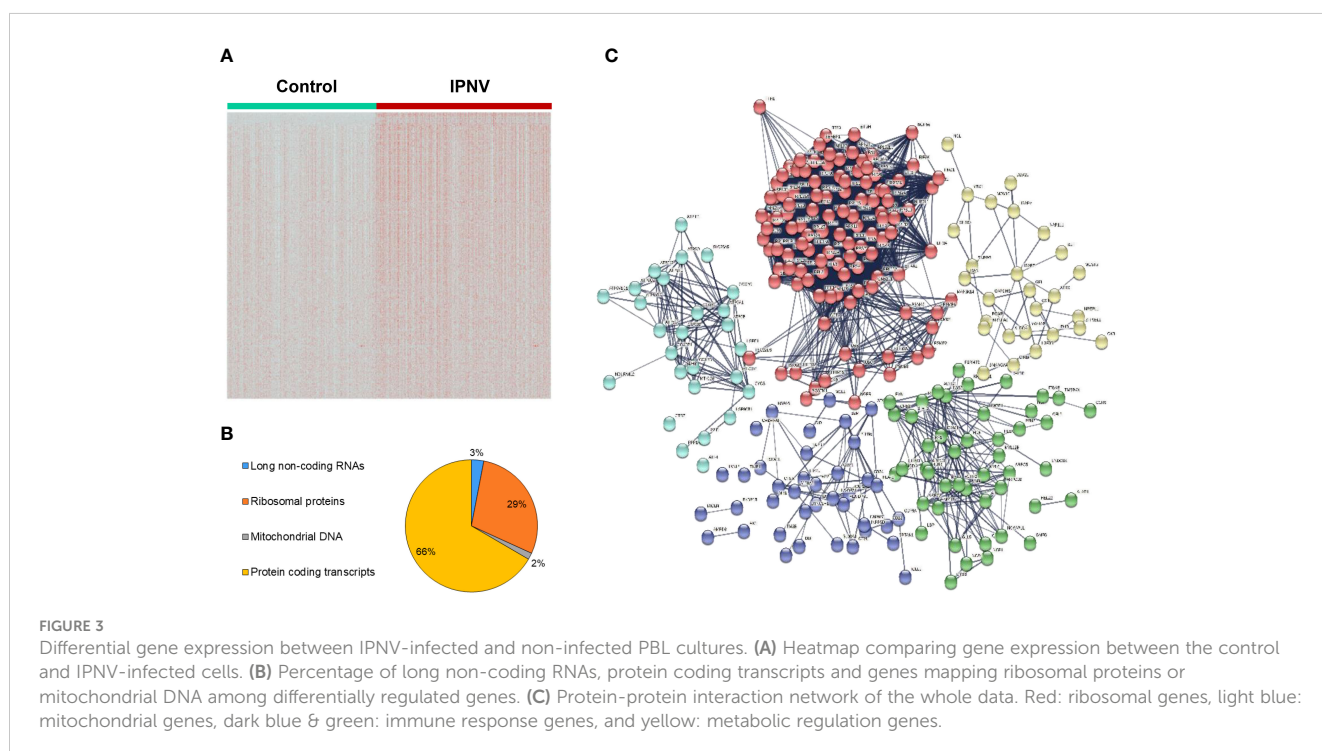
TABLE 2 Continued

PBL population	Gene	avg_logFC	pct.1	pct.2	p_val_adj
	Uncharacterized LOC110493925				
Neutrophils	Uncharacterized LOC110519496	1.559	0.403	0.029	0
	Uncharacterized LOC110537289	1.000	0.366	0.068	6.271E-273
	Uncharacterized LOC110529372	1.409	0.297	0.001	0
Thrombocytes	Uncharacterized LOC110527700	1.582	0.433	0.003	0
	Uncharacterized LOC110519161	0.553	0.274	0.013	0
	Uncharacterized LOC110537023	0.567	0.279	0.046	0

respectively (Figure 3B). This distribution was also observed in the protein-protein interaction network obtained (Figure 3C), where a condensed cluster (red nodes) corresponds to different components of ribosomes together with other proteins mainly annotated as elongation factors or proteasome subunits. Another cluster, represented by light blue nodes, grouped the different mitochondrial genes together with nuclear genes involved in mitochondrial functions, for instance ATP-synthases or cytochrome oxidase subunits. The dark blue cluster grouped proteins associated with immune response related to functions like antigen processing and presentation (*mhc I* and *mhc II*) and also genes involved in immune effector processes (*stat1*, *ifi44* or *ptpn6*, among others). The green cluster, also involved in the

immune response, grouped several genes involved in cell surface receptors signaling pathways (f.i. *stat3*, *irf1*, *ccr9*, *ilr6r* or *lcp1*) as well as a set of genes involved in phagocytosis including several family members of actin-related proteins. Finally, the yellow cluster joined several proteins involved in the regulation of gene expression as well as other metabolic processes (Figure 3C).

In a second step, the differential expression was studied within different leukocyte subpopulations. These studies rendered some interesting results concerning both shared and specific molecular responses. Monocytes and neutrophils showed the highest number of upregulated protein-coding genes in response to IPNV, with 267 and 233, respectively (Figure 4A). Both IgM⁺IgD⁺ and IgT⁺ B cells also experienced an important upregulation of genes (152 and 154,



respectively) in response to virus exposure (Figure 4A), whereas the B cells defined by *ccl4* transcription only up-regulated 13 protein-coding genes in response to IPNV. In T cells and thrombocytes, also a moderate number of protein-coding genes (94 and 112, respectively) experienced upregulation upon the *in vitro* infection (Figure 4A). The number of down-regulated genes in response to IPNV in all these populations was much lower, with no genes down-regulated in *ccl4*⁺ B cells (Figure 4A). Interestingly, plasma-like cells (*irf4*⁺ B cells), DC-like cell populations, NCC and HPCs did not experience regulation of any or a reduced number of genes in response to the virus within the established threshold (Figure 4A). Remarkably, the populations that up-regulated the higher number of protein-coding genes, also experienced important changes in the transcription levels of a high number of ribosomal proteins in response to the virus, with IgM⁺IgD⁺ and IgT⁺ B cells experiencing changes in a higher number of genes (Figure 4A). Finally, some long non-coding RNAs were also modulated in response to IPNV in these same populations (Figure 4A).

