LOS ERITROCITOS NUCLEADOS DE PECES EN LA RESPUESTA INMUNE FRENTE A INFECCIONES VÍRICAS

Iván Nombela Díaz

Tesis doctoral

DIRECTORA: María del Mar Ortega-Villaizán Romo

CODIRECTOR: Julio Coll Morales

Programa de Doctorado en Biología Molecular y Celular

Instituto en Investigación, Desarrollo e Innovación en Biotecnología Sanitaria de Elche (IDiBE)

Universidad Miguel Hernández de Elche

Esta tesis se presenta como un compendio de trabajos previamente publicados, o aceptados para revisión, que se citan a continuación:

Nombela, I., Puente-Marin, S., Chico, V., Villena, A. J., Carracedo, B., Ciordia, S., ... & Estepa, A. (2017). Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus. *F1000Research*, *6*. DOI: 10.12688/f1000research.12985.2. SJR: 0.93 (Q1/Q2).

Nombela, I., Carrion, A., Puente-Marin, S., Chico, V., Mercado, L., Perez, L., ... & Ortega-Villaizan, M. D. M. (2017). Infectious pancreatic necrosis virus triggers antiviral immune response in rainbow trout red blood cells, despite not being infective. *F1000Research*, *6*. DOI: 10.12688/f1000research.12994.2. SJR: 0.93 (Q1/Q2).

Nombela, I., Requena-Platek, R., Morales-Lange, B., Chico, V., Puente-Marin, S., Ciordia, S., ... & Ortega-Villaizan, M. D. M. (2019). 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*, *8*(5), 386. DOI: 10.3390/cells8050386. JCR: 4.829 (Q2)

Nombela, I., Lopez-Lorigados, M., Salvador-Mira, M.E., Puente-Marín, S., Chico, V., Ciordia, S., … & Ortega-Villaizan, M. D. M. (2019) Integrated transcriptomic and proteomic analysis of red blood cells from rainbow trout challenged with VHSV points towards novel immunomodulant targets. Enviado a *Vaccines*. SJR: 2.136 (Q1/Q2)

Nombela, I., & Ortega-Villaizan, M. D. M. (2018). Nucleated red blood cells: Immune cell mediators of the antiviral response. *PLoS pathogens*, *14*(4), e1006910. DOI: 10.1371/journal.ppat.1006910. JCR: 6,158 (Q1)

Empleados como indicio de calidad para la presentación de la tesis doctoral y cumpliendo así con la normativa del RD99/2011 y en la normativa vigente de la Universidad Miguel Hernández de Elche.

La financiación de esta tesis se ha desarrollado en el marco del proyecto ERC Starting Grant titulado "*The Crosstalk Between Red and White Blood Cells: The case of fish*". Referencia: GA639249. Acrónimo: BloodCellsCrosstalk.

APROBACIÓN DE LA COMISIÓN ACADÉMICA

Dr. Ricardo Mallavia Marín, Catedrático y Coordinador del Programa de Doctorado en Biología Molecular y Celular del Instituto de Investigación, Desarrollo e Innovación en Biotecnología Sanitaria de Elche de la Universidad Miguel Hernández (UMH) de Elche acredita que la Tesis Doctoral titulada "**LOS ERITROCITOS NUCLEADOS DE PECES EN LA RESPUESTA INMUNE FRENTE A INFECCIONES VÍRICAS**" realizada por el estudiante **Iván Nombela Díaz** y dirigida por la Dra. María del Mar Ortega-Villaizán Romo y el Dr. Julio Coll Morales reúne los indicios de calidad mínimos exigidos para el campo de evaluación.

Y para que así conste, da su conformidad a la lectura de esta tesis, firmando el presente escrito.

Fdo.:

En Elche, a de de 2019.

ABREVIATURAS

AMP: adenosín monofosfato

AMPK: quinasa dependiente de AMP

APCs: células presentadoras de antígenos

ATG: gen relacionado con autofagia

ATP: adenosín trifosfato

BD1: β-defensina 1

CD: cluster de diferenciación

cDNA: ADN complementario

CHSE-214: línea celular de embriones de salmón chinook

CR1: receptor de complemento 1

DCs: células dendríticas

DEGs: genes diferencialmente expresados

DEPs: proteínas diferencialmente expresadas

DNA: ácido desoxirribonucleico

dpe: días post-exposición

dpd: días post-desafío

ECL: quimioluminiscencia mejorada

eIF2α: subunidad α del factor eucariótico de iniciación de la traducción 2

ENV: virus de la necrosis eritrocítica

EPC: línea celular *Epithelioma papulosum cyprini*

FBS: suero bovino fetal

GO: ontología genica

GVHSV: gen o proteína G de VHSV

HK-RBCs: eritrocitos de riñón anterior

hpe: horas post-exposición

IFIT: proteína inducida por interferón con repeticiones de tetratricopéptidos

IFN: interferón

IFN1: interferón de tipo 1

IL: interleucina

IPNV: virus de la necrosis pancreática infecciosa

IRF: factores reguladores de interferón

ISAV: virus de la anemia infecciosa de salmónidos

ISGs: genes estimulados por interferón

LC3: cadena ligera 3

LC-MS/MS: espectometría de masas en tándem acoplada a cromatografía líquida

MHC: complejo mayor de histocompatibilidad

MHCI: MHC de clase I

MHCII: MHC de clase II

MOI: multiplicidad de infección

mTOR: diana de rapamicina en células de mamífero

Mx: dinamina con actividad GTPasa Mx

NKEF: factor potenciador de células NK

NLRs: receptores de tipo NOD

NVHSV: gen o proteína N de VHSV

PAMPs: patrones moleculares asociados a patógenos

PB-RBCs: eritrocitos de sangre periférica

PBS: búfer fosfato salino

PCR: reacción en cadena de la polimerasa

PFA: paraformaldehido

PIK3: fosfatidilinositol 3 quinasa

PLA: ensayo de ligación por proximidad

PPI: interacción proteína-proteína

PRRs: receptores de reconocimiento de patrones

PRV: ortoreovirus acuático

qPCR: PCR cuantitativa

RBCs: eritrocitos, del inglés red blood cells

RLRs: receptores semejantes a RIG-I

RNA: ácido ribonucleico

RNASeq: secuenciación del ARN

rpm: revoluciones por minuto

RPMI: medio de cultivo Roswell Park Memorial Institute

RTG-2: línea celular de gónada de trucha arcoíris

SLRs: receptores semejantes a secuestrosoma

TLR: receptor de tipo Toll

TOF: tiempo de vuelo

TSS: línea celular de estroma de bazo

UPS: sistema ubiquitina-proteasoma

VHSV: virus de la septicemia hemorrágica vírica

VSV: virus de la estomatitis vesicular

RABV: virus de la rabia

ÍNDICE

1. RESUMEN

El sistema inmune de los teleósteos no es tan conocido como el de mamíferos. En teleósteos, en comparación con mamíferos, existen un menor repertorio de inmunoglobulinas, menor afinidad de maduración y proliferación más lenta de linfocitos. En el mismo sentido, los eritrocitos o células rojas de la sangre (en inglés red blood cells, RBCs) de los teleósteos también se diferencian respecto a los de mamíferos en que poseen núcleo. Este hecho otorga a los RBCs de teleósteos la capacidad de crear respuestas celulares frente a una variedad de estímulos. Por ello, el objetivo de esta tesis consiste en el estudio de la respuesta inmune de RBCs frente a dos patógenos víricos de alta prevalencia en teleósteos, como son el virus de septicemia hemorrágica vírica (VHSV) y el virus de la necrosis pancreática infecciosa (IPNV). Teniendo en cuenta que el papel de los RBCs en las infecciones víricas es desconocido, la hipótesis de esta tesis es determinar si los RBCs tienen un papel importante como mediadores en la respuesta inmune durante una infección vírica. La metodología empleada en esta tesis se basó en analizar la respuesta inmune a nivel de expresión génica (mediante transcriptómica y PCR cuantitativa) y a nivel de proteína (mediante proteómica, western blot e inmunofluorescencia) de RBCs de trucha arcoíris (*Onchorhynchus mykiss*) expuestos a VHSV o IPNV en condiciones *ex vivo* e *in vivo*. Los resultados obtenidos muestran que tanto VHSV como IPNV no son capaces de replicar en RBCs, pero a pesar de ello pueden inducir una respuesta inmune. En el caso de VHSV, los RBCs expuestos al virus a tiempos cortos producen una activación de la autofagia junto con un incremento en los marcadores celulares de presentación de antígenos. A tiempos más largos, a 3 días post-exposición, los RBCs sufren un apagado celular debido a la fosforilación del factor de traducción eucariótico eIF2α, sin embargo, ello no impide la expresión de algunas proteínas como IL8 y el péptido antimicrobiano β-defensina 1. En contraste a estos resultados, los RBCs provenientes de truchas infectadas con VHSV analizados mediante transcriptómica y proteómica muestran un incremento en la expresión de interferón de tipo I, genes estimulados por interferón como Mx, presentación de antígenos y reguladores del sistema del complemento. En el caso de IPNV, la respuesta inmune de los RBCs expuestos al virus *ex vivo* se basa en la expresión del gen de interferón de tipo I, Mx y el receptor de tipo Toll 3, entre otros. También se detectó la expresión de Mx en RBCs expuestos a IPNV tras 6 días post-exposición. En conclusión, en esta tesis se describen los mecanismos de los RBCs en la respuesta antiviral.

