The Megalocytivirus RBIV Induces Apoptosis and MHC Class I Presentation in Rock Bream (Oplegnathus fasciatus) Red Blood Cells

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Rock bream iridovirus (RBIV) causes severe mass mortality in Korean rock bream (Oplegnathus fasciatus) populations. To date, immune defense mechanisms of rock bream against RBIV are unclear. While red blood cells (RBCs) are known to be involved in the immune response against viral infections, the participation of rock bream RBCs in the immune response against RBIV has not been studied yet. In this study, we examined induction of the immune response in rock bream RBCs after RBIV infection. Each fish was injected with RBIV, and virus copy number in RBCs gradually increased from 4 days post-infection (dpi), peaking at 10 dpi. A total of 318 proteins were significantly regulated in RBCs from RBIV-infected individuals, 183 proteins were upregulated and 135 proteins were downregulated. Differentially upregulated proteins included those involved in cellular amino acid metabolic processes, cellular detoxification, snRNP assembly, and the spliceosome. Remarkably, the MHC class I-related protein pathway was upregulated during RBIV infection. Simultaneously, the regulation of apoptosis-related proteins, including caspase-6 (CASP6), caspase-9 (CASP9), Fas cell surface death receptor (FAS), desmoplakin (DSP), and p21 (RAC1)-activated kinase 2 (PAK2) changed with RBIV infection. Interestingly, the expression of genes within the ISG15 antiviral mechanism-related pathway, including filamin B (FLNB), interferon regulatory factor 3 (IRF3), nucleoporin 35 (NUP35), tripartite motif-containing 25 (TRIM25), and karyopherin subunit alpha 3 (KPN A3) were downregulated in RBCs from RBIV-infected individuals. Overall, these findings contribute to the understanding of RBIV pathogenesis and host interaction.

Keywords: rock bream, RBIV, red blood cells, erythrocyte, proteome, MHC class I, apoptosis, ISG15

INTRODUCTION

Rock bream iridovirus (RBIV) is a dsDNA virus that belongs to family Iridoviridae, genus Megalocytivirus (1). This virus causes severe mass mortality in Korean rock bream (Oplegnathus fasciatus) populations. RBIV was first reported in the summer of 1998 in southern coastal areas of Korea (2). Since then, high mortality resulting from RBIV occurs every year, causing important
economic losses in rock bream aquaculture. RBIV is known to cause strong pathogenicity in rock bream individuals (3–7). To date, the immune response of rock bream with RBIV infection remains unclear, although it represents an important aquaculture health concern. Therefore, it is necessary to further detail the immune response mechanisms underlying the RBIV infection process in rock bream. Over the years, a considerable number of studies have investigated the immune response of rock bream at both physiological and molecular levels by transcriptomic and microarray analyses (8, 9). Recently, an increasing number of studies have been focused on the transcriptional immune responses of rock bream against RBIV (10–15). However, most have focused on kidney-mediated immune responses to determine the pathways responsible for fish mortality or survivability. Therefore, evaluation of the immune response or immune defense mechanisms in different organs is useful for the understanding host-RBIV interactions.

In contrast to mammalian red blood cells (RBCs) or erythrocytes, which lack a cell nucleus and organelles (16), nonmammalian RBCs are nucleated and contain organelles in their cytoplasm (17). Although the main physiological role for RBCs is the transportation of respiratory gases, their role in the antiviral response has recently been uncovered (18). Importantly, teleost RBCs can induce toll-like receptor (TLR) and peptidoglycan recognition protein (PGRP) receptor families (19), pathogen presentation to macrophages (20), and cytokine or interferon production (21–25). In addition, transcriptomic and proteomic studies of rainbow trout (Oncorhynchus mykiss) showed that nucleated RBCs contribute to several immune functions such as antigen presentation, leukocyte activation or immune cytokine production (26, 27).

To date, the impact of RBIV on rock bream RBCs in the global fish immune response has not been studied yet. In the present study, we aimed to investigate the differentially expressed proteins (DEPs) in rock bream RBCs upon RBIV in vivo infection in order to understand the molecular contribution of this cell type in the fish immune response against RBIV infection. Proteomic profiling of RBCs from RBIV-infected fish revealed upregulation of apoptosis, antigen processing, and presentation of peptide antigen via MHC class I (MHC-I) pathways. However, the ISG15 antiviral mechanism pathway appeared to be downregulated.

**MATERIALS AND METHODS**

**Isolation of RBIV**

RBIV was obtained from naturally infected rock bream individuals as previously described (11). RBIV major capsid protein (MCP) gene copy number was quantified from supernatant preparations by quantitative real-time polymerase chain reaction (RT-qPCR). Virus titer was calculated as $1.1 \times 10^{7}$ MCP gene copies. Although some studies have demonstrated the use of cell lines to culture Megalocytivirus (28, 29), RBIV does not replicate well in in vitro cell culture conditions, so the TCID$_{50}$ method was not used in this study.

**Quantification of RBIV Viral Copy Number**

RBIV-free rock bream individuals were obtained from a local farm. Thirty fish (11.2 ± 1.2 cm, 28.1 ± 3.2 g) were maintained at 23°C in an aquarium containing 250 L of UV-treated seawater. Fish were injected intraperitoneally (i.p.) with RBIV (100 µL/fish, $1.1 \times 10^{7}$ MCP gene copies) or phosphate-buffered saline (PBS) (100 µL/fish) as a control. Blood (200 µL/fish) and organs (spleen, kidney, and liver) were collected from RBIV-infected rock bream individuals at 1, 2, 4, 7, and 10 days post infection (dpi) (4 fish per time point). RBCs were isolated from blood (100 µL/fish) and purified by 2 consecutive density gradient centrifugations (7,206 g, Ficoll 1.007, Sigma-Aldrich). For RBIV copy number analysis, genomic DNA was isolated from the RBCs, blood, spleen, kidney, and liver of each fish using High Pure PCR Template Preparation Kit (Roche) following standard protocol. A standard curve was generated to determine RBIV MCP gene copy number by RT-qPCR as described previously (11). Virus copy number was determined from 100 µL of total genomic DNA. Statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software, USA). One-way analysis of variance (ANOVA) was performed between conditions, with Tukey’s multiple comparison test. $P \leq 0.05$ were considered to indicate statistical significance.