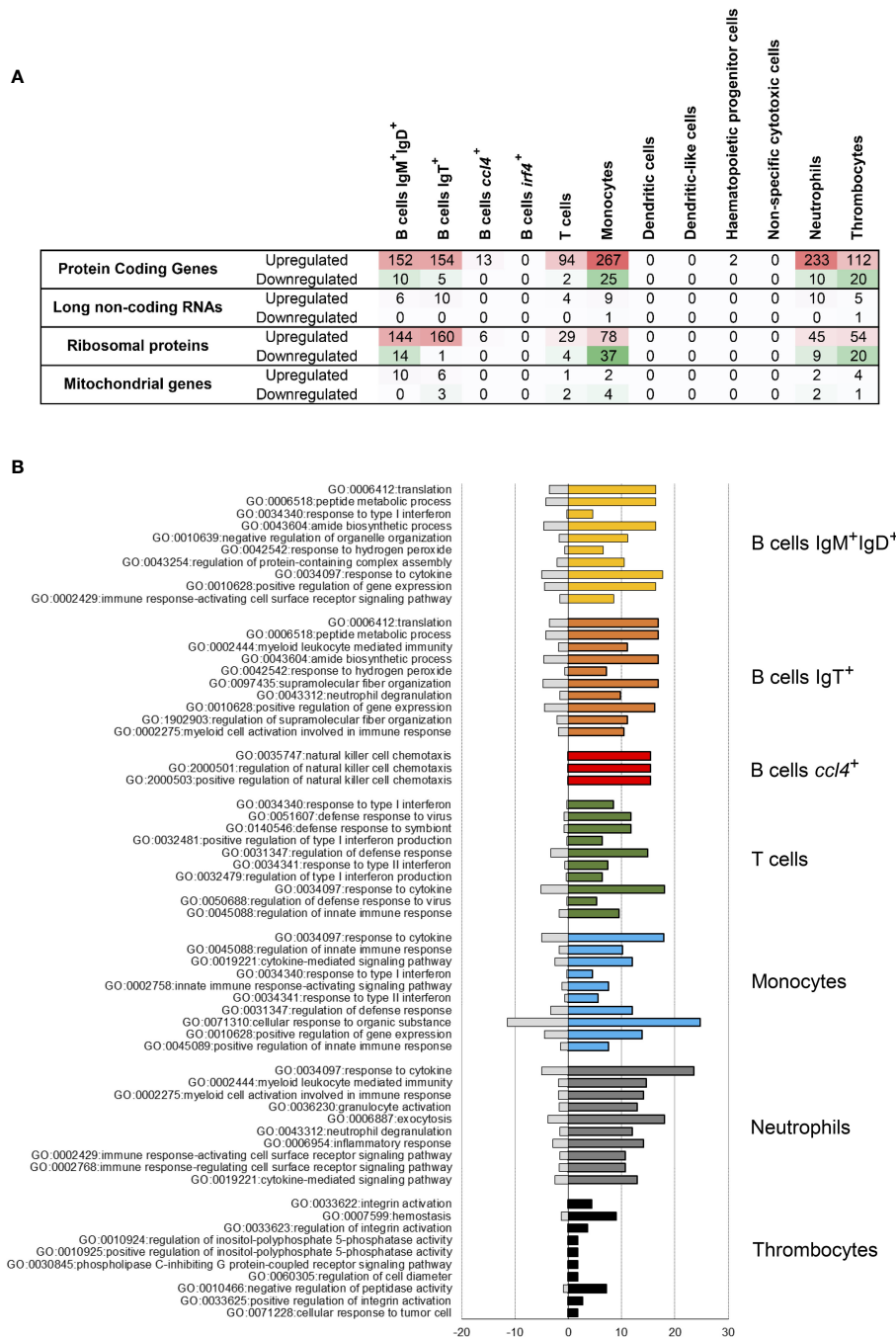
A functional enrichment analysis of the genes modulated in response to the virus pointed to several functionalities within upregulated genes identified in the PBL populations that were mainly affected by IPNV (Figure 4B, Supplementary Table S4). Interestingly, genes related to response to cytokines (GO:0034097) are among the most regulated genes for all the cell types mentioned above. Additionally, the genes regulated in both IgM⁺IgD⁺ and IgT⁺ B cells share some functions, such as peptide metabolic process (GO:0006518), amide biosynthetic process (GO:0043604) and positive regulation of gene expression (GO:0010628). Nonetheless, IgT⁺ B cells show a high expression of genes related to myeloid leukocyte mediated immunity (GO:0002444) or myeloid cell activation involved in immune response (GO:0002275). On the other hand, some of the genes regulated in *ccl4*⁺ B cells are connected to the natural killer cell chemotaxis (GO:0035747, GO:2000501, GO:2000503). The functional adscription of genes regulated in T cells in response to the virus suggest an important role in the antiviral defense, with genes ascribed to response to virus (GO:0051607), type I-II interferon (IFN) (GO:0034340, GO:0032481, GO:0034341) and response to symbiont (GO:0140546). In addition, genes related to the regulation of defense response (GO:0031347) and innate immune response (GO:0045088) are also upregulated in T cells. Monocytes stand out because of their high expression of genes involved in the cellular response to organic substance (GO:0071310), but also those related to innate immune responses (GO:0045088, GO:0045089, GO:0002758). As expected, most of the genes regulated in neutrophils are associated with inflammatory response (GO:0006954), as well as with neutrophil degranulation (GO:0043312). Granulocyte activation (GO:0036230) and exocytosis (GO:0006887) genes are also strongly represented among the regulated genes in neutrophils. Finally, the genes regulated in thrombocytes in response to the virus are functionally related to hemostasis (GO:0007599), negative regulation of peptidase activity (GO:0010466) and integrin activation (GO:0033622, GO:0033623, GO:0033625). Other functions shared among the genes regulated by several cell types include myeloid leukocyte mediated immunity (GO:0002444,

GO:0002275) and immune response-activating cell surface receptor signaling pathway (GO:0002768) (Supplementary Table S4).

When we performed a focused analysis on upregulated protein-coding genes among the most responsive populations, we identified a set of transcripts that was significantly upregulated at the same time in several PBL subpopulations indicating that the virus is able to induce a common molecular pathway (Figures 5A, B). These eight genes widely induced in cells from all affected PBL subpopulations included genes coding for two helicase with zinc finger 2 (*helz2* and LOC110528534), a CD9 antigen-like (LOC110486460), a E3 ubiquitin-protein ligase rnf213-beta-like (LOC110502787), a tripartite motif-containing protein 16-like (LOC110513115), a sascin-like (LOC110505631), a galectin-9-like (LOC110533869) and an IFN-induced very large GTPase 1 (LOC110486424) (Figure 5B). Another set of genes was widely stimulated in response to virus in at least three different PBL populations. These genes were grouped in important gene families such as, for instance, VHSV-induced proteins (LOC100135997 and LOC100136003), polyubiquitin-like proteins (LOC110536126, LOC110536134 and LOC100135966), different E3 ubiquitin-protein ligases rnf213-type alpha-like (LOC110528546, LOC110508243 and LOC110485301), RNF144A (LOC110500498), E3 ubiquitin/ISG15 ligase TRIM25-like (LOC110485965, LOC110516270 and LOC110518678) and additional genes encoding tripartite motif-containing protein 16-like (LOC110516150 and LOC110521165) and 47-like (LOC110522087), among others (Supplementary Table S5).

Analyzing each PBL subpopulation, some of the genes significantly upregulated at transcriptional level in response to IPNV were exclusive to only one or two of them (Figures 5A, C). In an overview, a set of 34 genes was exclusively upregulated in IgM⁺IgD⁺ B cells, whereas a total of 43 genes were singularly upregulated in IgT⁺ B cells. Both B cell groups share 31 upregulated genes. T cells show only 14 unique, upregulated genes, increasing this quantity up to 36 together with monocytes. Additionally, monocytes upregulated 129 distinct genes. A similar number of unique, upregulated genes were found in neutrophils. Finally, 52 of these genes were uniquely upregulated in thrombocytes (Figure 5A).

Several genes previously identified as markers for B cells showed a significant increase at transcriptional level in response to IPNV, such as the Ig mu and delta heavy chains in IgM⁺IgD⁺ B cells, and the immunoglobulins tau 1, tau 2 and tau 3 heavy chains in IgT⁺ B cells, or the B cell receptor (LOC110537828) and CD79b (*cd79b*) in both. Similarly, different Ig light chains increased their levels of transcription in response to IPNV, such as kappa G1, kappa G3 and kappa F2 in both groups of B cells and sigma in IgT⁺ B cells. Interestingly, kappa F1 and kappa G2 showed an opposite trend in these B cell populations, with the first one increasing its transcriptional level in IgM⁺IgD⁺ B cells and decreasing it in IgT⁺ B cells upon virus exposure, and the second one showing an opposite tendency. The interferon inducible protein (*iip*) also stands up in both groups while the interferon alpha/beta receptor 2 (LOC110501424) only in IgM⁺IgD⁺ B cells. Related to elongation factor activity, several elongation factors (LOC110496143, LOC110538825, LOC110489228, LOC110524284, LOC110508425,



LOC110521428, LOC110530688) were upregulated in both B cell types by the virus (Figure 5C, Supplementary Tables S5, S6). Regarding *ccl4*⁺ B cells, the most significant markers for this B cell population, *CCL4* protein (*ccl4*) and C-C motif chemokine 4 (LOC110494096) also experienced a remarkable transcriptional upregulation in response to IPNV (Table 3). The interferon-induced protein 44-like was also highly expressed in this group, as in monocytes. In this last group, several genes encoding

interferon-induced proteins (LOC110498001, LOC110538600, *mx*, LOC110494492, LOC110494493, LOC110488243, LOC110522342, and LOC110522343), E3 ubiquitin-protein ligases (DTX3L – LOC110494241, TRIM39 – LOC110500407, and LOC110531526), and tripartite motif-containing protein 16 (TRIM16 – LOC110489932, LOC110499822, LOC110513989, LOC110518413, and LOC110521260) or 47 (TRIM47 – LOC110503178) were also transcriptionally regulated (Supplementary Table S5). In a similar