The immune system of teleosts is not as known as the one of mammals. In teleosts, in comparison with mammals, there is a minor antibody repertoire, low affinity maturation and slower T cell proliferation. In this sense, erythrocytes or red blood cells (RBCs) of teleosts are also different in comparison with those of mammals. Teleost RBCs are nucleated and this fact allows them to generate responses against a variety of stimulus. For that, the objective of this thesis consists in the study of the immune response of RBCs against two viral pathogens with high prominence in teleost species, the viral hemorrhagic septicemia virus (VHSV) and infectious pancreatic necrosis virus (IPNV). Taking into account that the role of RBCs during viral infections is unknown, the hypothesis of this thesis is that RBCs play an important role as mediators of the immune response during viral infections. The methodology used in this thesis was based on analyzing the immune response at gene expression level (through transcriptomics and quantitative PCR) and protein level (through proteomics, western blot and immunostaining) of rainbow trout (*Onchorhynchus mykiss*) RBCs exposed to VHSV or IPNV in *ex vivo* and *in vivo* conditions. The results show that both VHSV and IPNV cannot replicate inside RBCs but they can induce an immune response. For VHSV, at short times post-exposure *ex vivo*, there is an induction of autophagy together with an increase in antigen presenting cell markers. At longer times post-exposure, RBCs suffer a cell shut-off due to phosphorylation of translation eukaryotic factor eIF2α, but this does not affect the expression of some proteins such as IL8 and β-defensin 1. In contrast with these results, RBCs from VHSV-challenged rainbow trout analyzed by transcriptomics and proteomics show an increase in the expression of type I interferon, interferon-stimulated genes such as Mx, antigen presentation and regulators of complement system. For IPNV, immune response to *ex vivo* viral exposure increase expression of type I interferon, Mx and Toll-like receptor 3 genes, among others. Also, Mx protein expression was detected in RBCs exposed to IPNV at 6 days post-exposure. In conclusion, this thesis describes mechanisms of RBCs involved in antiviral response.

2. INTRODUCCIÓN

2.1. El sistema inmune de los teleósteos

El sistema inmune de los teleósteos no está tan investigado en comparación con el sistema inmune de los mamíferos. Además, existen grandes diferencias entre el sistema inmune de los mamíferos y el de teleósteos, especialmente en la inmunidad adaptativa. Es sabido que los linfocitos B de teleósteos no poseen el mismo repertorio de inmunoglobulinas que los humanos [1], además tienen unos procesos de maduración de la afinidad y proliferación linfocítica más lentos [2]. Se ha hipotetizado que esto pudiera ser debido al carácter poiquilotermo de los teleósteos y a que estos son evolutivamente uno de los primeros grupos de vertebrados en los que las células y las moléculas pertenecientes a la inmunidad adaptativa aparecen por primera vez. Algunas de estas diferencias se muestran comparadas en la Tabla 1.

	Teleósteos	**Mamíferos**
Inmunoglobulinas	IgM, IgD e IgT/Z	IgM, IgG, IgA, IgD e IgE
Recombinación de cambio de clase	No	Si
Maduración de la afinidad	Deficiente	Muy elevada
Linfocitos T de memoria	Escasos	Elevados
T_H1, T_H2, T_H17 y citoquinas	Si	Si
MHC I y MHC II	Si	Si
Tejidos linfoides	Bazo, timo y riñón anterior	Bazo, timo y médula ósea

Tabla 1. Comparativa entre teleósteos y mamíferos de elementos clave en las respuesta inmune adaptativa [3].

La respuesta inmune innata se encarga de ejecutar los mecanismos de defensa frente a patógenos de forma no específica, es decir, de una forma genérica y sin generar una inmunidad a largo plazo. En este tipo de inmunidad en teleósteos se ven involucrados desde macrófagos, monocitos, células semejantes a NK y granulocitos [4]. Dentro de la inmunidad innata destaca el papel del interferón (IFN) de tipo I (IFN1), o IFNα/β, en la defensa antiviral de las células de vertebrados infectadas por virus [5]. El IFN1 secretado tiene una señalización autocrina y paracrina, protegiendo así a las células adyacentes de la infección vírica mediante la unión a sus distintos receptores de IFN1 que resultan en la inducción de una variedad de genes estimulados por IFN1 (ISGs). Entre estos ISGs se puede encontrar la dinamina con actividad GTPasa Mx (Mx) o la proteína quinasa activada por DNA de doble cadena (PKR). Diversas moléculas de IFN han sido

clonadas y secuenciadas en teleósteos, incluyéndose el salmón del Atlántico [6] y la trucha arcoíris [7] y además su actividad antiviral ya ha sido estudiada [8, 9]. La Figura 1 muestra un esquema de la cascada de señalización de IFN1.

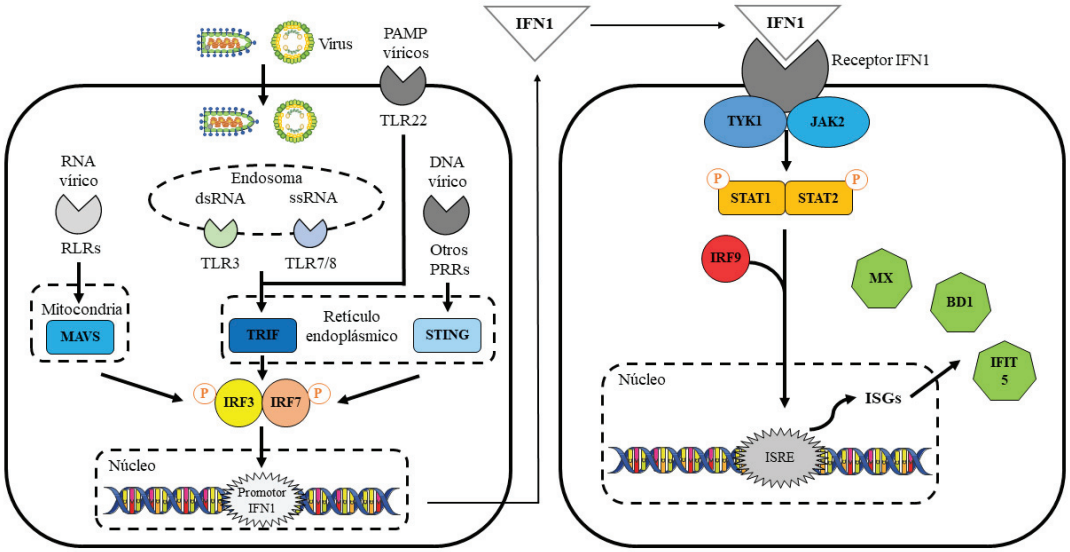


Figura 1. Esquema de la activación de IFN1 y de la cascada de señalización en células adyacentes. Modificado de Robertsen (2006) [5] y Zou & Secombes (2011) [10]

2.2. Los eritrocitos de teleósteos

El tejido sanguíneo se encuentra principalmente conformado por células, proteínas plasmáticas y agua, la cual supone aproximadamente un 90% del volumen de la sangre. El tipo celular más abundante en la sangre son los RBCs cuya abundancia varía desde uno a cinco millones de células por milímetro cúbico, disminuyendo conforme avanza en la escala evolutiva donde en los teleósteos presentan la menor concentración [11]. Los RBCs se caracterizan generalmente por su forma ovalada y plana, además de poseer un característico color rojo intenso debido a los pigmentos respiratorios que conforman las globinas, donde destaca la hemoglobina, principalmente. Por tanto, la función de estas células está principalmente enmarcada en el transporte de oxígeno y dióxido de carbono por los distintos tejidos del organismo [12]. En vertebrados no mamíferos, excluyendo algunas excepciones aisladas, los RBCs son nucleados y poseen orgánulos como mitocondrias, aparato de Golgi y ribosomas en su interior [11]. El núcleo de RBCs se diferencia del núcleo de otros tipos celulares debido a que presenta una continuidad del contenido citoplasmático en el interior del núcleo a través de poros, haciendo que la hemoglobina intracelular se extienda hasta el interior nuclear [13]. Respecto a la estructura celular de los RBCs nucleados (Figuras 2 y 3), destaca la presencia de una banda marginal de microtúbulos justo detrás de la membrana plasmática, cuya función se presupone que es el mantenimiento de la forma discoidal del RBC [14]. En el torrente sanguíneo, los RBCs presentan una variedad de estadios de maduración que afectan a la morfología, la densidad de la cromatina y el tamaño nuclear. La longevidad media de los RBCs nucleados de teleósteos es de 51 días aproximadamente, aunque se ha visto que algunos RBCs han presentado una supervivencia de hasta 270 días en el torrente sanguíneo [15]. También, se ha visto que existe una relación directa entre