**Experimental Infection for RBC Proteomic Analysis**

Fish (11.0 ± 0.8 cm, 29.3 ± 4.7 g) were randomly divided into two groups (20 fish per group): a virus-injected group and a PBS-injected group. The experimental group was injected i.p. with RBIV (100 µL/fish) containing $1.1 \times 10^{7}$ MCP gene copies, and the control group was injected i.p. with PBS (100 µL/fish). Each group of fish were maintained at 23°C in the aquarium containing 250 L of UV-treated seawater. Blood (100 µL/fish) was collected from 8 fish at 7 dpi. Then, RBCs were purified by 2 consecutive density gradient centrifugations (7,206 g, Ficoll 1.007, Sigma-Aldrich). All rock bream experiments were carried out in strict accordance with the recommendations of the Institutional Animal Care and Use Committee of Chonnam National University (permit number: CNU IACUC-YS-2015-4).

**TABLE 1 | List of primers used.**

| Name | Sequence | Accession number |
|------|----------|-----------------|
| β-actin | F CAGGGAAGAAATGCCCCAGA R CATAGATGAGGACCTGTGG | FJ975145 |
| MCP | F GTGCTCACAAGGACTGACATCG R CCCCCAACGTACTGATGCA | AY494994 |
| IFP3 | F TGGAGGTACCGTCATTGCTCG R CTTCTCTGTCTGTCTTCCTCTG | KF267453.1 |
| MHC class I | F AGATACTGAGAAAGACGCAA R TACAGCTTTCTTCCATCGATGTC | KC193802 |
| Fas | F GTTGGACTGTCCTGGTCATCA | AB619804 |
| Caspase 9 | F TCTGGGAGCAACACCGAGCG | KF501038 |
Proteomic Analysis
Ficoll-purified RBCs from 5 fish in each group were pelletized by centrifugation (1,600 rpm). The cell pellet was washed with PBS, digested, cleaned-up/desalted, and pooled for each group (2 control groups and 2 RBIV-infected fish groups). Then, samples were subjected to liquid chromatography and mass spectrometry analysis (LC-MS) as previously described (26), except that the Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific, Inc.) was used and 3 peptide fractions were collected. Progenesis QI v4.0 (Nonlinear Dynamics, Newcastle, UK) was used for protein differential expression analysis according to “between-subject design.” Log_2 peptide ratios followed a normal distribution that was fitted using least squares regression. Mean and standard deviation values were derived from Gaussian fit and were used to estimate P-values and false discovery rates (FDRs). The confidence interval for protein identification was set to ≥95% (P ≤ 0.05). Only proteins having ≥2 quantitated peptides were considered. Peptides with an individual ion score above the 1% FDR threshold were considered correctly identified.

Pathway Enrichment Analysis
DEP pathway enrichment analysis was performed using ClueGO (30), CluePedia (31), and Cytoscape (32). The GO Biological Process, GO Immune Process, Kegg, Reactome, and Wikipathways databases were used. A P ≤ 0.05 and Kappa score of 0.4 were used as threshold values. Proteins were identified by sequence homology with Homo sapiens using Blast2GO version 4.1.9 (33).

Quantitative Real-Time PCR Analysis of Gene Expression
For immune gene expression analysis, total RNA was extracted from RBCs using RNAiso Plus reagent (TaKaRa) following standard protocol. Total RNA was treated with DNase I (TaKaRa) and reverse transcribed using a ReverTra Ace qPCR RT Kit (Toyobo) according to manufacturer’s protocol. Real-time PCR was carried out in an Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer) using an AccuPre® 2x Greenstar qPCR Master Mix (Bioneer) as described previously (11). Each assay was performed in duplicate using β-actin genes as the

**FIGURE 1 |** RBIV MCP gene copy number in different rock bream organs. Fish i.p. injected with RBIV (1.1 × 10^7) were maintained at 23°C. Virus copy number in spleen (A), kidney (B), liver (C), blood (D), and RBCs (E) were analyzed at 1, 2, 4, 7, and 10 days post infection (dpi). One-way analysis of variance (ANOVA) was performed between conditions, with Tukey’s multiple comparison test. Different superscript letters denote significant differences (P < 0.05). a≠ b. Data are represented as individual values. Line represents mean value.
FIGURE 2 | Cytoscape network analysis of differentially expressed protein (DEPs) in RBCs from RBIV-infected rock bream. DEPs in RBCs from RBIV-infected rock bream at 7 dpi, with $-1.5 < \log_2 \text{FC} < 1.5$ and FDR $P < 0.001$. Overrepresented terms were identified by the Cytoscape ClueGo app, with GO Biological Process, Kegg, Reactome, and Wikipathways term databases. Red circles indicate upregulated/overrepresented terms, and green circles indicate downregulated/overrepresented terms. Gray circles indicate unspecific regulation. Color intensity represents the degree of overrepresentation.

FIGURE 3 | Upregulated functional pathways in the proteome profile of RBIV-infected RBCs. Upregulated/overrepresented terms in DEPs of RBCs from RBIV-infected rock bream at 7 dpi, with $-1.5 < \log_2 \text{FC} < 1.5$ and FDR $P < 0.001$. (A) Bar graph and (B) multilevel pie chart. Overrepresented terms were identified by the Cytoscape ClueGo app, with GO Biological Process, Kegg, Reactome, and Wikipathways term databases. Asterisks denote GO-term significance (* $P < 0.05$ and ** $P < 0.01$).
endogenous control. The primers used are listed in Table 1. Relative gene expression was determined by the $2^{-\Delta\Delta C_{t}}$ method (34). Statistical analyses were performed using GraphPad Prism software. Unpaired T-tests were performed between conditions. P < 0.05 were considered to indicate statistical significance. Data are represented as mean ± standard deviation.