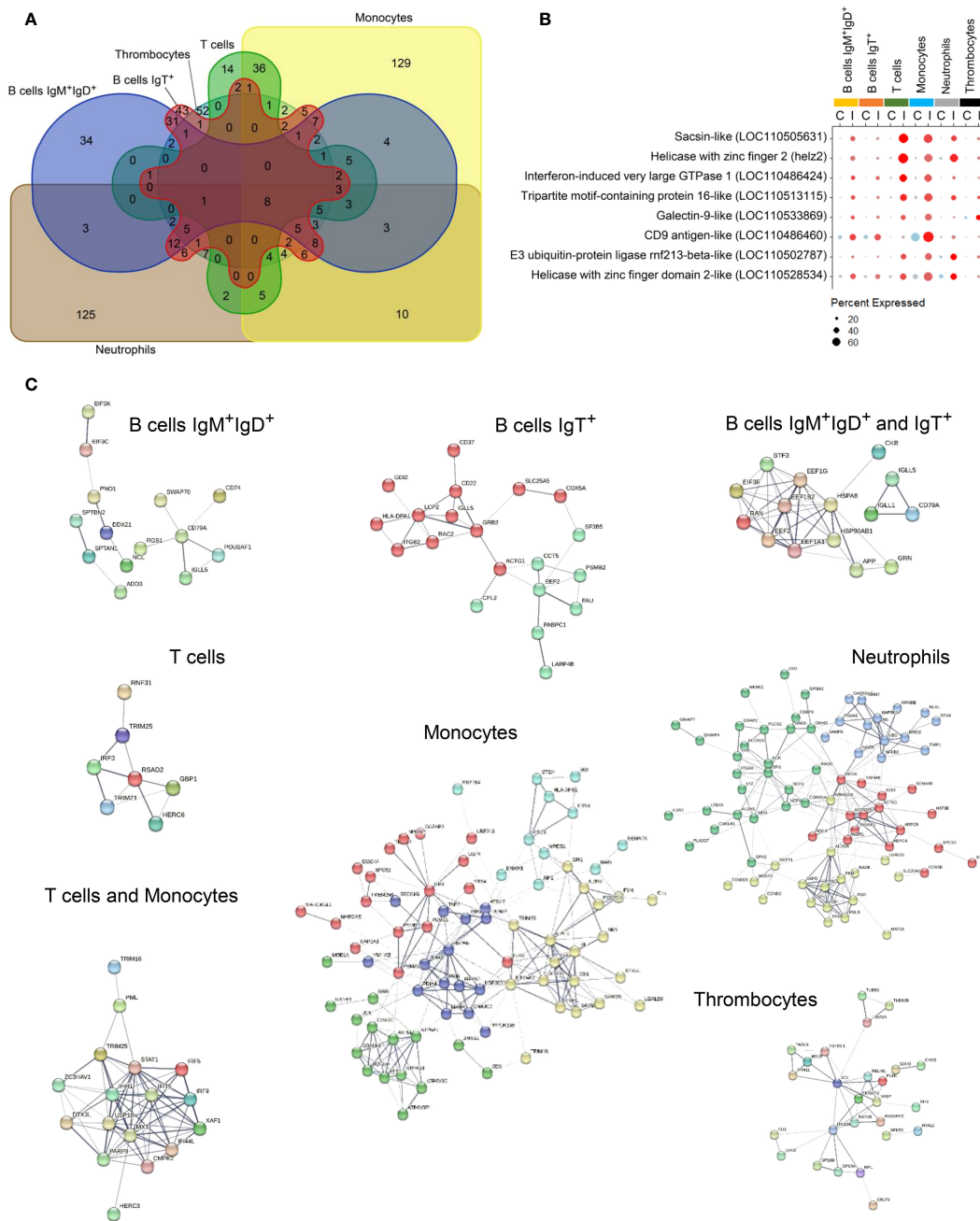


FIGURE 5 Upregulated gene distribution through those clusters with different gene expression after the infection. **(A)** Venn diagram comparing the number of genes unique to each cluster and those shared between two or more subpopulations. **(B)** Dotplot showing the transcription levels of genes commonly upregulated in response to IPNV in IgM⁺IgD⁺ B cells, IgT⁺ B cells, ccl4⁺ B cells, T cells, monocytes, neutrophils, and thrombocytes. **(C)** Protein-protein interaction network of genes upregulated in one or more clusters.

way, genes shared between monocytes and T cells included those encoding E3 ubiquitin-protein ligases (DTX3L – LOC110528193, RNF144A – LOC110537403, and TRIM25 – LOC110515277) together with other genes annotated as VHSV-induced proteins (VIG) (4 – LOC100135996, 9 – *Igals9*, and 10 – LOC100135999) and VIG-2 protein (*vig2*), and other important genes involved in the interferon-mediated response to viruses such as that coding for the Mx2 protein (*mx2*) or the interferon regulatory factor 3 (*irf3*), 8 (LOC110535315), and the interferon-induced protein 44 (*ifi44l* and

LOC110491927), among others (Figure 5C, Supplementary Tables S5, S6). T cells alone regulate in response to the virus E3 ubiquitin-protein ligase HERC6 (LOC110498430, and LOC110498455), TRIM16 (LOC110491260, and LOC110517410), TRIM39 (LOC110531504), TRIM47 (LOC110503177), RNF31 (LOC110489697), and interferon-induced protein 44 (LOC110494012) (Supplementary Table S5). In neutrophils, upregulated genes encode proteins related to leukocyte activation/degranulation, such as interleukin-1 receptor type II (*il-1rii*),

TABLE 3 Top 5 protein coding genes differentially expressed in response to IPNV specifically in one cell type.

ID Rainbow trout	Best Hit Human Proteins	Description	avg logFC	pct.1	pct.2	p_val_adj
B cells IgM⁺IgD⁺						
XM_021578985.1	NP_055247.3	Interferon alpha/beta receptor 2 (LOC110501424)	-3.16	0.043	0.211	9.02E-64
XM_021563839.1	NP_073587.1	Poly [ADP-ribose] polymerase 12-like (LOC110490445)	-0.27	0.05	0.139	9.92E-22
IgD		Immunoglobulin delta heavy chain constant region	-3.62	0.561	0.642	3.74E-11
XM_021576525.1	NP_004719.2	Nucleolar RNA helicase 2-like (LOC110499400)	-0.33	0.229	0.319	6.34E-09
XM_021566733.1	NP_659443.1	Uncharacterized LOC110492423 (LOC110492423)	-0.28	0.286	0.377	7.22E-08
B cells IgT⁺						
Igsigma	NP_001243225.1	Immunoglobulin light chain sigma constant region	-2.02	0.872	0.913	1.18E-09
XM_021606086.1	NP_002769.1	Prosaposin-like (LOC110525711)	-0.88	0.663	0.762	7.46E-08
XM_021563840.1	NP_085116.2	Zinc finger protein 35-like (LOC110490446)	-1.81	0.645	0.757	2.63E-07
NM_001165059.1	NP_001302.1	Cysteine-rich protein 1 (<i>crip1</i>)	-1.67	0.516	0.625	3.87E-06
XM_021569416.1	NP_291032.2	H-2 class II histocompatibility antigen, A-B alpha chain-like (LOC110494409)	-0.92	0.457	0.552	1.16E-05
B cells ccl4⁺						
XM_021568818.1	NP_066286.1	C-C motif chemokine 4-like (LOC110494096)	-232.23	0.265	0.677	2.10E-09
NM_001124489.2	NP_066286.1	CCL4 protein (<i>ccl4</i>)	-610.10	0.529	0.741	4.08E-06
T cells						
XM_021568674.1	XP_011538818.1	Interferon-induced protein 44-like (LOC110494012)	-16.69	0.19	0.453	1.84E-14
XM_021575046.1	NP_060382.3	Probable E3 ubiquitin-protein ligase HERC6 (LOC110498430)	-0.47	0.008	0.189	1.60E-13
XM_021575077.1	NP_060382.3	Probable E3 ubiquitin-protein ligase HERC6 (LOC110498455)	-0.62	0	0.161	1.64E-12
NM_001124253.1	NP_542388.2	Viperin (<i>vig1</i>)	-0.96	0	0.152	1.50E-11
XM_021573594.1		Uncharacterized LOC110497472 (LOC110497472)	-17.55	0.575	0.731	4.91E-09
Monocytes						
NM_001124324.1	NP_001760.1	CD9 protein (LOC100135986)	-5.03	0.15	0.505	1.67E-23
XM_021577767.1	NP_742013.1	E3 ubiquitin-protein ligase TRIM39-like (LOC110500407)	-0.90	0.006	0.286	1.59E-21
XM_021587750.1	NP_000557.1	High affinity immunoglobulin gamma Fc receptor I-like (LOC110507636)	-23.28	0.303	0.616	2.61E-18
XM_021573049.1	NP_004020.1	Interferon regulatory factor 3-like (LOC110497044)	-1.23	0.02	0.262	3.45E-16
XM_021600673.1	NP_006811.2	Interferon-induced protein 44-like (LOC110522343)	-0.53	0.025	0.249	1.13E-13
Neutrophils						
XM_021561680.1	XP_005268438.1	PLAC8-like protein 1 (LOC110489105)	-12.85	0.726	0.868	4.21E-17
NM_001124479.1		CC chemokine CK-2.1 (<i>ck-2.1</i>)	-22.35	0.127	0.344	5.38E-17
XM_021574196.1	NP_006282.2	Tumor necrosis factor alpha-induced protein 2-like (LOC110497812)	-1.26	0.148	0.323	1.27E-10
XM_021603966.1	NP_951009.1	MAP kinase-interacting serine/threonine-protein kinase 2-like (LOC110524378)	-0.80	0.085	0.244	1.71E-10
XM_021591098.1	NP_000241.1	Eosinophil peroxidase-like (LOC110509901)	-49.80	0.989	0.993	7.75E-10