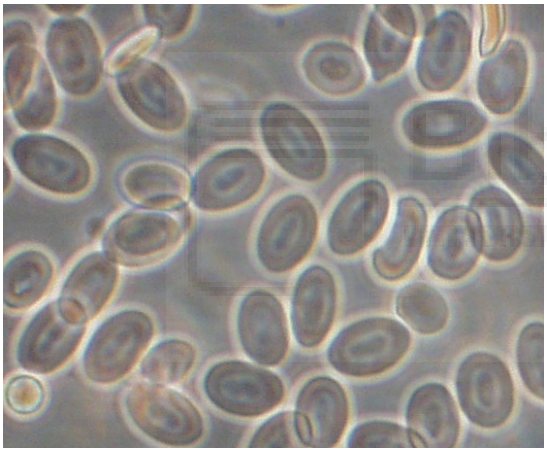


Figura 2. Imagen representativa de un cultivo celular de RBCs de trucha arcoíris

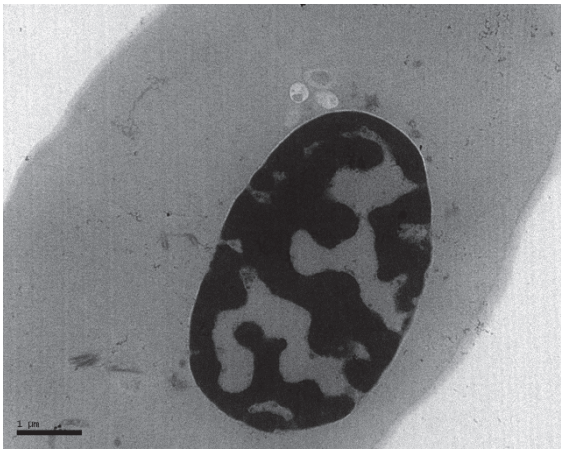


Figura 3. Imagen mediante microscopía electrónica de transmisión de un RBC de trucha arcoíris

la capacidad metabólica y de síntesis del RBC con la longevidad, donde los RBCs más jóvenes presentan una mayor cantidad de RNA y de orgánulos intracelulares, como mitocondrias y ribosomas, que los eritrocitos más longevos [16].

El motivo por el cual los RBCs de vertebrados no mamíferos mantienen su núcleo todavía es desconocido. Es sabido que los RBCs de mamíferos pierden su núcleo durante el proceso de hematopoyesis en la fase de enucleación [17]. La hematopoyesis se define como el proceso formador de células sanguíneas a partir de células troncales multipotentes, caracterizadas por una capacidad de autorrenovación y diferenciación a distintos tipos celulares [18]. La hematopoyesis en mamíferos consta de dos fases: una primera fase de proliferación donde las células troncales son inducidas a expandirse y activar sus mecanismos de diferenciación por la acción de factores extrínsecos o intrínsecos; y una segunda fase de maduración donde el eritroblasto progenitor se vuelve incapaz de proliferar y es sometido a cambios citoplasmáticos y nucleares [17]. Esta maduración implica pérdida de mitocondrias, reducción en el número de ribosomas y reorganización de los filamentos de actina, todo ello mediado por un proceso de autofagia. Entre los cambios nucleares destaca la condensación de los cromosomas y la preparación para la enucleación, que se ha presentado como un caso extremo de división asimétrica [17]. A pesar de que este proceso es el encargado de producir la enucleación de los eritroblastos en mamíferos, se ha visto que los mecanismos moleculares por los que se produce la hematopoyesis se encuentran conservados entre humanos y teleósteos [19, 20].

El hecho de que los RBCs de teleósteos posean núcleo y orgánulos les otorga la capacidad de poseer un mayor número de funciones, por ejemplo, la capacidad de responder frente a un estímulo con una respuesta *de novo* y coordinar procesos celulares. Además de su función respiratoria, se ha visto que los RBCs nucleados poseen otras funciones fisiológicas dentro del organismo como son el transporte y metabolismo de glucosa [21], homeóstasis del calcio [22] y regulación de los procesos redox del óxido nítrico [23]. Recientemente, existen estudios centrados en determinar la implicación de los RBCs en la respuesta inmune, demostrando que es un tema emergente de investigación [24-27].

2.3. Patógenos de eritrocitos

Los RBCs de teleósteos, al igual que cualquier otro tipo celular, presentan patógenos intracelulares. En humanos, el patógeno de RBCs más conocido es el protozoo *Plasmodium falciparum*, que causa la enfermedad de la malaria. Los RBCs de teleósteos presentan un variado conjunto de patógenos que son capaces de parasitar intracelularmente a los RBCs. Entre ellos, se encuentran los micoplasmas del género *Haemobartonella* y *Eperythrozoon*, la familia de protistas parásitos *Haemogregarinidae* y la familia de alveolados *Dactylosomatidae* [28]. En el caso de los patógenos víricos, hasta la fecha se conocen tres virus que tienen como diana este tipo celular: el virus de la necrosis eritrocítica (ENV) [29], el virus de la anemia infecciosa de salmónidos (ISAV) [30] y el ortoreovirus acuático (PRV) [31]. El primer virus que se descubrió que era capaz de infectar a los RBCs de teleósteos fue el ENV [32]. La infectividad del ENV en los RBCs se evaluó mediante microscopía electrónica, donde se pudieron observar viriones en el interior celular junto lesiones características de la patología que produce el ENV en aproximadamente un 15% de los RBCs. A pesar de esto, la respuesta antiviral que el RBC realiza para la defensa frente a este virus no ha sido objeto de estudio todavía [29]. Por el contrario, la respuesta antiviral tanto para ISAV como PRV sí ha sido estudiada en los RBCs de salmónidos. En el caso de la infección por ISAV, los RBCs del salmón del Atlántico presentan un incremento en la expresión de IFNα, una subfamilia de IFN1 [30]. La expresión de IFNα también se encuentra incrementada en los RBCs de salmón del Atlántico tras la infección por PRV, además de otros genes como proteína quinasa R (*pkr*), *mx*, gen estimulado por interferón 15 (*isg15*) o viperina [33]. Los RBCs son permisivos para las infecciones de PRV o ISAV, esto indica que el virus es capaz de replicarse en el interior del RBC de manera que se observa un incremento del título vírico de los sobrenadantes de RBCs infectados por estos virus [30].

2.4. Antecedentes de la respuesta inmune en eritrocitos de teleósteos

La implicación de los RBCs nucleados en las funciones del sistema inmune se han determinado en distintos procesos:

2.4.1. Expresión de receptores de tipo Toll (TLR): se ha determinado la expresión de TLR3 y TLR9 en RBCs de teleósteos, capaces de unirse a RNA de doble cadena y a DNA de bacterias o virus con motivos CpG no metilados, respectivamente [26]. Por otra parte, en los RBCs nucleados de pollo se encontró la expresión de TLR21 [26]. Los TLRs se encargan de reconocer patrones moleculares asociados a patógenos e iniciar los mecanismos de respuesta inmune innata. En teleósteos, hasta 20 tipos de TLRs han sido descritos [34], aunque el número de TLRs puede variar según la especie. Los TLRs se encuentran conservados estructuralmente entre los distintos grupos de vertebrados donde se han identificado, lo que sugiere que los mecanismos de señalización de los TLRs en teleósteos son semejantes a los descritos ya en mamíferos [35].

2.4.2. Secreción de moléculas señalizadoras: se ha descrito que los RBCs nucleados de ave [36] y teleósteos [37] son capaces de secretar factores semejantes a interleucinas, como el factor inhibidor de macrófagos, cuando se encontraban estimulados por el patógeno *Candida albicans* [36]. En los RBCs de teleósteos, se ha observado que la expresión de IFN1 es incrementada frente a diversos patógenos víricos [30, 33]. Las moléculas de IFN se dividen en dos familias: de tipo I y de tipo II. La familia de interferón tipo I consiste en varias moléculas (IFNα, IFNβ, IFNω; entre otras) que se encuentran relacionadas con la respuesta antiviral mientras que la familia de interferón de tipo II consta sólo de una molécula (IFNγ) secretada principalmente por linfocitos T y células NK con el objetivo de coordinar diferentes procesos de la inmunidad innata y adaptativa [38]. Por otro lado, otras moléculas relacionadas con la señalización de citoquinas como el supresor de la señalización de citoquinas 1 (SOCS1) y el receptor de IL10 (IL10R), ambos reguladores negativos de la respuesta inmune, se han visto inducidos en RBCs de salmón del Atlántico desafiados con PRV [39] .

2.4.3. Fagocitosis y formación de rosetas: la fagocitosis se define como un proceso defensivo mediante el cual una célula es capaz de engullir un patógeno con el objetivo de dirigirlo a fagosomas y de este modo eliminar potenciales amenazas [40]. Este proceso se encuentra mediado por macrófagos, aunque también se ha visto en otros tipos celulares como linfocitos B [41]. Tanto en teleósteos como en mamíferos los macrófagos son las células profesionales encargadas de realizar la fagocitosis [42]. Se ha descrito que los RBCs de trucha arcoíris son capaces de unir y engullir el hongo

parásito *Candida albicans* [43]. Este estudio además plantea que los RBCs son capaces de formar rosetas para facilitar la eliminación de patógenos por los macrófagos.