RESULTS

RBIV Levels in Rock Bream RBCs

RBIV copy number was quantified in RBC, blood, spleen, kidney, and liver samples. At 2, 4, 7, and 10 dpi, increased viral copy numbers were observed in the spleen, kidney, and liver. The maximum copy number for all samples was reached at 10 dpi (average value of $4.99 \times 10^{7}$ in the spleen, $2.56 \times 10^{7}$ in the kidney, and $2.44 \times 10^{7}/100 \mu L$ in the liver) (Figures 1A–C).

In blood samples, the viral transcription level was $7.16 \times 10^{1}/100 \mu L$ at 1 dpi, gradually increased to $3.81 \times 10^{2}/100 \mu L$ at 2 dpi, and reached maximum values of $9.36 \times 10^{2}/100 \mu L$ at 7 dpi and $2.04 \times 10^{3}/100 \mu L$ at 10 dpi (Figure 1D). In Ficoll-purified RBCs from fish at 1, 2, 4, 7, and 10 dpi, virus copy numbers gradually increased with time; the average number of virus copies was $1.25 \times 10^{2}$, $2.31 \times 10^{2}$, $8.42 \times 10^{2}$, $9.22 \times 10^{3}$, and $3.54 \times 10^{4}/100 \mu L$, respectively (Figure 1E).

Protein Profiling of RBCs From RBIV-Infected Rock Bream

Cytoscape pathway enrichment analysis was performed in order to evaluate the functional pathways involved in the response of rock bream RBCs to RBIV (Figure 2). Proteins with a FDR <0.001 and $-1.5<\log_{2}$ Fold Change (FC)<1.5 were selected for functional network analysis. A total of 318 proteins were differentially regulated at a significant level.
in RBCs from RBIV-infected individuals: 183 proteins were upregulated and 135 were downregulated. Uregulated pathways were categorized into 13 main categories, while downregulated pathways were categorized into 2 (Figures 2–6 and Tables 2–4). Within upregulated pathways, proteins were involved in synthesis of active ubiquitin, E1 and E2 enzymatic roles, pyridine-containing compound metabolic processes, RNA transport, the spliceosome, cytosolic tRNA aminoacylation, the vitamin B6 biosynthetic process, snRNP assembly, and cellular detoxification, the cholesterol biosynthetic process, the Parkin-ubiquitin proteasomal system pathway, apoptosis, and antigen processing and presentation of peptide antigen via MHC class I (Figures 2–4 and Tables 2, 3). Within downregulated pathways, proteins were mainly involved in the ISG15 antiviral mechanism and p130Cas linkage to MAPK signaling for integrins (Figures 2, 5, 6 and Table 4).

Differentially Expressed Proteins Related to the Apoptosis Functional Pathway
A total of 36 apoptosis-related proteins were differentially regulated in RBCs from RBIV-infected individuals: 26 proteins were upregulated and 10 were downregulated (Figure 6). Among them, caspase-6 (CASP6), caspase-9 (CASP9), fas cell surface death receptor (FAS), and desmoplakin (DSP) were upregulated at 1.65, 5.35, 5.89, and 2.26 log2FC, respectively (Table 2). p21 (RAC1)-activated kinase 2 (PAK2) was downregulated at −2.39 log2FC (Table 2).

Differentially Expressed Proteins Related to the Spliceosome and snRNP Assembly Functional Pathways
Ten spliceosome-related proteins were differentially regulated in RBCs from RBIV-infected individuals: 7 proteins were upregulated and 3 were downregulated (Figure 6 and Table 2). Moreover, 6 snRNP assembly-related proteins were differentially expressed: 5 proteins upregulated and 1 protein downregulated (Figure 6 and Table 2). Among upregulated proteins, the top-scored was small nuclear ribonucleoprotein polypeptide F (SNRPF), with 8.79 log2FC. In addition, small nuclear ribonucleoprotein D1 polypeptide (SNRPD1) and small nuclear ribonucleoprotein polypeptide G (SNRPG) were highly upregulated (Table 2).

Differentially Expressed Proteins Related to Cellular Amino Acid Metabolic Processes and Cellular Detoxification Pathways
A total of 28 DEPs in RBCs from RBIV-infected individuals were involved in cellular amino acid metabolic processes, including 22 upregulated and 6 downregulated proteins (Figure 6 and Table 2). Among upregulated proteins, histamine N-methyltransferase (HNMT), aldehyde dehydrogenase 9 family member A1 (ALDH9A1), glutamate-cysteine ligase catalytic subunit (GCLC), phosphoglycerate dehydrogenase (PHGDH), ribosome maturation factor (SBDS), and pyrroline-5-carboxylate reductase 3 (PYCR3) were highly upregulated with log2FC of 7.33, 7.08, 7.06, 5.96, 5.38, and 4.14, respectively (Table 2). Of the 15 DEPs involved in cellular detoxification, 10 were upregulated (from 1.50 to 6.94 log2FC) and 5 were downregulated (from −2.90 to −5.96 log2FC) (Table 2). Of note, upregulated proteins included antioxidant enzymes such as glutathione S-transferase mu 3 (GSTM3), superoxide dismutase 1 (SOD1), and thioredoxin reductase 3 (TXNRD3).