(Continued)

TABLE 3 Continued

ID Rainbow trout	Best Hit Human Proteins	Description	avg logFC	pct.1	pct.2	p_val_adj
Thrombocytes						
XM_021606237.1	NP_000398.1	Platelet glycoprotein Ib beta chain-like (LOC110525802)	-1.97	0.955	0.973	1.39E-26
XM_021624569.1	NP_000410.2	Integrin alpha-IIb-like (LOC110538030)	-1.30	0.984	0.991	9.84E-22
XM_021566541.1	NP_003764.3	Hyaluronidase-3-like (LOC110492323)	-0.76	0.972	0.983	1.33E-20
XM_021570096.1	NP_821080.1	Tubulin beta-6 chain-like (LOC110494765)	-4.81	0.8	0.859	1.34E-19
XM_021560543.1		CXADR-like membrane protein (LOC110488335)	-1.05	0.759	0.828	5.45E-15

Fold change (FC) values are obtained dividing control by infected values, therefore negative logFC indicate overexpression of genes in infected samples.

chemokine receptor 1 (LOC110538268), CC chemokine CK-2.1 (*ck-2.1*) or platelet-activating factor (LOC110529826), as well as transcripts for NF-kappa-B p100 subunit (LOC110502378, and LOC110527634), proteasome subunits (alpha 5 - *psma5*, and beta 7 - LOC110488346), polyubiquitin-B (LOC110523403) or E3 ubiquitin-protein ligase DTX1 (LOC110525574). Finally, thrombocytes showed increased expression levels of genes encoding platelet glycoproteins (LOC110500812, and LOC110525802), thrombopoietin receptor (LOC110521821, and LOC110524756), cytokine receptor factor 3 (LOC110539053) or tubulin (α - LOC110501960, LOC110511912; β -3 - LOC110526471; β -6 - LOC110494765) (Supplementary Table S5).

4 Discussion

4.1 Single-cell atlas or rainbow trout PBLs

In the current study, we have used total rainbow trout PBLs to establish a transcriptional atlas of a range of rainbow trout leukocyte subsets, to then study the early response of all these leukocyte subtypes to a viral encounter. Because in previous single-cell sequencing experiments in which we analysed blood B cells in rainbow trout by means of single cell transcriptomics, we sorted cells by means of small size, low complexity and high MHC II expression to focus our analysis on B cells (9, 10), these previous studies never identified transcriptional patterns associated with plasma-like cells. In the current study, a small population identified clearly corresponds to this cell type, showing a high expression of Ig mu heavy chain (IgM) and *irf4*, but no expression of the Ig delta heavy chain (IgD). We had previously determined by means of flow cytometry that fish B cells lose surface IgD upon differentiation (26), similarly to mammalian B cells (27). Remarkably, an enrichment analysis highlighted the transcription of genes related with GO terms associated with endoplasmic reticulum development and differentiation. Again, flow cytometry studies have performed in rainbow trout recently have also established endoplasmic reticulum expansion as a characteristic trait of differentiated plasmablasts/plasma cells (28). An additional B cell subset was identified among PBLs, characterized by the transcription of *ccl4*. This *ccl4*⁺ B cell subset had been also

reported in previous single-cell studies performed with rainbow trout blood B cells by our group (9) and in carp (29). As *CCL4* seems to be closely related to mammalian *CCL5*, which is known to have the capacity to attract Th cells, it has been suggested that this population might specifically be focused on doing this, also in teleost fish. Regarding IgT⁺ B cells, the majority of cells expressed a prosaposin-like coding gene (LOC110509903). The protein synthesized, prosaposin, is a precursor of four different saposins. In the tongue sole (*Cynoglossus semilaevis*), a peptide derived from saposins was shown to exhibit a significant antimicrobial activity (30), suggesting a possible antimicrobial role for IgT⁺ B cells. Additionally, these cells are also characterized by the transcription of myocyte enhancer factor 2C (*mef2c*). In mice, the protein synthesized is important for B cell proliferation after BCR engagement, upon phosphorylation by p38 MAPK (31).

Among the different markers identified in B cells in this study, one gene stands up for its broad transcription in all B cells in comparison to other populations, the phospholipase A and acyltransferase 1-like. The role of this gene in the synthesis of N-acyl ethanolamines (NAEs) has been studied in humans and mice (32), yet their role in B cell function is still not clear. Other genes expressed at significantly higher levels in B cells when compared to other cell types include the zinc finger proteins 34-like (*znf34*) and 35-like (*znf35*). The first one has been shown to play an important role in the immune response against viruses by promoting the expression of type I IFN. Thus, for example, PRRSV (Porcine Reproductive and Respiratory Syndrome Virus) was shown to inhibit a long non-coding RNA and, consequently ZNF34, not allowing type I IFN expression and facilitating viral replication (33). ZNF35, on the other hand, binds to the rs10924104-A allele and promotes the expression of CD58 in B lymphocytes (34). Finally, the uncharacterized gene (LOC110533868), another highly expressed transcript in rainbow trout B cells, codes for EVI2A. Even though the function of this protein is still not clearly defined, it is thought to be related to BCR function as a lymphocyte-specific tumor suppressor (35).

In T cells, the most highly expressed gene was the one encoding the S100-A5-like protein. In humans, this protein has been identified in epithelial cells as a target for immunotherapy against bladder cancer. It is known to inhibit the secretion of inflammatory-related chemokines and the recruitment of CD8⁺ T cells (36).

However, this protein was residually expressed in human peripheral blood cells (36), therefore, the biological role it plays in rainbow trout T cells remains to be defined. Another gene, which synthesizes the SH2 domain-containing protein 1A-like, is also overexpressed in T cells when compared to other cell subsets. This protein is present in activated T cells and NK cells in humans, regulating their immune functions (37). The gene encoding PLAC8 protein, also highly expressed in rainbow trout T cells, has been reported to play a role in inflammatory response by Th cells (38). Granzymes are known to play an important role in cell-mediated cytotoxic capacities of T cells and NK cells, in both mammals and fish (39). In this study, a granzyme K-like was also highly transcribed in T cells.