2.4.4. Autofagia: previamente se ha mencionado la autofagia como un proceso que interviene en la formación de eritropoyesis [44]. En RBCs de rodaballo (*Scophthalmus maxima*), se ha sugerido la implicación de la autofagia en respuesta al virus de la septicemia hemorrágica vírica (VHSV) [25]. La autofagia es un proceso catabólico celular altamente conservado a lo largo de la evolución por el cual una célula es capaz de mantener la homeostasis celular debido a la degradación de orgánulos, proteínas y otras macromoléculas celulares en vesículas con doble membrana denominadas autofagosomas que posteriormente se fusionan con lisosomas para completar la degradación [45]. Los estímulos que desencadenan la autofagia son de variada naturaleza y origen. La vía canónica de activación de la autofagia es iniciada por las quinasas de fosfatidilinositol (PIK3), diana de rapamicina en células de mamíferos (mTOR) y quinasa dependiente de AMP (AMPK), como consecuencia de un déficit de nutrientes y energía para que la célula pueda subsistir, que acaba desembocando en la formación de autofagosomas y finalmente en la activación de autofagia [46, 47]. La Figura 4 muestra un esquema de la formación del autofagosoma.

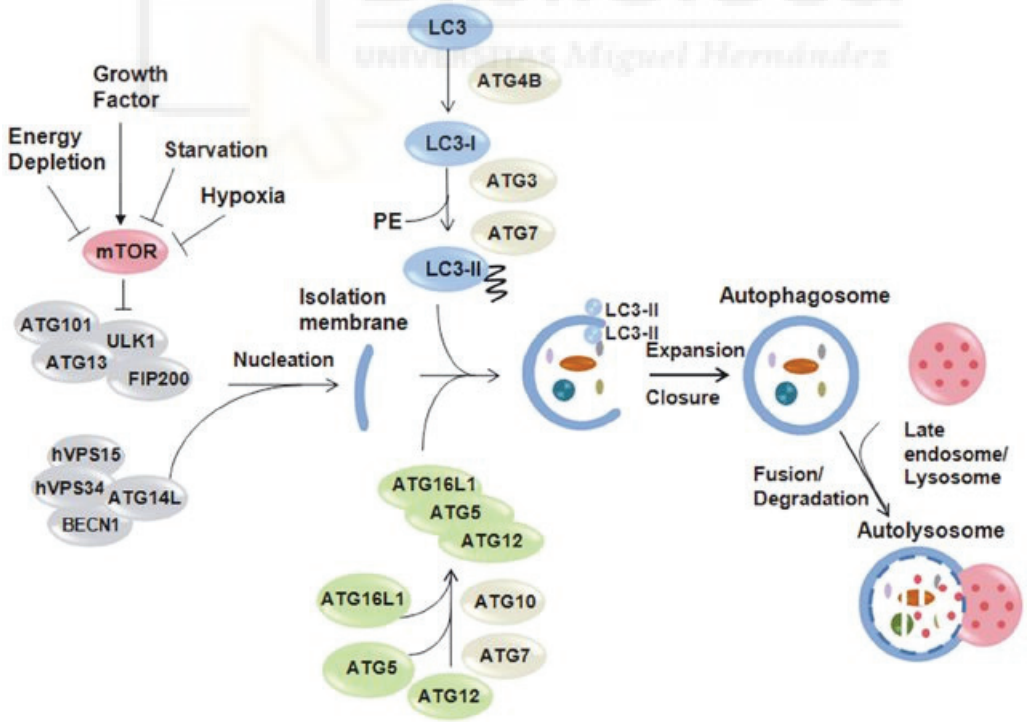


Figura 4. Mecanismos de regulación clave en la formación del autofagosoma y de la activación de la autofagia [48].

En el mecanismo de activación de autofagia y formación de autofagosomas están implicadas varias moléculas. Cuando la célula detecta falta de energía (la ratio

AMP/ATP es alta), se produce una activación de la AMPK que induce la inhibición de la diana de rapamicina en células de mamífero (mTOR) y activa al complejo formado por ULK1/2 (quinasa semejante a Unc-51), ATG13 (gen relacionado con autofagia 13), FIP200 (familia de proteínas de interacción de 200 kDa) y ATG101 (gen relacionado con autofagia 101) [49]. El complejo ULK1 colabora con el complejo III PIK3 en la formación inicial del autofagosoma [50]. Este complejo se encuentra formado por la beclina 1 (BECN1), entre otras proteínas. Su principal función es la fosforilación del fosfatidilinositol, dando lugar a fosfatidilinositol 3-fosafto, que será el principal componente de la membrana del autofagosoma [50]. También intervienen otras proteínas involucradas en la formación y desarrollo del autofagosoma como son WIPI1 (proteína de interacción con fosfoinositido con dominios de repetición WD) y el complejo ATG12-ATG5-ATG16L1 (genes relacionados con autofagia 12, 5 y semejante a 16, respectivamente) [51], así como proteínas involucradas en el proceso de maduración del autofagosoma, como la proteína asociada al receptor de ácido gamma-aminobutírico (GABARAP) ([52]. La proteína de cadena ligera 3 (LC3) es una proteína clave en este proceso. LC3 se encuentra en el citosol de la célula, en forma LC3-I, pero cuando se activa el proceso de autofagia una serie de proteínas (ATG3, ATG4, ATG7), que poseen actividad enzimática, actúan sobre LC3I eliminando la glicina del extremo C-terminal y conjugando la proteína con fosfatidiletanolamina, dando lugar a una forma de LC3 lipidada denominada LC3II, que se va a unir a la membrana del autofagosoma y va a jugar un papel clave en la formación, elongación y fusión del autofagosoma con el lisosoma [53].

La autofagia, aparte de poseer un papel en la supervivencia y el mantenimiento celular, también presenta un rol importante frente a la acción de los virus en sus hospedadores celulares. La autofagia sirve como un mecanismo de defensa antiviral, aunque también se ha descrito que es capaz de promover la replicación vírica [54], como ocurre en el caso de algunos virus RNA que modifican el sistema de endomembranas con el objetivo de crear sitios de replicación denominados factorías [55], y también la supresión de la inmunidad innata antivírica, ya que se ha visto que algunos virus pueden manipular la autofagia para eliminar moléculas clave de la respuesta inmune innata e inflamatoria [56]. Similarmente a las funciones provirales de la autofagia, los efectos antivirales de la autofagia ocurren a varios niveles. En primer lugar, es sabido que existe una relación entre la autofagia y PRRs como receptores de tipo NOD (NLRs), receptores de tipo RIG-I (RLRs), o los ya mencionados TLR3, 7/8 y 9 que reconocen patrones moleculares asociados a patógenos (PAMPs) de patógenos víricos [57]. La gran mayoría de los virus RNA libera su genoma al citoplasma para establecer sitios de replicación, y por tanto,

posibilitando la activación de los TLRs [58]. A pesar de esto, la activación puede ser limitada porque el RNA vírico está empaquetado en la nucleocápsida o secuestrado en complejos de replicación asociados a membranas donde quedan protegidos del ambiente celular [54]. Otro nivel de implicación antiviral de la autofagia se encuentra a través de la degradación de virus mediada por receptores parecidos a secuestrosoma 1/p62, como son NBR1, NDP52 y optineurina denominados SLRs [54]. Típicamente, estas moléculas presentan dominios que reconocen y capturan variedad de moléculas, y dominios que interaccionan con LC3 para dirigir las moléculas capturadas hacia los autofagosomas. En el caso de p62, este presenta un dominio de interacción con proteínas ubiquitinadas [59] aunque también se ha descrito interacciones directas con virus, como por ejemplo, el virus sindbis [60]. Por último, se ha implicado a la autofagia en el procesamiento de antígenos víricos y su presentación por las moléculas del complejo mayor de histocompatibilidad (MHC) de tipo I y MHCII, como es el caso del virus del herpes simple 1 mediante MHCI [61] y Epstein Barr virus mediante MHCII [62].

2.4.5. Expresión de péptidos antimicrobianos: los péptidos antimicrobianos son moléculas pequeñas de 12 a 50 aminoácidos que tienen un amplio espectro de actividad frente a bacterias, virus, hongos e incluso protozoos. Suelen localizarse en la superficie de células epiteliales como primera línea de defensa frente a los patógenos. Además de su función microbicida, los péptidos antimicrobianos se han visto implicados en otros procesos de la respuesta inmune como potenciadores de fagocitosis y desgranulación de basófilos, así como en otras funciones para prevenir daños en los tejidos afectados por una respuesta inflamatoria crónica como por ejemplo la apoptosis de células infectadas, el descenso en la producción de citoquinas y la neutralización del lipopolisacárido [63-65]. Se ha descrito que los RBCs nucleados de teleósteos poseen péptidos antimicrobianos de una gran variedad de procedencias y orgánulos celulares [66]. Una de las principales fuentes es la hemoglobina, proteína mayoritaria en RBCs. En el pez gato americano (*Ictalurus punctatus*) se observó la inducción y la expresión de péptidos antimicrobianos homólogos a la hemoglobina durante una infección parasitaria de *Ichthyophthirius multifiliis* [67]. Otro péptido antimicrobiano, ortólogo de la granulisina de humanos, denominado NK-lisina, se ha observado colocalizado y sobreexpresado en las membranas de autofagosomas de RBCs de rodaballo cuando estaban expuestos a VHSV [25].