Differentially Expressed Proteins Involved in Antigen Processing and Presentation of Peptide Antigen Via MHC Class I
Of 9 DEPs in RBCs from RBIV-infected individuals involved in antigen processing and presentation of peptide antigen...
### TABLE 2 | List of upregulated pathways in RBCs from RBIV-infected rock bream.

| Category                                      | Accession | Protein name | Protein description                                      | Log2FC |
|-----------------------------------------------|-----------|--------------|----------------------------------------------------------|---------|
| Synthesis of active ubiquitin: roles of E1 and E2 enzymes | A0A096M453 | UCHL3        | Ubiquitin C-terminal hydrolase L3                         | +4.54169 |
|                                               | A0A060YC09 | UBE2L3       | Ubiquitin conjugating enzyme E2 L3                        | +3.28977 |
|                                               | E7EXC7    | USP9X        | Ubiquitin specific peptidase 9 X-linked                  | +1.88619 |
|                                               | A0A1A8BMV9 | USP5         | Ubiquitin specific peptidase 5                           | +1.73518 |
|                                               | A0A1A7XFZ1 | UBA6         | Ubiquitin like modifier activating enzyme 6              | −5.67014 |
| Pyridine-containing compound metabolic process | A0A0P7UQB0 | NUP98        | Nucleoprin 98                                             | +6.56510 |
|                                               | A0A060W490 | PNPO         | Pyridoxamine 5'-phosphate oxidase                        | +6.54472 |
|                                               | A0A1A8DQA8 | PHGDH        | Phosphoglycerate dehydrogenase                            | +5.96297 |
|                                               | A0A060XS34 | PDIXK        | Pyridoxal kinase                                         | +3.52413 |
| RNA transport                                 | A0A060Y2P7 | MCPP2        | Mitochondrial pyruvate carrier 2                         | +2.35431 |
|                                               | A0A087XLW0 | PGAM1        | Phosphoglycerate mutase                                  | +2.21249 |
|                                               | J3QRQ2    | DCXR         | Dicarbonyl and L-xylulose reductase                      | +1.89013 |
|                                               | A0A087Y9K3 | PSAT1        | Phosphoserine aminotransferase 1                         | +1.65664 |
|                                               | A0A087Y9G8 | TP1          | Triosephosphate isomerase 1                              | −2.53308 |
|                                               | A0A1A7XVE8 | MDH1         | Malate dehydrogenase 1                                   | −7.96449 |
| Spliceosome                                    | A0A0P7UQB0 | NUP98        | Nucleoprin 98                                             | +6.56510 |
|                                               | A0A146RA28 | EIF5B        | Eukaryotic translation initiation factor 5B               | +4.94553 |
|                                               | A0A087XUQ0 | PYM1         | PYM homolog 1, exon junction complex associated factor    | +4.08632 |
|                                               | A0A060UX2R3 | NUP93      | Nucleoprin 93                                            | +3.41500 |
|                                               | C9KH96    | RBM8         | RNA binding motif protein 8A                              | +3.16004 |
|                                               | A0A060WH91 | PABPC1       | Poly(A) binding protein cytoplasmic 1                     | +2.51108 |
|                                               | A0A1A7XKU0 | RANGAP1      | Ran GTPase activating protein 1                           | +2.40663 |
|                                               | A0A087XK21 | TRNT1        | tRNA nucleotidyl transferase 1                           | +1.52564 |
|                                               | A0A087XJ99 | EIF3I        | Eukaryotic translation initiation factor 3 subunit I      | −2.81793 |
|                                               | A0A060XCL3 | ALYREF       | Aly/REF export factor                                    | −3.15159 |
|                                               | H2LP66    | EIF3J        | Eukaryotic translation initiation factor 3 subunit J      | −4.12793 |
|                                               | A0A146MRi7 | NUP35        | Nucleoprin 35                                            | −6.06319 |
| Cytosolic tRNA aminoacylation                   | A0A0P7X7D4 | SNPRF        | Small nuclear ribonucleoprotein poly peptide F            | +8.79320 |
|                                               | A0A087Y346 | SNRPD1       | Small nuclear ribonucleoprotein D1 poly peptide           | +4.98734 |
|                                               | I3KXZ4    | LSM3         | LSM3 homolog, U6 small nuclear RNA and mRNA degradation    | +3.60900 |
|                                               |           |              |                                                          |         |
|                                               | C9KH96    | RBM8         | RNA binding motif protein 8A                              | +3.16004 |
|                                               | A0A060XGY3 | SFS3A        | Splicing factor 3a subunit 3                              | +2.23314 |
|                                               | A0A1L3A6A6 | HSPA8        | Heat shock protein family A (Hsp70) member 8              | +1.81502 |
|                                               | A0A0P7UL65 | SNRPG        | Small nuclear ribonucleoprotein polypeptide G             | +1.70328 |
|                                               | A0A087YO9  | PP1H         | Peptidylprolyl isomerase H                                | −3.11189 |
|                                               | A0A060XCL3 | ALYREF       | Aly/REF export factor                                    | −3.15159 |
|                                               | H2RJ37    | SNRPAP1      | Small nuclear ribonucleoprotein polypeptide A'            | −3.29532 |
|                                               | G3NS19    | FARS1A       | Phenylalanyl-tRNA synthetase subunit alpha                 | +3.43435 |
|                                               | A0A1A7ZJC0 | MARS         | Methionyl-tRNA synthetase                                 | +3.27543 |
|                                               | A0A087YJF0 | EPRS         | Glutamyl-prolyl-tRNA synthetase                           | +2.78295 |
|                                               | A0A060YC35 | SARS         | Seryl-tRNA synthetase                                    | +2.61934 |
|                                               | A0A060WOF7 | LARS         | Leucyl-tRNA synthetase                                    | −1.93372 |
|                                               | A0A060W490 | PNPO         | Pyridoxamine 5'-phosphate oxidase                        | +6.54472 |