Rainbow trout monocytes highly transcribed olfactomedin-4, which has been reported to be present in a specific subset of human and murine neutrophils (40). Additionally, these cells transcribe lysozyme C-II, a c-type lysozyme with described roles in the immune response against a wide range of pathogens in fish (41, 42), as well as the KRT79 protein, reported in monocytes and macrophages in humans (43).

The most characteristic gene transcribed by DCs was the macrophage expressed 1 (*mpeg1*), used as a marker for macrophages in vertebrates and for which antimicrobial activity has been reported in fish (44). In the other DC-like subpopulation, some additional genes are detected, such as the ribonucleoside-diphosphate reductase subunit M2 (*rrm2*) or the ribonucleotide reductase catalytic subunit M1 (*rrm1*), known to alter the DNA damage response and the p53 pathway in humans (45).

HPCs were characterized by the high expression of genes encoding beta-galactoside-binding lectins, tetraspanin-8-like proteins, a low affinity immunoglobulin gamma Fc region receptor II-like protein and an osteoclast stimulatory transmembrane protein. Among beta-galactoside-binding lectins, we can find galectins, which have an important role in innate immunity against infections also in fish (46). Tetraspanin-8 has also been shown to act as a key component of the pro-inflammatory signaling cascade in cancer and viral infections due to its interaction with other proteins (47). On the other hand, in humans, the low affinity immunoglobulin gamma Fc region receptor IIa is related to phagocytosis and has been identified in neutrophils, monocytes, macrophages and DCs (48). Therefore, this group of HPC seems to represent a precursor of the myeloblastic line, as it shares additional markers also found in monocyte-macrophage precursors, such as for example the osteoclast stimulatory transmembrane protein.

In NCCs, the transcription of an eotaxin-like gene was the most outstanding characteristic. Its product is the cytokine CCL11, which interestingly, has been shown to inhibit the differentiation of DCs and promote the development of Th2 cells in humans (49). Neutrophils, on the other hand, were characterized by a high expression of complement factor D (*cfD*), also designated as adipsin, a well-known adipokine (a cytokine secreted by adipose tissue), also identified in fish (50). Although its precise role in the functionality of fish neutrophils is unknown, in mammals, adipsin secreted from adipose tissue seemed associated to the neutrophil infiltration that takes place during inflammatory arthritis (51), suggesting a potential autocrine role of this molecule. Finally,

thrombocytes were characterized by the transcription of genes coding integrin α IIB and β 3, which were also reported as thrombocyte-markers in fish in a previous study (52). Additionally, the gene coding for the coagulation factor XIII A chain was also identified as a thrombocyte marker in this study and before (53).

4.2 Early transcriptional response of PBLs to IPNV exposure

The increase observed in the number of genes transcribed in PBLs after the IPNV infection demonstrates that these cells respond to the viral encounter. Although the major percentage of transcripts upregulated corresponds to protein coding genes, the amount of transcripts mapping ribosomal genes is also strongly regulated, especially in some specific PBL subsets.

Interestingly, plasmablasts/plasma cells (*irf4*⁺ B cells), DC-like cell populations, NCC and HPCs were not significantly affected by virus encounter at this very early time point, while the other B cell subsets, T cells, DCs, thrombocytes and monocytes were the main cell types that responded to IPNV recognition. Surprisingly, some of the upregulated genes were common to all these affected PBL subpopulations after the IPNV infection, being all of them related to the immune system. These included genes like the saccin-like, which codes for a protein that regulates the Hsp70 chaperone machinery (54) and has been previously seen to be upregulated in fish response to reovirus (55). Two other commonly regulated genes were those coding for helicases with zinc finger 2 (*helz2* and LOC110528534), known to be involved in antiviral responses to the viral mimic polyriboinosinic polyribocytidylic acid (pIC) (56). This protein is also a transcriptional co-activator of several nuclear receptors, such as the peroxisome proliferator-activated receptor α (PPARA) and γ (PPARG), being this a mechanism by which it exerts antiviral effects (57). A gene coding for the IFN-induced very large GTPase 1 (GVIN1) protein, which participates in the IFN-mediated immunity (58), was also up-regulated by IPNV in all affected subsets. Interestingly, a recent study identified GVIN1 among the transcripts differentially expressed in bacterial cold-water disease (BCWD) resistant rainbow trout (59). A gene coding for TRIM16 was also in the group of commonly up-regulated genes. This protein is part of the TRIM superfamily, greatly involved in the cellular response to type I and II IFN-mediated immunity (60). A gene coding for a galectin-9-like protein was also up-regulated by IPNV in all affected subsets. Similarly, in red sea bream (*Pagrus major*), galectin-9-like transcription was up-regulated in response to either a viral or a bacterial pathogen (61). Interestingly, galectin-9 has been shown to induce the apoptosis of activated T cells (62) and to work as a chemoattractant for eosinophils (63). In mammals, galectin-9 has been demonstrated to induce platelet adhesion (64). The CD9 antigen-like, an abundant transcript in rainbow trout PBLs that seems to participate in the immune response to pathogenic encounter or vaccination (65, 66), was also among the genes commonly modified by IPNV. Yet, in contrast to what we have observed in the current study, a previous work from our group determined that the levels of transcription of CD9 decreased in

IgM⁺ B cells in response to viral hemorrhagic septicemia virus (VHSV) (66). Another commonly regulated gene is that coding for the E3 ubiquitin protein ligase rnf213-beta-like, a key factor for ubiquitin-dependent autophagy that usually shows higher levels of expression in cell types with a phagocytic capacity (67). Interestingly, Nombela et al. demonstrated that VHSV induced protein ubiquitination in rainbow trout (68). Therefore, it might be possible that IPNV stimulates a similar pathway.

Nonetheless, in this study, we have also detected a series of genes that are exclusively regulated in each of these specific leukocyte subsets in response to the virus, pointing to differences in how these cells respond to the virus. For example, B cells showed a response to the virus focused on both type-I IFN and cytokine-mediated signaling, yet the overexpression of genes encoding different types of Igs, revealed that these cells also increase Ig production in response to the virus. The *ccl4*⁺ B cells, thought to be involved in attracting Th cells (9), upregulated a group of genes related to chemotaxis, in addition to genes related to the IFN response. In T cells, one of the genes exclusively regulated was that coding for the IFN-induced protein 44-like, known to be induced by IFN γ in teleosts (69). Although its precise function is still unknown in fish species, it has been suggested to act as a viral replication suppressor, like in mammals (69). T cells also seem to regulate the transcription of different types of E3 ubiquitin-protein ligases upon IPNV encounter. Many of these ligases are well-known IFN-activated proteins that regulate the NF- κ B signaling pathway (70). Some TRIM ligases were also specifically regulated in monocytes by the virus, in addition to IRF-3 and IRF-8 which also control the NF- κ B signaling pathway in teleost fish (71). In neutrophils, the up-regulation of the levels of transcription of the NF- κ B p100 subunit seems to indicate an activation of the non-canonical NF- κ B pathway, which was also shown to be activated in human neutrophils (72). In concordance, the up-regulation of IL-1RII transcription should avoid the docking between IL-1 β and IL-1RI, negatively regulating the canonical NF- κ B pathway in these cells (73). Finally, in addition to the genes commonly regulated in all affected leukocyte subsets, thrombocytes specifically upregulates genes that code for proteins related to the adhesion and mobility.

In conclusion, in this study, we have obtained a single cell transcriptome of rainbow trout PBLs in control culture conditions and in cultures exposed to IPNV. This set of samples has allowed us to obtain a transcriptional atlas of rainbow trout PBL populations, identifying several novel genes for each of them. When the transcriptional profile of these cells was compared in control and infected conditions, we found that plasmablasts/plasma cells (*irf4*⁺ B cells), DC-like cell populations, NCC and HPCs were not significantly affected by the virus at this early time point, while the other B cell subsets, T cells, DCs, thrombocytes and monocytes were the main cell types that responded. Interestingly, eight genes were transcriptionally up-regulated by the virus in all of these cell types, genes mostly related to IFN signaling. Nonetheless, each of these populations also regulated a set of specific genes that revealed an exclusive response to the viral infection. Thus, for example, B cells regulated Ig production, while thrombocytes regulated genes associated with adhesion. These exclusive profiles also were in many cases associated to activation of the NF- κ B pathway, yet revealed specific

ways to achieve this activation in some cases. The data generated will be useful to better understand the functionality of teleost leukocyte subpopulations, and how they respond to a viral encounter.