2.4.6. Expresión de moléculas del complejo mayor de histocompatibilidad: diversos estudios han descrito la expresión de MHC de tipo I en RBCs de trucha arcoíris [68, 69], además de ser el factor determinante para la transfusión de eritrocitos entre individuos [70]. Se ha determinado inducción de la expresión de MHCI en una línea celular de riñón

anterior de salmón del Atlántico por ISAV [71] y en RBCs de salmón del Atlántico por PRV [39]. Por otro lado, también se ha descrito la expresión de MHCII a nivel de RNA en RBCs de ave [72]. Las moléculas del MHC participan en el proceso de presentación de antígenos para modular una respuesta adaptativa acorde a la naturaleza del patógeno [73]. Típicamente, MHCI presenta antígenos endógenos que son degradados por el proteasoma y transportados al retículo endoplasmático, donde se unen a MHCI. Después son transportadas a la superficie celular, donde las moléculas MHCI cargadas estimulan una respuesta de linfocitos T clúster de diferenciación (CD) 8+ para controlar la infección [74]. En cambio, las moléculas MHCII presentan péptidos de antígenos exógenos que son procesados por degradación lisosomal. Los péptidos se unen a en MHCII y son transportados a la superficie celular donde estimulan una respuesta de células T CD4+ [75]. Notablemente, antígenos exógenos también pueden ser presentados a través de MHCI para mediar una respuesta CD8+, en un mecanismo conocido como presentación cruzada [76]. La expresión de las moléculas MHC tradicionalmente ha sido asociada a las células presentadoras de antígenos (APCs). Recientemente, una nueva clasificación de APCs ha sido propuesta [77]. Las APCs podrían dividirse como APCs típicas, capaces de expresar moléculas MHC y estimular la diferenciación de células T *naïve*; mientras que las APCs atípicas podrían incrementar la expresión de las moléculas MHC bajo ciertas condiciones, pero su capacidad de estimular células T *naïve* es desconocida [77]. En mamíferos, entre las APCs típicas se encontrarían las células dendríticas (DCs), sin embargo las funciones de APCs en teleósteos todavía no están completamente dilucidadas [78]. Entre las APCs atípicas se encontrarían poblaciones de células epiteliales, endoteliales y de estroma [77].

2.4.7. Activación del sistema de complemento y formación de inmunocomplejos: el sistema del complemento en teleósteos se conforma de aproximadamente 35 proteínas solubles y de membrana cuya principal función es la eliminación de patógenos [79]. La mayoría de los componentes del sistema del complemento de mamíferos pueden ser encontrados en teleósteos. Del mismo modo, la vía clásica de señalización del complemento, cuya activación es dependiente de la unión de un anticuerpo frente a un antígeno, apareció por primera vez en teleósteos como consecuencia de la aparición de la inmunidad adaptativa y las inmunoglobulinas [80]. Además, los teleósteos presentan una característica única que es la presencia de un mayor número de isoformas de la proteína C3 [79]. La unión del anticuerpo con su antígeno forma un inmunocomplejo, el cual es capaz de activar la vía clásica del sistema del complemento que resulta en la unión covalente de fragmentos de los componentes del complemento, principalmente C3b. Se ha descrito que los RBCs de trucha arcoíris son capaces de unirse a dichos

complejos a través de la unión al receptor del complemento 1 (llamado CR1) presente en los RBCs. Dicho receptor es capaz de unir fragmentos de C3b y C4b activos [81]. Por tanto, los RBCs se encargarían de dirigir los inmunocomplejos fuera de la circulación del sistema reticuloendotelial [82]. Además, Nelson, en 1953, describió el fenómeno de inmunoadherencia de los RBCs a patógenos, mediado por proteínas del complemento presentes en el suero y anticuerpos [83].

2.5. La problemática de las infecciones víricas en acuicultura de salmónidos

La acuicultura comenzó su actividad hace 4000 años en la China previa a la época del feudalismo. A pesar de su comienzo temprano en la historia de la humanidad, apareció como una actividad con un perfil de subsistencia [84] en la mitad del siglo XX cuando las primeras especies de salmón y trucha fueron cultivadas a modo de práctica experimental. Actualmente la acuicultura supone una actividad cuya producción total anual excede los 50 millones de toneladas y posee un valor estimado de 80.000 millones de dólares en todo el mundo [85]. Hasta 2007, la acuicultura poseía un crecimiento anual del 6.9% y recientemente superó a la pesca como sector de producción de alimentos marinos [86]. A pesar de este crecimiento, en los últimos años ha habido un incremento en la aparición de nuevas enfermedades de peces debido a la acuicultura intensiva, movimiento internacional de peces y sus productos, además de diversas fuentes de estrés antropogénico en los ecosistemas acuáticos [87]. Estas enfermedades causan pérdidas substanciales entre la población de peces resultando en pérdidas económicas para la acuicultura comercial [87]. Gran parte de estos patógenos emergentes corresponden a virus, siendo el virus de la septicemia hemorrágica vírica (VHSV) y el virus de la necrosis pancreática infecciosa (IPNV), dos virus causantes de grandes pérdidas económicas en la acuicultura de salmónidos.

2.5.1. Virus de la septicemia hemorrágica vírica (VHSV): el VHSV pertenece a la familia *Rhabdoviridae*, dentro del género *Novirhabdovirus*. Los viriones poseen forma de bala y presentan un genoma de RNA de cadena única con polaridad negativa que contiene los genes víricos en el orden 3’ hacia 5’: N (nucleocápsida), P (fosfoproteína), M (proteína de la matriz), G (glicoproteína) y L (polimerasa) [88] (Figura 5). VHSV es el agente infeccioso causante de la septicemia hemorrágica vírica, caracterizada por la alta mortalidad entre las especies infectadas [89] y ha sido catalogada como la enfermedad de mayor relevancia en cultivos de trucha arcoíris en Europa. Típicamente, VHSV se consideraba patógeno de teleósteos de la familia de los salmónidos pero en diversos estudios se ha detectado una enorme expansión hacia otras familias de teleósteos como *Esocidae*, *Gadidae* o *Pleuronectidae* [90], y se cree que su expansión puede abarcar incluso a más familias de las que actualmente se conocen.

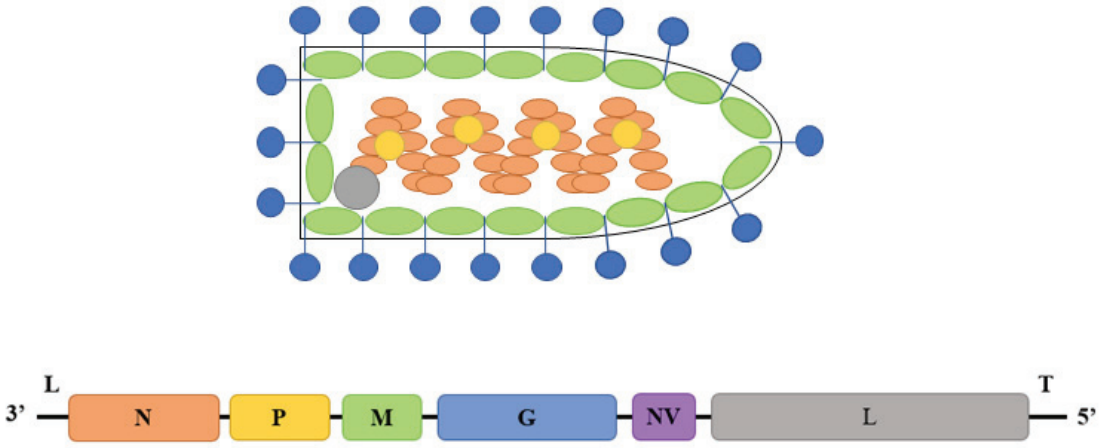


Figura 5. Representación esquemática de la partícula vírica de VHSV junto con su genoma. N (nucleocápsida), P (fosfoproteína), M (proteína de la matriz), G (glicoproteína) y L (polimerasa), NV (proteína no vírica).

El ciclo de replicación de VHSV se inicia con la unión de la proteína G del virión de VHSV a un receptor celular que todavía es desconocido, aunque existen diversas evidencias sobre su naturaleza [91]. Entre ellos, la fosfatidilserina ha sido propuesta como receptor de otros rhabdovirus como el virus de la estomatitis vesicular (VSV) [92] o virus de la rabia (RABV) [93]. Además de la fosfatidilserina, el receptor nicotínico de la acetilcolina se ha propuesto como receptor de estos virus debido a la capacidad de unión de la G de RABV a dicho receptor [94]. Una vez que el virus se ha unido a la superficie celular a través del receptor, ocurre el proceso de endocitosis. La liberación de la nucleocápsida vírica al interior celular ocurre por la acidificación del endosoma, que induce la fusión de la membrana del endosoma con la membrana vírica [95]. Una vez liberada la nucleocápsida en el interior celular, se inicia la transcripción primaria del RNA vírico por la propia RNA polimerasa del virus [95]. La síntesis de RNA comienza en el extremo 3' del genoma generando un RNA "líder". Después, se transcriben individualmente los RNA mensajeros de los genes N, P, M, G y L mediante un mecanismo de "parada y arranque" debido a que cada uno de ellos cuenta con un promotor exclusivo. De este modo, la polimerasa transcribe hasta alcanzar una región intergénica que contiene una señal de parada. Posteriormente, la polimerasa continúa hasta alcanzar la señal de arranque y continúa la transcripción del siguiente gen. Este fenómeno produce que exista una atenuación en la eficiencia de la transcripción de los genes, de modo que se transcriben más aquellos que se encuentran más cercanos al extremo 3', en este caso la N. El resultado de la transcripción genera un gradiente en el número de copias de RNA mensajero del siguiente modo: N > P > M > G > L. Aparte de la transcripción de los genes víricos, también es necesaria la replicación de RNA que conforma el genoma

vírico. Para ello, la polimerasa debe ignorar las señales de arranque y parada con el objetivo de formar una copia única. Este se consigue mediante la unión selectiva de la proteína N recién traducida a la secuencia líder del RNA vírico. La traducción de los RNA mensajeros víricos generan las proteínas que conforman al virión mientras que las copias únicas del genoma completo permiten la formación del genoma RNA de cadena única con polaridad negativa. Este proceso de replicación se encuentra esquematizado en la Figura 6.