(Continued)
| Category | Accession | Protein name | Protein description | Log$_2$FC |
|----------|-----------|--------------|---------------------|-----------|
| snRNP Assembly | A0A060X3S4 | PDXK | Pyridoxal kinase | +3.52413 |
| | A0A087YOK3 | PSAT1 | Phosphoserine aminotransferase 1 | +1.65664 |
| | A0A097XD74 | SNRPF | Small nuclear ribonucleoprotein polypeptide F | +8.79320 |
| | A0A087Y34B | NJR98 | Nucleoporin 98 | +6.56510 |
| | A0A087Y34B | SNRPD1 | Small nuclear ribonucleoprotein D1 polypeptide | +4.98734 |
| | A0A080X2R3 | NJR93 | Nucleoporin 93 | +3.41500 |
| | A0A087UL65 | SNRPG | Small nuclear ribonucleoprotein polypeptide G | +1.70328 |
| | A0A16MR7 | NJR35 | Nucleoporin 35 | -6.06319 |
| Cellular detoxification | A0A087YGW8 | CLIC2 | Chloride intracellular channel 2 | +6.00740 |
| | H2RV4 | GSTM3 | Glutathione S-transferase mu 3 | +5.94070 |
| | I3IV50 | FAS | Fas cell surface death receptor | +5.88751 |
| | W5KQL6 | APOE | Apolipoprotein E | +4.62692 |
| | A0A0S7HPB7 | FAM213B | Family with sequence similarity 213 member B | +4.13534 |
| | B9MSR2 | SOD1 | Superoxide dismutase 1 | +2.53220 |
| | A0A087X9L9 | TXNRD3 | Thioredoxin reductase 3 | +2.07857 |
| | A0A060VRY4 | XPA | XPA, DNA damage recognition and repair factor | +1.76996 |
| | A0A087YMH6 | ADH5 | Alcohol dehydrogenase 5 (class III), chi polypeptide | +1.57015 |
| | W5NF82 | NEFL | Neurofilament light | +1.50524 |
| | A0A087YDB9 | TRPM6 | Transient receptor potential cation channel subfamily M member 6 | -2.90258 |
| | B3VTP4 | APOA4 | Apolipoprotein A4 | -3.50052 |
| | A0A087WSO8 | TXNRD1 | Thioredoxin reductase 1 | -3.51362 |
| | C9DTM8 | EPX | Eosinophil peroxidase | -5.96703 |
| | A0A0P7XV8 | MPO | Myeloperoxidase | -5.96703 |
| | W5KQL6 | APOE | Apolipoprotein E | +4.62692 |
| | W5NG17 | GGPS1 | Geranylgeranyl diphosphate synthase 1 | +3.68607 |
| | A0A0S7LJM9 | CNBP | CCHC-type zinc finger nucleic acid binding protein | +3.65477 |
| | A0A060XEO0 | ERLIN2 | ER lipid raft associated 2 | +3.09663 |
| | A0A060WKOS | PMVK | Phosphomevalonate kinase | +3.00278 |
| | C1BJ00 | VDAC2 | Voltage dependent anion channel 2 | +2.78431 |
| | B9MSR2 | SOD1 | Superoxide dismutase 1 | +2.53220 |
| | B3VTP4 | APOA4 | Apolipoprotein A4 | -3.50052 |
| | C1BJM7 | APOA1 | Apolipoprotein A1 | -3.58118 |
| | I6QFY3 | CFTR | Cystic fibrosis transmembrane conductance regulator | -3.85295 |
| | A0A146NL6 | HNMT | Histamine N-methyltransferase | +7.33475 |
| | Q19A30 | ALDH9A1 | Aldehyde dehydrogenase 9 family member A1 | +7.08477 |
| | H2M1L3 | GCLC | Glutamate-cysteine ligase catalytic subunit | +7.05565 |
| | A0A1A8DA99 | PHGDH | Phosphoglycerate dehydrogenase | +5.96297 |
| | A0A087YC2 | SBDS | SBDS, ribosome maturation factor | +5.38398 |
| | H2SS02 | PYCR3 | Pyrroline-5-carboxylate reductase 3 | +4.13757 |
| | A0A097USQ3 | PSMD1 | Proteasome 26S subunit, non-ATPase 11 | +3.93617 |
| | W5ULA8 | GSS | Glutathione synthetase | +3.49635 |
| | A0A087X9P9 | RPS5B | Ribosomal protein S28 | +3.46599 |
| | G3SNS9 | FARS1 | Phenylalanyl-tRNA synthetase subunit alpha | +3.43435 |
| | A0A1A7ZC8 | MARS | Methionyl-tRNA synthetase | +3.27543 |
| | A0A147AH6 | PSMB6 | Proteasome subunit beta 6 | +3.23477 |
| | Q66H0W | COASY | Coenzyme A synthase | +2.88808 |
| | A0A087YF0 | EPSR | Glutaminyl-prolyl-tRNA synthetase | +2.78295 |
| | A0A087WUL2 | PSMB3 | Proteasome subunit beta 3 | +2.74293 |