5 Limitations of the study

The results obtained in this paper regarding the classification of leukocyte subsets is based on the transcriptional profiles obtained and should be further validated in future studies by complementary imaging, functional or flow cytometry experiments. Furthermore, it should be taken into account that small changes in the data could be obtained if the results were compared with an updated reference genome. Finally, the differential response to the virus was obtained studying only two fish and incubating the leukocytes with the virus *in vitro*. Slightly different results might be obtained if the infection was to be performed *in vivo*. Finally, it might be possible that throughout the incubation period, control leukocytes slightly modify their transcriptional pattern to the one that may had been obtained in freshly isolated leukocytes.

Data availability statement

The data discussed in this publication has been deposited in NCBI's Gene Expression Omnibus and is accessible through the GEO Series accession number GSE260891 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE260891>).

Ethics statement

The animal study was approved by CSIC (Consejo Superior de Investigaciones Científicas) Ethics Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

PP: Data curation, Formal analysis, Writing – original draft. PJ-B: Visualization, Writing – original draft. EM: Investigation, Writing – review & editing. BA: Writing – original draft. CT: Conceptualization, Funding acquisition, Project administration, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the European Research Council (ERC Consolidator Grant 2016 725061 TEMUBLYM) and by the Comunidad de Madrid (grant 2016-T1/BIO-1672). This work was also part of the project PID2020-113268RB-I00 funded by MCNI/AEI/10.13039/501100011033.

Acknowledgments

Lucia González and Silvia Nogales are greatly acknowledged for technical assistance.

Conflict of interest

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

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1404209/full#supplementary-material>

References

- Hu CB, Wang J, Hong Y, Li H, Fan DD, Lin AF, et al. Single-cell transcriptome profiling reveals diverse immune cell populations and their responses to viral infection in the spleen of zebrafish. *FASEB J.* (2023) 37:e22951. doi: 10.1096/fj.2022011505RRRR
- Han X, Zhou Z, Fei L, Sun H, Wang R, Chen Y, et al. Construction of a human cell landscape at single-cell level. *Nature.* (2020) 581:303–9. doi: 10.1038/s41586-020-2157-4
- Carmona SJ, Teichmann SA, Ferreira L, Macaulay IC, Stubbington MJ, Cvejic A, et al. Single-cell transcriptome analysis of fish immune cells provides insight into the evolution of vertebrate immune cell types. *Genome Res.* (2017) 27:451–61. doi: 10.1101/gr.207704.116
- Tang Q, Iyer S, Lobardi R, Moore JC, Chen H, Lareau C, et al. Dissecting hematopoietic and renal cell heterogeneity in adult zebrafish at single-cell resolution using rna sequencing. *J Exp Med.* (2017) 214:2875–87. doi: 10.1084/jem.20170976
- Hernandez PP, Strzelecka PM, Athanasiadis EI, Hall D, Robalo AF, Collins CM, et al. Single-cell transcriptional analysis reveals ilc-like cells in zebrafish. *Sci Immunol.* (2018) 3(29):eaau5265. doi: 10.1126/sciimmunol.aau5265
- Guslund NC, Solbakken MH, Briec MSO, Jentoft S, Jakobsen KS, Qiao SW. Single-cell transcriptome profiling of immune cell repertoire of the atlantic cod which naturally lacks the major histocompatibility class ii system. *Front Immunol.* (2020) 11:559555. doi: 10.3389/fimmu.2020.559555
- Niu J, Huang Y, Liu X, Zhang Z, Tang J, Wang B, et al. Single-cell rna-seq reveals different subsets of non-specific cytotoxic cells in teleost. *Genomics.* (2020) 112:5170–9. doi: 10.1016/j.ygeno.2020.09.031
- Taylor RS, Ruiz Daniels R, Dobie R, Naseer S, Clark TC, Henderson NC, et al. Single cell transcriptomics of atlantic salmon (*Salmo salar*) liver reveals cellular heterogeneity and immunological responses to challenge by *Aeromonas salmonicida*. *Front Immunol.* (2022) 13:984799. doi: 10.3389/fimmu.2022.984799
- Perdiguero P, Morel E, Tafalla C. Diversity of rainbow trout blood B cells revealed by single cell RNA sequencing. *Biol (Basel).* (2021) 10(6):511. doi: 10.3390/biology10060511
- Perdiguero P, Morel E, Diaz-Rosales P, Tafalla C. Individual B cells transcribe multiple rearranged immunoglobulin light chains in teleost fish. *iScience.* (2021) 24:102615. doi: 10.1016/j.isci.2021.102615
- Rato S, Rausell A, Munoz M, Telenti A, Ciuffi A. Single-cell analysis identifies cellular markers of the hiv permissive cell. *PLoS Pathog.* (2017) 13:e1006678. doi: 10.1371/journal.ppat.1006678
- Zanini F, Robinson ML, Croote D, Sahoo MK, Sanz AM, Ortiz-Lasso E, et al. Virus-inclusive single-cell RNA sequencing reveals the molecular signature of progression to severe dengue. *Proc Natl Acad Sci U S A.* (2018) 115:E12363–9. doi: 10.1073/pnas.1813819115
- Abbas A, Vu Manh TP, Valente M, Collinet N, Attaf N, Dong C, et al. The activation trajectory of plasmacytoid dendritic cells *in vivo* during a viral infection. *Nat Immunol.* (2020) 21:983–97. doi: 10.1038/s41590-020-0731-4
- McWilliam Leitch EC, McLauchlan J. Determining the cellular diversity of hepatitis c virus quasispecies by single-cell viral sequencing. *J Virol.* (2013) 87:12648–55. doi: 10.1128/JVI.01602-13
- Wang Q, Peng C, Yang M, Huang F, Duan X, Wang S, et al. Single-cell rna-seq landscape midbrain cell responses to red spotted grouper nervous necrosis virus infection. *PLoS Pathog.* (2021) 17:e1009665. doi: 10.1371/journal.ppat.1009665
- Gervais O, Penalzoza C, Gratacap R, Papadopoulou A, Beltran M, Henderson NC, et al. Understanding host response to infectious salmon anaemia virus in an Atlantic salmon cell line using single-cell rna sequencing. *BMC Genomics.* (2023) 24:161. doi: 10.1186/s12864-023-09254-z
- Dopazo CP. The infectious pancreatic necrosis virus (IPNV) and its virulence determinants: What is known and what should be known. *Pathogens.* (2020) 9(2):94. doi: 10.3390/pathogens9020094
- Yu KK, MacDonald RD, Moore AR. Replication of infectious pancreatic necrosis virus in trout leucocytes and detection of the carrier status. *J Fish Dis.* (1982) 5:401–10. doi: 10.1111/j.1365-2761.1982.tb00496.x
- Wolf K, Quimby MC. Established eurythermic line of fish cells *in vitro*. *Science.* (1962) 135:1065–6. doi: 10.1126/science.135.3508.1065

SUPPLEMENTARY TABLE S1

Results from the marker identification analysis. Table shows all transcript with significant differential expression for each cell type showing an adjusted $p < 0.001$ and with an average fold change in logarithmic scale > 0.25 . Potential gene markers for each cell type are included in the corresponding spreadsheet. For 'B cells ALL', all populations of cells representing B cells were grouped together prior to the analysis.

SUPPLEMENTARY TABLE S2

Results from GO term single enrichment analysis using protein coding genes markers. The table shows all GO terms for each Gene Ontology category ('Biological process', 'Molecular function' and 'Cellular component') enriched within protein coding genes identified as markers from each cell type. Results for each cell type are included in the corresponding spreadsheet.

SUPPLEMENTARY TABLE S3

Results from differential expression analysis between control vs IPNV treated cells. Table shows all transcript with significant differential expression for each cell type showing an adjusted $p < 0.001$ and with an average fold change in logarithmic scale > 0.25 . Potential gene markers for each cell type are included in the corresponding spreadsheet. For 'B cells ALL', all populations of cells representing B cells were grouped together prior to the analysis.