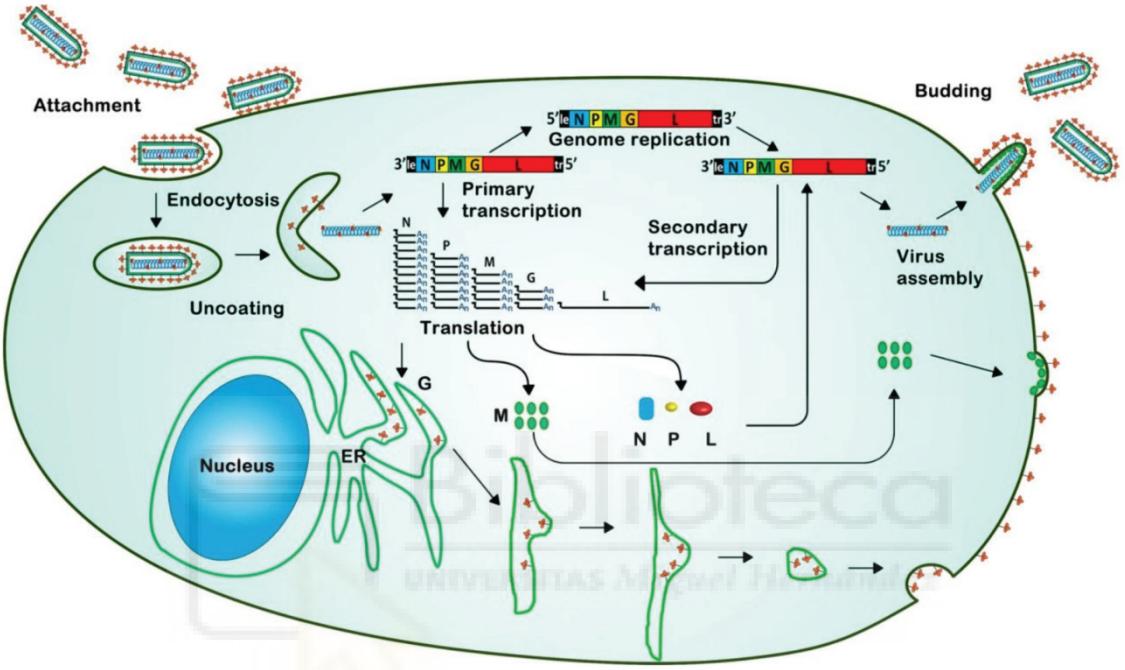


Figura 6. Esquema del ciclo de replicación de un rhabdovirus. Los distintos pasos del ciclo constan de: adhesión a la membrana celular, endocitosis, desenvoltura, replicación del genoma, transcripción del RNA mensajero, traducción de las proteínas víricas, ensamblaje y liberación del virión [95].

2.5.2. Virus de la necrosis pancreática infecciosa (IPNV): IPNV pertenece a la familia *Birnaviridae.* IPNV fue el primer virus de teleósteos aislado en cultivo celular [96] y hasta día de hoy es considerado como uno de los virus de teleósteos más estudiados y sigue siendo una amenaza para el cultivo de trucha arcoíris en la actualidad [97]. Los viriones de IPNV tienen forma icosaédrica sin envuelta de 60 nm de diámetro, conteniendo un genoma que consta de dos segmentos (denominados A y B) de RNA de doble cadena (Figura 7). El segmento A codifica para una poli proteína que es cortada post-traduccionalmente en tres proteínas víricas llamadas VP2, VP3 y VP4 [98]. VP2 es la proteína responsable de la especificidad del serotipo y la diana de los anticuerpos que se encargan de neutralizar al virus. Por otra parte, el segmento B codifica para una RNA

polimerasa dependiente de RNA [99]. La patología asociada a IPNV se trata de la necrosis pancreática infecciosa (IPN) que afecta a salmónidos principalmente [100]. La enfermedad IPN fue por primera vez descubierta como una enfermedad contagiosa aguda que podía causar hasta una mortalidad de un 100% en el cultivo de salmónidos. Posteriormente se han notificado mortalidades que varían desde el 5% hasta el 100% [101]. Actualmente, se han propuesto cuatro serogrupos (A, B, C y D) para la clasificación de los virus catalogados como *Aquabirnavirus* [102]. Los serogrupos están más relacionados con la distribución geográfica que con las especies hospedadoras.

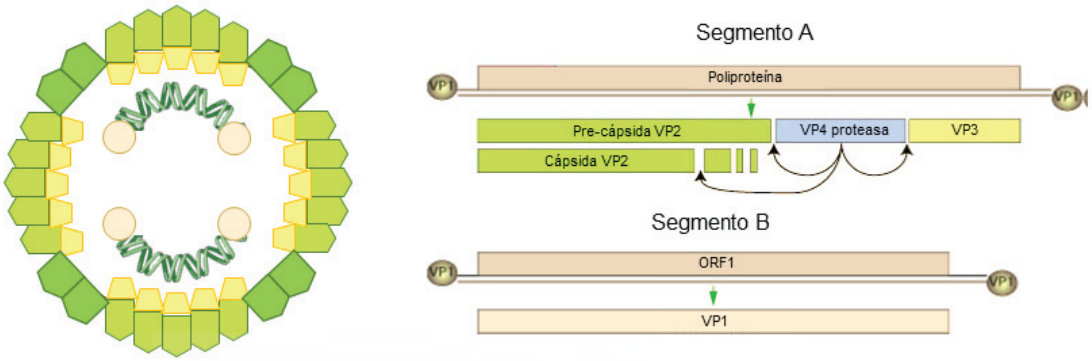


Figura 7. Representación esquemática de la partícula vírica de IPNV junto con su genoma segmentado. Modificado de Dobos & Roberts (1983) [98].

2.5.3. Medidas de control y prevención de las infecciones víricas: la principal estrategia para el control de las enfermedades víricas en la acuicultura es evitar el contacto con el virus. Dichas medidas de bioseguridad se han utilizado durante décadas y actualmente están bien establecidas. Sin embargo, los brotes víricos siguen ocurriendo y la investigación dirigida al desarrollo de vacunas contra enfermedades víricas sigue siendo necesaria. En la actualidad existen vacunas para IPNV pero la falta de estudios *in vivo* con desafíos empleando IPNV ralentizaron su investigación [103]. Los resultados más recientes muestran pasos favorables hacia la creación de una vacuna de DNA frente a IPNV [104]. En el caso de VHSV, debido a la resistencia generada a una reinfección en supervivientes de brotes de VHSV [105], siempre ha existido investigación en el desarrollo de una vacuna frente a este virus [106]. Diversos estudios han demostrado la capacidad de diversas vacunas de conferir protección frente a VHSV [107-110].

2.6. Justificación de la unidad temática

El conocimiento sobre el sistema inmune de teleósteos todavía no está tan completo como el conocimiento que existe sobre el sistema inmune de los mamíferos. Además, ambos presentan numerosas diferencias significativas en su respuesta inmune tanto a nivel celular como molecular. Como se ha descrito anteriormente, existen patógenos víricos que tienen como diana a los RBCs, los cuáles son capaces de desarrollar una respuesta inmune frente a dicha infección, además de participar en múltiples procesos de carácter inmune. A pesar de esto, hasta el momento se desconoce si los RBCs pueden desarrollar mecanismos que interfieran en el progreso de la infección.

Por tanto, los objetivos de esta tesis doctoral son:

1. Dilucidar la respuesta inmune de los RBCs de trucha arcoíris frente a VHSV e IPNV en condiciones *ex vivo*.
2. Estudiar la modulación de los marcadores de las APCs (MHCI, MHCII, CD86 y CD83) en RBCs de trucha arcoíris tras ser expuestos a VHSV, así como los procesos implicados en el procesamiento de antígenos.
3. Analizar la respuesta inmune *in vivo* de RBCs procedentes de truchas arcoíris desafiadas con VHSV.