(Continued)
| Category | Accession | Protein name | Protein description | Log2FC |
|----------|-----------|--------------|---------------------|--------|
| A0A060YC3S | SARS | Seryl-tRNA synthetase | +2.61934 |
| H2VBD9 | PSMD5 | Proteasome 26S subunit, non-ATPase 5 | +2.46929 |
| A0A060Y2H5 | RPS21 | Ribosomal protein S21 | +2.03250 |
| A0A0N8K350 | ARG2 | Arginase 2 | +1.90686 |
| H2MN42 | NIT2 | Nitrilase family member 2 | +1.87753 |
| Q4SVN8 | PSMB4 | Proteasome subunit beta 4 | +1.84703 |
| A0A0B7Y0K3 | PSAT1 | Phosphoserine aminotransferase 1 | +1.65684 |
| A0A0R7KX8C8 | ALDH4A1 | Aldehyde dehydrogenase 4 family member A1 | −1.60365 |
| A0A0F8Q9Q0 | AASDHPT | Aminoacylase-semialdehyde dehydrogenase-phosphopantetheinyl transferase | −1.8438 |
| W5M476 | SARDH | Sarcosine dehydrogenase | −1.86083 |
| A0A060WOQ7 | LARS | Leucyl-tRNA synthetase | −1.93372 |
| A0A060237T | MRI1 | Methylthioribose-1-phosphate isomerase 1 | −2.64492 |
| A0A087WSW9 | TXNRD1 | Thioredoxin reductase 1 | −3.51362 |
| A0A146UVQ0 | CCT3 | Chaperonin containing TCP1 subunit 3 | −4.20768 |
| A0A17P7USQ3 | PSMD11 | Proteasome 26S subunit, non-ATPase 11 | −3.83617 |
| A0A060Y2H5 | UBE2L3 | Ubiquitin conjugating enzyme E2 L3 | −3.28977 |
| A0A147AH96 | PSMB6 | Proteasome subunit beta 6 | −3.23477 |
| A0A146VFH4 | TUBA4A | Tubulin alpha-4A chain | −2.86588 |
| A0A060WLR9 | TUBA3C | Tubulin alpha 3C | −2.86588 |
| A0A0R7WUL2 | PSMB3 | Proteasome subunit beta 3 | −2.74293 |
| H2VBD9 | PSMD5 | Proteasome 26S subunit, non-ATPase 5 | −2.49629 |
| Q4SVN8 | PSMB4 | Proteasome subunit beta 4 | −1.84703 |
| A0A146PU69 | ACTB | Actin beta | −1.83645 |
| A0A189JAM4 | TUBA1C | Tubulin alpha 1c | −2.55283 |
| H6QXT0 | CASP1 | Caspase 1 | −2.90548 |
| F2Z2E2 | IQGAP3 | IQ motif containing GTPase activating protein 3 | −4.82941 |
| A0A0P7UB0 | NUP98 | Nucleoporin 98 | +6.56510 |
| I3Y5O | FAS | Fas cell surface death receptor | +5.88751 |
| A0A060WPW9 | RUVBL1 | RuvB like AAA ATPase 1 | +5.68115 |
| A0A060X866 | CASP9 | Caspase 9 | +5.36463 |
| A0A099M453 | UCHL3 | Ubiquitin C-terminal hydrolase L3 | +4.54169 |
| W5L34 | ABCB1 | ATP binding cassette subfamily B member 1 | +4.23220 |
| A0A146UQZ0 | CCT3 | Chaperonin containing TCP1 subunit 3 | +4.20768 |
| A0A0P7USQ3 | PSMD11 | Proteasome 26S subunit, non-ATPase 11 | +3.83617 |
| A0A060X2R3 | NUP93 | Nucleoporin 93 | +3.41500 |
| A0A060YC09 | UBE2L3 | Ubiquitin conjugating enzyme E2 L3 | +3.28977 |
| A0A147AH96 | PSMB6 | Proteasome subunit beta 6 | +3.23477 |
| A0A060X866 | ERLIN2 | ER lipid raft associated 2 | +3.09663 |
| C1BJ00 | VDAC2 | Voltage dependent anion channel 2 | +2.78431 |
| A0A087WUL2 | PSMB3 | Proteasome subunit beta 3 | +2.74293 |
| A0A060X866 | RP2 | Ribophorin II | +2.51942 |
| A0A060WH91 | PABPC1 | Poly(A) binding protein cytoplasmic 1 | +2.51108 |
| H2VBD9 | PSMD5 | Proteasome 26S subunit, non-ATPase 5 | +2.49629 |
| A0A0P7USQ3 | PSMD5 | Proteasome 26S subunit, non-ATPase 5 | +2.49629 |
| A0A17XKJU0 | RANGAP1 | Ran GTPase activating protein 1 | +2.40663 |
| A0A146RM67 | DSP | Desmoplakin | +2.25988 |

(Continued)
TABLE 2 | Continued

| Category | Accession | Protein name | Protein description | Log$_{2}$FC |
|----------|-----------|--------------|---------------------|------------|
|          | A0A060UK9 | ACTL6A       | Actin like 6A       | +1.92039   |
|          | E7EXG7   | USP9X        | Ubiquitin specific peptidase 9 X-linked | +1.88819   |
|          | Q45VN8   | PSMB4        | Proteasome subunit beta 4 | +1.84703   |
|          | A0A1L3A8A6 | HSP90   | Heat shock protein family A (Hsp70) member 8 | +1.81502   |
|          | A0A1A8BMM9 | USP5     | Ubiquitin specific peptidase 5 | +1.73S18   |
|          | H2MXM9   | CASP6        | Caspase 6            | +1.65460   |
|          | A0A060W5L7 | USP47   | Ubiquitin specific peptidase 47 | −1.85717   |
|          | A0A1A8GUB0 | YWHAB     | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein beta | −2.17782   |
|          | A0A060W5K5 | PAK2      | p21 (RAC1) activated kinase 2 | −2.39132   |
|          | G3NDG3   | PLEC         | Plectin              | −3.20510   |
|          | G3NRU2   | RNF146       | Ring finger protein 146 | −3.25047   |
|          | C1BKM7   | APOA1        | Apolipoprotein A1    | −3.58118   |
|          | I6QFY3   | CFTR         | Cystic fibrosis transmembrane conductance regulator | −3.85294   |
|          | F2Z2E2   | IQGAP3       | IQ motif containing GTPase activating protein 3 | −4.82941   |
|          | X1WEE8   | TRIM25       | Tripartite motif containing 25 | −5.61605   |
|          | A0A146MR17 | NUP35    | Nucleoporin 35       | −6.06319   |

TABLE 3 | List of identified proteins related to antigen processing and presentation of peptide antigen via MHC class I.