SUPPLEMENTARY TABLE S4

Results from GO term single enrichment analysis using protein coding genes significantly induced in response to *in vitro* exposure to IPNV. The table shows all GO terms for each Gene Ontology category ('Biological process', 'Molecular function' and 'Cellular component') enriched within protein coding genes identified as markers from each cell type. Results for each cell type are included in the corresponding spreadsheet.

SUPPLEMENTARY TABLE S5

Genes grouped according to the Venn diagram (Figure 5A).

SUPPLEMENTARY TABLE S6

Protein-protein interaction analysis using String database.

20. Reed LJ, Muench A. A simple method of estimating fifty per cent end points. *Am J Epidemiol.* (1938) 27:493–7. doi: 10.1093/oxfordjournals.aje.a118408
21. Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J R Stat Soc Ser B (Methodological).* (1995) 57(1):289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x
22. Sciammas R, Shaffer AL, Schatz JH, Zhao H, Staudt LM, Singh H. Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. *Immunity.* (2006) 25:225–36. doi: 10.1016/j.immuni.2006.07.009
23. Paivandy A, Akula S, Lara S, Fu Z, Olsson AK, Kleinau S, et al. Quantitative in-depth transcriptome analysis implicates peritoneal macrophages as important players in the complement and coagulation systems. *Int J Mol Sci.* (2022) 23(1):211. doi: 10.3390/ijms23031185
24. Chopin M, Lun AT, Zhan Y, Schreuder J, Coughlan H, D'Amico A, et al. Transcription factor pu.1 promotes conventional dendritic cell identity and function via induction of transcriptional regulator dc-script. *Immunity.* (2019) 50:77–90. doi: 10.1016/j.immuni.2018.11.010
25. Karsunky H, Merad M, Cozzio A, Weissman IL, Manz MG. Flt3 ligand regulates dendritic cell development from flt3+ lymphoid and myeloid-committed progenitors to flt3+ dendritic cells in vivo. *J Exp Med.* (2003) 198:305–13. doi: 10.1084/jem.20030323
26. Granja AG, Tafalla C. Different igm(+) B cell subpopulations residing within the peritoneal cavity of vaccinated rainbow trout are differently regulated by BAFF. *Fish Shellfish Immunol.* (2019) 85:9–17. doi: 10.1016/j.fsi.2017.10.003
27. Calame KL. Plasma cells: Finding new light at the end of B cell development. *Nat Immunol.* (2001) 2:1103–8. doi: 10.1038/ni1201-1103
28. Morel E, Herranz-Jusado JG, Simon R, Abos B, Perdiguero P, Martin-Martin A, et al. Endoplasmic reticulum expansion throughout the differentiation of teletost B cells to plasmablasts. *iScience.* (2023) 26:105854. doi: 10.1016/j.isci.2022.105854
29. Pan YR, Wu CS, Zhong YQ, Zhang YA, Zhang XJ. An atlas of grass carp IgM+ B cells in homeostasis and bacterial infection helps to reveal the unique heterogeneity of B cells in early vertebrates. *J Immunol.* (2023) 211:964–80. doi: 10.4049/jimmunol.2300052
30. Tong J, Guan X, Jiang S, Sun L. A saposin domain-containing protein of tongue sole *Cynoglossus semilaevis*: Antimicrobial activity and mechanism. *Dev Comp Immunol.* (2023) 141:104633. doi: 10.1016/j.dci.2023.104633
31. Khiem D, Cyster JG, Schwarz JJ, Black BL. A p38 mapk-mef2c pathway regulates B-cell proliferation. *Proc Natl Acad Sci U S A.* (2008) 105:17067–72. doi: 10.1073/pnas.0804868105
32. Uyama T, Inoue M, Okamoto Y, Shinohara N, Tai T, Tsuboi K, et al. Involvement of phospholipase A2 acyltransferase-1 in n-acylphosphatidylethanolamine generation. *Biochim Biophys Acta.* (2013) 1831:1690–701. doi: 10.1016/j.bbali.2013.08.017
33. Liu Y, Liu X, Bai J, Sun Y, Nauwynck H, Wang X, et al. A new long noncoding RNA, mahat, inhibits replication of porcine reproductive and respiratory syndrome virus by recruiting ddx6 to bind to znf34 and promote an innate immune response. *J Virol.* (2022) 96:e0115422. doi: 10.1128/jvi.01154-22
34. Hitomi Y, Ueno K, Aiba Y, Nishida N, Kawai Y, Kawashima M, et al. Rs10924104 in the expression enhancer motif of CD58 confers susceptibility to human autoimmune diseases. *Hum Genet.* (2024) 143:19–33. doi: 10.1007/s00439-023-02617-2
35. Li XW, Rees JS, Xue P, Zhang H, Hamaia SW, Sanderson B, et al. New insights into the DT40 B cell receptor cluster using a proteomic proximity labeling assay. *J Biol Chem.* (2014) 289:14434–47. doi: 10.1074/jbc.M113.529578
36. Li H, Chen J, Li Z, Chen M, Ou Z, Mo M, et al. S100a5 attenuates efficiency of anti-pd-1/pd-1 immunotherapy by inhibiting cd8(+) t cell-mediated anti-cancer immunity in bladder carcinoma. *Adv Sci (Weinh).* (2023) 10:e2300110. doi: 10.1002/advs.202300110
37. Xu P, Zhou J, Xiao Y, Miao H. Association of sh2 domain-containing protein 1a, immunoglobulins and T lymphocyte subsets with Epstein-Barr virus infections. *Turk J Pediatr.* (2023) 65:603–10. doi: 10.24953/turkped.2022.1128
38. Yang X, Shui X, Dai X, Hao S, Ke F, Zhu L, et al. Plac8 promotes ev71 infected inflammatory lesion by disturbing Th-cell-related cytokines release in neonatal mouse. *Virology.* (2021) 564:39–45. doi: 10.1016/j.virol.2021.10.001
39. Huang R, Zhong S, Liu H, Kong R, Wang Y, Hu W, et al. Identification and characterization of common carp (*Cyprinus carpio*) granzyme A/K, a cytotoxic cell granule-associated serine protease. *Fish Shellfish Immunol.* (2010) 29:388–98. doi: 10.1016/j.fsi.2010.04.002
40. Alder MN, Mallela J, Opoka AM, Lahni P, Hildeman DA, Wong HR. Olfactomedin 4 marks a subset of neutrophils in mice. *Innate Immun.* (2019) 25:22–33. doi: 10.1177/1753425918817611
41. Siwicki A, Studnicka M. The phagocytes ability of neutrophils and serum lysozyme activity in experimentally infected carp, *Cyprinus carpio* L. *J Fish Biol.* (1987) 31:57–60. doi: 10.1111/j.1095-8649.1987.tb05293.x
42. Wang RX, Huang Y, Shi Y, Jiang FH, Gao Y, Liu X, et al. Characterization and functional analysis of a c-type lysozyme gene from obscure puffer *Takifugu obscurus*. *Dev Comp Immunol.* (2022) 133:104412. doi: 10.1016/j.dci.2022.104412
43. Zilionis R, Engblom C, Pfirschke C, Savova V, Zemmour D, Saaticoglu HD, et al. Single-cell transcriptomics of human and mouse lung cancers reveals conserved myeloid populations across individuals and species. *Immunity.* (2019) 50:1317–1334. doi: 10.1016/j.immuni.2019.03.009
44. Ni LY, Han Q, Chen HP, Luo XC, Li AX, Dan XM, et al. Grouper (*Epinephelus coioides*) mpeg1s: Molecular identification, expression analysis, and antimicrobial activity. *Fish Shellfish Immunol.* (2019) 92:690–7. doi: 10.1016/j.fsi.2019.06.060
45. Sagawa M, Ohguchi H, Harada T, Samur MK, Tai YT, Munshi NC, et al. Ribonucleotide reductase catalytic subunit m1 (rrm1) as a novel therapeutic target in multiple myeloma. *Clin Cancer Res.* (2017) 23:5225–37. doi: 10.1158/1078-0432.CCR-17-0263
46. Tian M, Xu D, Fu Q, Zhang L, Yang N, Xue T, et al. Galectins in turbot (*Scophthalmus maximus* L.): Characterization and expression profiling in mucosal tissues. *Fish Shellfish Immunol.* (2021) 109:71–81. doi: 10.1016/j.fsi.2020.12.004
47. Muller M, Saunders C, Senftleben A, Heidbuechel JPW, Halwachs B, Bolik J, et al. Tetraspanin 8 subfamily members regulate substrate-specificity of a disintegrin and metalloprotease 17. *Cells.* (2022) 11(17):2683. doi: 10.3390/cells11172683
48. Dai Y, Chen W, Huang J, Cui T. Fcgr2a could function as a prognostic marker and correlate with immune infiltration in head and neck squamous cell carcinoma. *BioMed Res Int.* (2021) 2021:8874578. doi: 10.1155/2021/8874578
49. Yang J, Hawkins OE, Barham W, Gilchuk P, Boothby M, Ayers GD, et al. Myeloid IKKbeta promotes antitumor immunity by modulating ccl11 and the innate immune response. *Cancer Res.* (2014) 74:7274–84. doi: 10.1158/0008-5472.CAN-14-1091
50. Hue I, Capilla E, Rosell-Moll E, Balbuena-Pecino S, Goffette V, Gabillard JC, et al. Recent advances in the crosstalk between adipose, muscle and bone tissues in fish. *Front Endocrinol (Lausanne).* (2023) 14:1155202. doi: 10.3389/fendo.2023.1155202
51. Li Y, Zou W, Brestoff JR, Rohatgi N, Wu X, Atkinson JP, et al. Fat-produced adipsin regulates inflammatory arthritis. *Cell Rep.* (2019) 27:2809–2816. doi: 10.1016/j.celrep.2019.05.032
52. Stosik M, Tokarz-Deptula B, Deptula W. Characterisation of thrombocytes in osteichthyes. *J Vet Res.* (2019) 63:123–31. doi: 10.2478/jvetres-2019-0017
53. Ali A, Rexroad CE, Thorgaard GH, Yao J, Salem M. Characterization of the rainbow trout spleen transcriptome and identification of immune-related genes. *Front Genet.* (2014) 5:348. doi: 10.3389/fgene.2014.00348
54. Parfitt AA, Michael GJ, Vermeulen EG, Prodrromou NV, Webb TR, Gallo JM, et al. The dietary protein saccin is a functional co-chaperone that protects against polyglutamine-expanded ataxin-1. *Hum Mol Genet.* (2009) 18:1556–65. doi: 10.1093/hmg/ddp067
55. Dai Z, Li J, Hu C, Wang F, Wang B, Shi X, et al. Transcriptome data analysis of grass carp (*Ctenopharyngodon idella*) infected by reovirus provides insights into two immune-related genes. *Fish Shellfish Immunol.* (2017) 64:68–77. doi: 10.1016/j.fsi.2017.03.008
56. Caballero-Solares A, Hall JR, Xue X, Eszlamo K, Taylor RG, Parrish CC, et al. The dietary replacement of marine ingredients by terrestrial animal and plant alternatives modulates the antiviral immune response of atlantic salmon (*Salmo salar*). *Fish Shellfish Immunol.* (2017) 64:24–38. doi: 10.1016/j.fsi.2017.02.040
57. Fusco DN, Pratt H, Kandilas S, Cheon SS, Lin W, Cronkite DA, et al. Helz2 is an IFN effector mediating suppression of dengue virus. *Front Microbiol.* (2017) 8:240. doi: 10.3389/fmicb.2017.00240
58. Klamp T, Boehm U, Schenk D, Pfeffer K, Howard JC. A giant gtpase, very large inducible gtpase-1, is inducible by IFNs. *J Immunol.* (2003) 171:1255–65. doi: 10.4049/jimmunol.171.3.1255
59. Ali A, Thorgaard GH, Salem M. Pacbio iso-seq improves the rainbow trout genome annotation and identifies alternative splicing associated with economically important phenotypes. *Front Genet.* (2021) 12:683408. doi: 10.3389/fgene.2021.683408
60. Ozato K, Shin DM, Chang TH, Morse HC 3rd. Trim family proteins and their emerging roles in innate immunity. *Nat Rev Immunol.* (2008) 8:849–60. doi: 10.1038/nri2413
61. Choi KM, Joo MS, Cho DH, Woo WS, Kang G, Heo MJ, et al. Molecular identification and mrna expression profiles of galectin-9 gene in red sea bream (*Pagrus major*) infected with pathogens. *Anim (Basel).* (2021) 11(1):139. doi: 10.3390/ani11010139
62. Wada J, Ota K, Kumar A, Wallner EI, Kanwar YS. Developmental regulation, expression, and apoptotic potential of galectin-9, a beta-galactoside binding lectin. *J Clin Invest.* (1997) 99:2452–61. doi: 10.1172/JCI119429
63. Hirashima M. Ecalectin/galectin-9, a novel eosinophil chemoattractant: Its function and production. *Int Arch Allergy Immunol.* (2000) 122 Suppl 1:6–9. doi: 10.1159/000053623
64. Zhi Z, Jooss NJ, Sun Y, Colicchia M, Slater A, Moran LA, et al. Galectin-9 activates platelet ITAM receptors glycoprotein vi and c-type lectin-like receptor-2. *J Thromb Haemost.* (2022) 20:936–50. doi: 10.1111/jth.15625
65. Fujiki K, Gauley J, Bols N, Dixon B. Cloning and characterization of cDNA clones encoding CD9 from atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *Immunogenetics.* (2002) 54:604–9. doi: 10.1007/s00251-002-0506-0
66. Castro R, Abos B, Gonzalez L, Aquilino C, Pignatelli J, Tafalla C. Molecular characterization of cd9 and cd63, two tetraspanin family members expressed in trout B lymphocytes. *Dev Comp Immunol.* (2015) 51:116–25. doi: 10.1016/j.dci.2015.03.002