Por tanto, en esta tesis trabajamos sobre la hipótesis de que los RBCs nucleados de los teleósteos pueden jugar un papel clave para combatir las infecciones víricas. Conocer el papel de los RBCs en la respuesta antiviral permitirá, además de ampliar el conocimiento básico sobre las funciones desconocidas de los RBCs nucleados, iniciar estudios sobre nuevas estrategias para la creación de inmunoestimulantes o vacunas DNA de nueva generación dirigidas a los RBCs para combatir enfermedades víricas en acuicultura.

Para realizar este estudio, hemos empleado un modelo de trabajo compuesto por:

- RBCs de trucha arcoíris (*Oncorhynchus mykiss*), especie modelo en acuicultura, susceptible de ser infectada por VHSV e IPNV.
- Virus de la septicemia hemorrágica vírica, cepa 07.71 [111], virus RNA de cadena única.
- Virus de la necrosis pancreática infecciosa, cepa Sp [112], virus RNA de cadena doble.

3. MATERIALES Y MÉTODOS

3.1 Animales

Los individuos de trucha arcoíris (*Oncorhynchus mykiss*) empleados en este estudio fueron obtenidos de una piscifactoría comercial libre de patógenos y mantenidos en las instalaciones del animalario de la Universidad Miguel Hernández (UMH) de Elche en un sistema de agua declorada recirculante a 14 ºC con una densidad de un ejemplar por cada 3 litros. Los individuos se alimentaron diariamente empleando un pienso comercial. Previamente a realizar los experimentos, los individuos fueron aclimatados a las condiciones de laboratorio durante 2 semanas. Todos los protocolos, procedimientos y métodos de experimentación animal indicados en esta tesis fueron revisados y aprobados por la Oficina de Investigación Responsable de la UMH y la autoridad competente de la Administración provincial. Los procedimientos fueron realizados en acuerdo a las pautas establecidas en el Real Decreto 53/2013 y la directiva europea 2010/63/EU para la protección de animales empleados para experimentación y otros fines científicos.

3.2. Cultivo celular

La composición del medio de cultivo empleado para cultivar RBCs fue RPMI-1640 suplementado con un 10% suero bovino fetal (FBS), 1 mM piruvato, 2 mM L-glutamina, 50 µg/mL gentamicina, 2 µg/mL fungizona, 100 U/mL penicilina y 100 µg/mL estreptomicina. Los RBCs se cultivaron a 14 ºC. Las líneas celulares TSS (células de estroma de bazo), RTG-2 (células de gónada de trucha arcoíris, ATCC® CCL-55), EPC (*Epithelioma papulosum cyprini*, ATCC® CRL-2872) y CHSE-214 (células de embriones de salmón chinook, ATCC CRL-1681) también fueron empleadas en esta tesis. La línea celular TSS (donada por el Dr. Prof. Alberto Villena) fue mantenida en RPMI-1640 suplementado con 20% FBS, 1 mM piruvato, 2 mM L-glutamina, 50 µg/mL gentamicina y 2 µg/mL fungizona a 21 ºC. La línea celular RTG-2 fue mantenida en medio MEM conteniendo un 10% FBS, 1 mM piruvato, 2 mM L-glutamina, 50 µg/mL gentamicina y 2 µg/mL fungizona a 21 ºC. La línea celular EPC fue mantenidas a 28°C, en RPMI-1640 con 10% FBS, 1 mM piruvato, 2 mM L-glutamina, 50 µg/mL gentamicina y 2 µg/mL fungizona. La línea celular CHSE-214 fue mantenida en RPMI-1640 con 10% FBS 1 mM piruvato, 2 mM L-glutamina, 50 µg/mL gentamicina, 2 µg/mL fungizona, 100 U/mL penicilina y 100 µg/mL estreptomicina.

3.3. Purificación de eritrocitos de sangre periférica

La purificación de RBCs extraída de sangre periférica se realizó mediante un gradiente de densidad en Histopaque 1077. Brevemente, aproximadamente 100 µL de sangre de trucha arcoíris fueron diluidos en 2 mL de RPMI con 10% FBS y

penicilina/estreptomicina. La sangre diluida en RPMI fue depositada lentamente en capa sobre un volumen equivolumétrico de Histopaque® 1077 y las células fueron centrifugadas a 1600 rpm durante 30 minutos. Posteriormente, un segundo centrifugado fue realizado con Histopaque 1077 tal y como se ha descrito anteriormente para aumentar la pureza del cultivo celular de RBCs. Después, los RBCs fueron lavados con RPMI 2% FBS dos veces y se cultivaron en un flask de 25 mL. Al día siguiente, se contaron y emplearon para realizar los distintos ensayos. La purificación de RBCs siguiendo este protocolo se realizó en todas las Publicaciones que conforman en esta tesis.

3.4. VHSV e IPNV

Los virus utilizados en esta tesis fueron el virus de la septicemia hemorrágica vírica (VHSV) y virus necrosis pancreática infecciosa (IPNV). La cepa de VHSV 07.71 fue aislada en Francia y se adquirió de la ATCC (ATCC VR-1388) y se propagó en la línea celular EPC a 14 ºC como queda descrito en detalle en la Publicación 1. Los sobrenadantes de las células EPC infectadas con VHSV fueron clarificados mediante centrifugación a 1500 rpm durante 5 minutos y se conservaron a -80 ºC hasta que fueron empleados. El VHSV se propagó en estas condiciones para los ensayos *ex vivo* de la Publicación 1 y 3, así como para la obtención de los diversos inóculos de los ensayos de infección *in vivo* de las Publicaciones 3 y 4. En el caso de IPNV, la cepa empleada fue Sp, que fue adquirida desde la ATCC (ATTC VR-1318). IPNV fue propagado en la línea celular CHSE-214 tal y cómo se describe en Publicación 2, y para su utilización en la misma publicación.

3.5. Ensayos de infección vírica

Los ensayos de infección vírica fueron realizados a la multiplicidad de infección (MOI) que se indica en cada uno de los experimentos siguiendo la siguiente fórmula:

$$\mathrm{MOI} = \frac{\text{Título del virus } (\mathrm{TCID}_{50}/\mathrm{mL}) \cdot \text{Volumen de la infección } (\mathrm{mL}) \cdot \mathrm{x}}{\text{Nº de células}}$$

Donde x es la dilución del sobrenadante con el virus que se va a emplear para infectar. Las células fueron infectadas incubando el virus durante los tiempos indicados y la MOI indicada en cada ensayo, a 14 ºC. Después, los RBCs fueron lavados con RPMI 2% FBS frío para eliminar el virus no adherente y finalmente se cultivaron con RPMI 2% FBS a los tiempos indicados en cada ensayo.

3.6. Extracción de RNA, síntesis de cDNA y PCR cuantitativa

La extracción de RNA fue realizada empleando el kit comercial E.Z.N.A ® Total RNA kit siguiendo las instrucciones del fabricante. Tras la extracción, la concentración de RNA fue cuantificada usando el espectrofotómetro NanoDrop®. El RNA se conservó a -80 ºC hasta su uso. Antes de sintetizar el DNA complementario (cDNA), el RNA fue tratado con desoxirribonucleasa para eliminar posible DNA genómico residual empleado el kit TURBO™ DNAse siguiendo las instrucciones adjuntas. La PCR con transcriptasa inversa para sintetizar cDNA fue realizada empleando la enzima M-MLV. La PCR cuantitativa (qPCR) fue realizada en un sistema ABI PRISM 7300. Las reacciones se realizaron en un volumen total de 20 µL con un total de 12 o 24 ng de cDNA según se indica en cada Publicación, 900 nM de cada primer, 10 µL de mezcla universal de TaqMan con 300 nM de sonda o 10 µL de mezcla maestra de SYBR Green. Los ciclos empleados fueron 50 ºC durante 2 minutos y 95 ºC durante 10 minutos, seguidos por 40 ciclos a 95 ºC durante 15 segundos y después 60 ºC durante un minuto. Los cebadores y sondas empleadas en cada ensayo se pueden encontrar en las tablas adjuntas a cada artículo. La expresión génica fue analizada utilizando el método $2^{-\Delta Ct}$ o $2^{-\Delta\Delta Ct}$ [113] según se indica en cada experimento. La qPCR y sus sucesivos cálculos fueron realizados de este modo para todas las Publicaciones que conforman en esta tesis.

3.7. Tinción intracelular y extracelular

La tinción de los RBCs con anticuerpos frente a marcadores inmunológicos se realizó a nivel intracelular y de membrana. Para la tinción intracelular, los RBCs se fijaron en RPMI con paraformaldehído (PFA) al 4% y glutaraldehído al 0.008% durante 20 minutos. Después, la membrana de los RBCs fue permeabilizada con saponina al 0.05% diluida en PBS durante 15 minutos. Tras permeabilizar la membrana, los anticuerpos primarios fueron diluidos en buffer permeabilizador a la dilución recomendada por el suministrador y se incubaron con los RBCs durante 60 minutos a temperatura ambiente. Los anticuerpos secundarios fueron incubados durante 30 minutos. Después de cada incubación de anticuerpos, los RBCs fueron lavados con buffer permeabilizador. Para realizar la tinción de membrana de los marcadores de membrana, los RBCs fueron previamente fijados con PFA al 4% y glutaraldehído al 0.008% durante 20 minutos. Los anticuerpos primarios y secundarios fueron diluidos en PBS e incubados con los RBCs durante 60 y 30 minutos, respectivamente. Finalmente, para ambas tinciones, los RBCs se mantuvieron en PBS con PFA al 1% hasta que fueron analizados mediante citometría de flujo usando el citómetro FACSCanto II y microscopía de fluorescencia mediante el sistema de imagen IN Cell Analyzer 6000. Para realizar la tinción intracelular de LC3,

los RBCs fueron tratados con metanol frío durante 15 minutos previamente a la fijación con PFA 4% y glutaraldehído 0.008%. Después, tanto el anticuerpo primario anti-LC3 como el anticuerpo secundario se diluyeron en PBS con Triton-X100 0.3%. La tinción intracelular fue empleada tal y como se ha explicado en las Publicaciones 1, 2, 3 y 4 mientras que la tinción extracelular se realizó en la Publicación 3.