| Category | Accession | Protein name | Protein description | Log$_{2}$FC |
|----------|-----------|--------------|---------------------|------------|
|          | A0A146MHT9 | MR1          | Major histocompatibility complex, class I-related | +4.08719   |
|          | A0A0P7USQ3 | PSMD11       | Proteasome 26S subunit, non-ATPase 11 | +3.83617   |
|          | Q5SRD4   | TAP2         | Transporter 2, ATP binding cassette subfamily B member | +3.83484   |
|          | A0A147AH6 | PSMB6        | Proteasome subunit beta 6 | +3.23477   |
|          | A0A087WUL2 | PSMB3        | Proteasome subunit beta 3 | +2.74293   |
|          | H2VB09   | PSMD5        | Proteasome 26S subunit, non-ATPase 5 | +2.46929   |
|          | Q45VN8   | PSMB4        | Proteasome subunit beta 4 | +1.84703   |
|          | I3J5Y7   | CANX         | Calnexin             | −1.55500   |
|          | A5A0E1   | SNAP23       | Synaptosome associated protein 23 | −2.80077   |

via MHC class I (Figure 4), 7 were upregulated and 2 were downregulated (Figure 6 and Table 3). Among the upregulated proteins (with log$_{2}$FC ranging from 1.85 to 4.08), were major histocompatibility complex class I-related protein (MR1), transporter 2 ATP binding cassette subfamily B member (TAP2), and 6 proteasome subunit proteins (proteasome 26S subunit non-ATPase 11 [PSMD11], proteasome subunit beta 6 [PSMB6], proteasome subunit beta 3 [PSMB3], proteasome 26S subunit non-ATPase 5 [PSMD5], and proteasome subunit beta 4 [PSMB4]).

Differentially Expressed Proteins Involved in ISG15 Antiviral Mechanism Pathway

The interferon-stimulated gene 15 (ISG15) antiviral mechanism pathway appeared to be mainly downregulated in RBCs from RBIV-infected rock bream (Figure 5). Within this pathway, 3 proteins were upregulated (signal transducer and activator of transcription 1 [STAT1], nucleoporin 93 [NUP93], and nucleoporin 98 [NUP98], with log$_{2}$FC ranging from 2.73 to 6.57), and 5 were downregulated (filamin B [FLNB], nucleoporin 35 [NUP35], interferon regulatory factor 3 [IRF3], tripartite...
TABLE 4 | List of downregulated pathways in RBCs from RBIV-infected rock bream.

| Category | Accession | Protein name | Protein description | Log₂FC |
|----------|-----------|--------------|---------------------|--------|
| ISG15 antiviral mechanism | A0A0P7UQB0 | NUP98 | Nucleoporin 98 | +6.56510 |
| | A0A060X2R3 | NUP98 | Nucleoporin 93 | +3.41500 |
| | C7AT20 | STAT1 | Signal transducer and activator of transcription 1 | +2.72893 |
| | A0A060W790 | KPNA3 | Karyopherin subunit alpha 3 | -1.55875 |
| | A0A067ZTD7 | IRF3 | Interferon regulatory factor 3 | -2.77578 |
| | X1WEE8 | TRIM25 | Tripartite motif containing 25 | -5.61605 |
| | A0A087X811 | FLNB | Filamin B | -5.77028 |
| | A0A146MR17 | NUP35 | Nucleoporin 35 | -6.06319 |

| p130Cas linkage to MAPK signaling for integrins | Q6PH06 | CRK | CRK proto-oncogene, adaptor protein | +3.83869 |
| | A0A146RM67 | DSP | Desmoplakin | +2.25958 |
| | A0A0F8ALN2 | FGA | Fibrinogen alpha chain | -1.84245 |
| | H2LW76 | FGG | Fibrinogen gamma chain | -3.25828 |
| | C1BKVM7 | APOA1 | Apolipoprotein A1 | -3.58117 |
| | A0A087X4W0 | FGB | Fibrinogen beta chain | -4.94492 |
| | A0A0R4ICS1 | ITGA4 | Integrin subunit alpha 4 | -5.35249 |

**FIGURE 7** | Relative mRNA and protein expression analysis of IRF3, MHCI, FAS, and CASP9. RBCs from RBIV-infected rock bream compared to PBS-injected rock bream (control). (A) Gene expression analysis, relative to control individuals (red line), evaluated by means of RT-qPCR. The β-actin gene was used as an endogenous control. Bars represent the mean ± standard deviation (SD) (n = 4 individuals). Unpaired T-tests were performed between conditions. *P < 0.05. (B) Quantitative protein expression values of selected proteins for pathway validation from proteomic analysis. Bars indicate log₂FC value. FDR values are indicated in Supplementary Table S1.

**DISCUSSION**

In this study, we report relevant findings in which RBIV, an economically important virus in rock bream aquaculture production, induce an immune response in RBCs. The spleen is one of the major target organs for RBIV replication (2–4, 7). However, we found similarities in RBIV level patterns in the spleen, kidneys, liver, blood, and RBCs. RBIV copy numbers were not as high as in RBCs as in other organs. Nonetheless, RBIV time-dependent increments were found in rock bream blood or Ficoll-purified RBCs.

Previous microarray analyses of kidney samples from RBIV-infected rock bream have shown that hemoglobin (α and β) expression gradually decreased after RBIV replication reached its maximum levels (around 10⁶ to 10⁷/µL) at 20 to 25 dpi.
expression were observed at 70 dpi when low viral loads were detected (below $10^7$ µL) (unpublished data). On the other hand, rock bream individuals treated with poly (I:C) exhibited high expression levels of irf3, isg15, and protein kinase RNA-activated (pkr) genes in blood samples, whereas no significant upregulation was observed in the spleen or kidney (6). Furthermore, the highest mhCI constitutive gene expression was detected in the blood of rock bream compared to other tissues such as spleen or kidney (10). Together, these findings emphasize the importance of evaluating blood-mediated immune responses in rock bream against RBIV infection.