67. Otten EG, Werner E, Crespillo-Casado A, Boyle KB, Dharamdasani V, Pathe C, et al. Ubiquitylation of lipopolysaccharide by rnf213 during bacterial infection. *Nature*. (2021) 594:111–6. doi: 10.1038/s41586-021-03566-4
68. Nombela I, Requena-Platek R, Morales-Lange B, Chico V, Puente-Marin S, Ciordia S, et al. Rainbow trout red blood cells exposed to viral hemorrhagic septicemia virus up-regulate antigen-processing mechanisms and MHC I&II, CD86, and CD83 antigen-presenting cell markers. *Cells*. (2019) 8(5):386. doi: 10.3390/cells8050386
69. Grimholt U, Fosse JH, Sundaram AYM. Selective stimulation of duplicated atlantic salmon MHC pathway genes by interferon-gamma. *Front Immunol*. (2020) 11:571650. doi: 10.3389/fimmu.2020.571650
70. Tomar D, Singh R. Trim family proteins: Emerging class of ring e3 ligases as regulator of NK-kappab pathway. *Biol Cell*. (2015) 107:22–40. doi: 10.1111/boc.201400046
71. Yan X, Zhao X, Huo R, Xu T. Irf3 and irf8 regulate nf-kappab signaling by targeting myd88 in teleost fish. *Front Immunol*. (2020) 11:606. doi: 10.3389/fimmu.2020.00606
72. Dimitrakopoulos FD, Antonacopoulou AG, Kottorou AE, Panagopoulos N, Kalofonou F, Sampsonas F, et al. Expression of intracellular components of the nf-kappab alternative pathway (nf-kappab2, relb, nik and bcl3) is associated with clinical outcome of nsclc patients. *Sci Rep*. (2019) 9:14299. doi: 10.1038/s41598-019-50528-y
73. Eggsetol HO, Lunde HS, Knutsen TM, Haugland GT. Interleukin-1 ligands and receptors in lumpfish (*Cyclopterus lumpus* L.): Molecular characterization, phylogeny, gene expression, and transcriptome analyses. *Front Immunol*. (2020) 11:502. doi: 10.3389/fimmu.2020.00502