RBCs are the most common cell type in the blood, so understanding their immune response will be essential to identify future strategies for controlling RBIV infection. In the present study, we evaluated the proteome of RBCs from RBIV-infected rock bream. Among the upregulated proteins, the MHCI and apoptosis-related pathways were the most overrepresented in RBCs from RBIV-infected rock bream. MHCI plays a crucial role in the presentation of antigen peptides, which are produced by the degradation of intracellular pathogens. These antigen peptides then bind to MHCI molecules and are presented to CD8+ T lymphocytes to trigger cellular immune responses and induce the elimination of infected or apoptotic cells (35, 36). Apoptosis is a process of programmed cell death known to prevent the transmission of infection to uninfected healthy cells by killing infected cells (37). Cytotoxic lymphocytes (CTL) kill infected cells by 2 main pathways: i) releasing cytolytic granules such as pore-forming protein perforin and serine protease granzymes (38, 39) and ii) activating the caspase-dependent Fas ligand pathway (40, 41). In the present study, antigen processing and presentation of peptide antigen via MHCI was upregulated in RBCs from RBIV-infected rock bream. Simultaneously, FAS and CASP9, two proteins implicated in the caspase-dependent Fas ligand pathway, were upregulated in RBCs from RBIV-infected rock bream. Indeed, it has been reported that cytotoxic effector cells induce apoptosis in response to RBIV infection (11). In addition, perforin- and granzyme-related apoptosis initiation signals have been reported to be activated in the kidneys of RBIV-infected rock bream. However, the authors also reported that the Fas-induced, caspase-dependent apoptosis pathway was barely induced based on only slight increases in fas, casp3, casp8, and casp9 gene expression (11, 13). Conversely, based on our proteomic results, both FAS and CASP9 proteins were upregulated in RBCs from RBIV-infected individuals, indicating that RBIV-activated apoptosis in rock bream RBCs could occur via the caspase-dependent Fas ligand pathway. These results could also suggest that apoptosis-related genes may be differentially expressed in kidneys and RBCs. Similarly, we have previously reported that a myristoylated membrane protein (MMP)-based DNA vaccine administered to rock bream triggered differential expression of apoptosis-related genes (including perforin, granzyme, Fas, Fas ligand, and caspases) depending on the tissue analyzed (spleen, kidney, liver, or muscle) (42). In addition, we have observed that other proteins involved in promoting or inducing apoptosis, such as DSP, PAK2, and heat shock protein family A (Hsp70) member 8 (HSPA8) proteins, were highly upregulated in rock bream RBCs upon RBIV infection. The induction of both the antigen processing and presentation via MHCI pathway and the apoptosis-related pathway against RBIV infection may indicate that RBCs attempt to activate CTLs and subsequently trigger them to induce apoptosis by perforin and granzyme production, which are critical factors for the inhibition of RBIV replication (13). Separately, MHCI-induced apoptosis has been also reported during differentiation and activation of certain hematopoietic cells (43).

Surprisingly, in the present study, proteins related to the ISG15 antiviral mechanism such as IRF3, NUP35, and TRIM25 were downregulated in RBCs from RBIV-infected individuals. In general, the first line of defense against viral infection is based on type I interferon (IFN) expression (44). ISG15 is known to play an antiviral role against different viral pathogens [reviewed in (45)]. In fish, the IFN-related immune response, as well as ISG15-related proteins, are known to exhibit an inhibitory effect on viral infections (46–53). In our previous studies, we have found that mx gene expression upregulation occurs soon after viral infection and is maintained in the kidneys of RBIV-infected rock bream at least till 10 dpi (15). However, the expression of the isg15 and pkr genes declined after 4 dpi. Therefore, type I IFN responses induced by RBIV infection seemed to be limited in time and were not able to maintain antiviral responses at later stages, leading to fish mortality (15). Many viruses have developed strategies to counteract the antiviral activity of ISG15 (54). In orange-spotted grouper (Epinephelus coioides) spleen cell line (GS), ISG15 was not significantly upregulated by Singapore grouper iridovirus (SGIV) infection, while it was overexpressed by grouper nervous necrosis virus (GNNV) (45). Moreover, SGIV infection could downregulate the expression of ISG15, IFN and Mx previously induced by poly I:C, suggesting that SGIV was able to counteract the cellular interferon-mediated antiviral activity. In this regard, the authors also speculated that SGIV encoded proteins could play vital roles in preventing ISG15 activity during SGIV infection. To our knowledge, nothing is known about the interactions between RBIV proteins and host innate immune responses, especially those related to IFN or ISG15 pathways proteins. Therefore, in light of evidences, further studies are needed to elucidate RBIV interactions and/or counteracting effects on rock bream innate immune response.

Finally, pathways related to the spliceosome, snRNP assembly, cellular amino acid metabolic processes, and cellular detoxification were differentially regulated in RBCs from RBIV-infected rock bream. In the same way, previous investigations by Nombela et al. have reported the regulation of proteins related to spliceosomal complex and antioxidant/antiviral response in RBCs exposed in vitro to VHSV (23). However, how these mechanisms contribute to rock bream immune response to RBIV remains to be studied.

In summary, we have demonstrated that rock bream RBCs are able to generate a response to RBIV infection. This response was characterized by the upregulation of apoptosis-, MHCI, cellular detoxification-, and spliceosome-related pathways and
the downregulation of ISG15 antiviral mechanisms. We have therefore identified novel target proteins in RBCs that will be valuable tools for future studies on the elucidation of RBIV-rock bream interaction mechanisms. These relevant findings will contribute to mitigate an economically important viral disease affecting rock bream aquaculture.

AUTHOR CONTRIBUTIONS

M-HJ performed experiments, analyzed data, and wrote the manuscript. VC performed experiments. SC and MM performed proteomic sequencing. MO-V conceived ideas, analyzed data, oversaw the research, and wrote the manuscript. VC and S-JJ contributed to the preparation of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00160/full#supplementary-material

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