3.8. Western blot

Para los ensayos de western blot, los pellets de RBCs y otros tipos celulares fueron lavados tres veces para eliminar proteínas del FBS empleado como componente del medio de cultivo. Tras el último lavado, las células fueron resuspendidas en PBS con un cóctel de inhibidor de proteasas. A continuación, se lisaron mediante 3 ciclos de congelación / descongelación y se emplearon micropistilos para completamente lisar los pellets de RBCs (>10^7 de células). Los sobrenadantes fueron clarificados mediante centrifugación a 12000 rpm a 4º C. Antes de cargar las muestras proteicas a los geles de poliacrilamida, se les añadió buffer de carga con β-mercaptoetanol para establecer condiciones reductoras. Además, las muestras fueron calentadas a 100 ºC durante 5 minutos para desnaturalizar completamente las proteínas. El porcentaje del gel de poliacrilamida Tris-glicina con SDS dependió del peso molecular de la proteína de interés analizada. Dicho porcentaje puede encontrarse detallado en cada uno de los distintos experimentos de Western blot. La electroforesis fue realizada a 100V o 150V, dependiendo del porcentaje del gel, durante 90 minutos. Para realizar el inmunoblot, las proteínas en el gel fueron transferidas a una membrana de nitrocelulosa en buffer de transferencia compuesto por 2.5 mM Tris, 9 mM glicina y 20% metanol durante 120 minutos a 100V. Las membranas de nitrocelulosa de 0.45 µm de tamaño de poro fueron bloqueadas con PBS 0.1% Tween-20 y 5% de leche desnatada durante 1 hora a temperatura ambiente. Los anticuerpos primarios fueron diluidos en PBS 0.1% Tween-20 a la dilución recomendada por el suministrador e incubados a 4 ºC durante toda la noche. Los anticuerpos secundarios se diluyeron en PBS 0.1% Tween-20 y fueron incubados a una dilución de 1/10000 durante 30 minutos. Se utilizaron anticuerpos secundarios hechos en cabra frente a conejo o ratón conjugados con peroxidasa. Tras cada incubación tanto de anticuerpo primario como secundario, la membrana fue lavada con PBS 0.1% Tween-20 durante 15 minutos repitiendo el lavado 3 veces. La actividad peroxidasa fue detectada empleando reactivos ECL de quimioluminiscencia y revelado mediante exposición a films de rayos X o empleando el sistema Chemidoc XRS (Biorad). La técnica de western blot se realizó de este modo en las Publicaciones 1, 2, 3 y 4.

3.9. Anticuerpos

Los anticuerpos primarios empleados en esta tesis tienen tres orígenes: comercial o producidos en laboratorio. Los anticuerpos comerciales se emplearon a las diluciones recomendadas por la casa comercial. La producción de anticuerpos se realizó en el laboratorio del Prof. Dr. Luis Mercado, el laboratorio del Dr. Julio Coll o el laboratorio de la Dra. Amparo Estepa. Los anticuerpos empleados en cada una de las Publicaciones quedan descritos en la correspondiente sección de Materiales y Métodos de la Publicación.

3.10. Reactivos

En el transcurso de esta tesis, los RBCs han sido tratados con distintos compuestos y enzimas con el objetivo de determinar la capacidad de entrada vírica, evaluación de autofagia, actividad proteasómica o respuesta antiviral. La neuroaminidasa de *Vibrio cholerae*, una enzima capaz de degradar el ácido siálico, fue empleada para evaluar la entrada de VHSV en los RBCs a concentraciones de 50 y 100 mU/mL a 21 ºC durante 30 minutos. Después del tratamiento, los RBCs fueron lavados para eliminar completamente la enzima. El cloruro de amonio (NH_4Cl) es un compuesto empleado para bloquear la acidificación del endosoma, y, por tanto, impedir la entrada del virión mediante la fusión con la membrana endosómica que resulta en la liberación de la nucleocápsida en el interior celular. Durante la infección de RBCs con VHSV, NH_4Cl fue añadido a una concentración de 7 mM. La autofagia fue evaluada empleando niclosamida a unas concentraciones finales de 10 y 20 µM, un compuesto que bloquea la degradación de autofagosomas evitando su fusión con lisosomas. La actividad del proteasoma fue bloqueada empleando el compuesto MG132, y dicha actividad del proteasoma se midió empleado un kit comercial. Los reactivos descritos se emplearon en las Publicaciones 1 y 3.

3.11. Análisis de proteómica

Los datos de proteómica presentados en esta tesis corresponden a una cromatografía liquida acoplada a espectrometría de masas clásica (LC-MS/MS) (Publicación 3 y 4) y un análisis mediante tecnología iTRAQ 4plex (Publicación 1). La diferencia entre ambas metodologías se centra en que, tras la digestión y procesado inicial de las proteínas de la muestra, en el análisis iTRAQ se realiza un marcaje individual de cada una de las muestras y después se crea una mezcla final sobre la que se realiza la espectrometría de masas. El marcaje otorgado a cada una de las muestras otorga un incremento en el valor m/z con una diferencia de 1 de las distintas muestras, de manera que la cantidad de proteína específica contenida en cada una de ellas se determina de manera

comparativa. Cada una de las condiciones o muestras de los estudios de proteómica corresponde a un grupo de muestras procedente de 8 individuos (8 millones de células por individuo, 64 millones de células en total). En total, se analizaron 4 grupos correspondientes a 2 grupos control negativo y 2 grupos de RBCs expuestos a VHSV (*ex vivo*) o procedentes de individuos desafiados con VHSV (*in vivo*). Las células se centrifugaron y sobre el pellet se añadió agua desionizada. Para lisar las células, se congelaron a -80 ºC. El lisado se centrifugó a 17000g durante 20 minutos para separar las fracciones de membrana y citosólica de las células lisadas. De la fracción citosólica, la hemoglobina se eliminó parcialmente empleando un kit comercial. Cada una de las fracciones fue analizada por separado, tal y como se describe en Puente-Marin *et al*. [114]. Brevemente, las muestras se analizaron con un nuevo método que combina fraccionamiento de las muestras entre fracción citosólica y de membrana, digestión de las proteínas, fraccionamiento de péptidos en pH de fase reversa y finalmente, cromatografía líquida acoplada un electrospray ionizante que permite analizar los péptidos a un sistema de detección MS/MS que emplea el tiempo de vuelo (TOF) para determinar los pesos moleculares. Los espectros MS/MS fueron exportados a formato MGF y fueron enfrentados en el servidor Mascot de Uniprot/Swissprot Knowledgebase junto a contaminantes comunes frente a una base de datos de secuencias de teleósteos para el análisis por LC-MS/MS y de *Oncorhynchus mykiss* para el análisis por iTRAQ. El análisis de las rutas funcionales reguladas positivamente o negativamente se realizó mediante los programas Cytoscape [115], ClueGO [116] y CluePedia [117]. El programa STRING [118] se utilizó para el análisis de interacción de proteínas. Los ensayos de proteómica se han utilizado en las Publicaciones 1, 3 y 4.

3.12. Análisis de transcriptómica

Los distintos estudios de transcriptómica de RBCs presentados en esta tesis se desarrollaron en condiciones *ex vivo* o *in vivo*. Los RBCs de cada una de las muestras fue añadida a un buffer de lisis y se mantuvo a - 80 ºC hasta que fue enviada para el análisis RNASeq. Se crearon grupos de RBCs para realizar el análisis en las condiciones: no expuestos y expuestos VHSV. La secuenciación y la preparación de la librería de RNASeq fueron llevadas a cabo por la empresa STABVida Lda. La identificación de los cóntigos se realizó por homología con secuencias de teleósteos mediante el software BLAST2GO tal y como se describe en Puente-Marin *et al*. [114]. El análisis de las rutas funcionales reguladas positivamente o negativamente se realizó mediante los programas Cytoscape [115], ClueGO [116]y CluePedia [117]. El programa STRING [118] se utilizó para el análisis de interacción de proteínas. Los datos obtenidos mediante transcriptómica se encuentran en las Publicaciones 3 y 4.

3.13. Aislamiento celular de eritrocitos

Para evaluar la respuesta a nivel *in vivo* mediante transcriptómica, los RBCs procedentes de truchas arcoíris, desafiadas o no con VHSV, se purificaron mediante un citómetro con capacidad de clasificación celular BD FacsJazz. Se aislaron RBCs de muestras de sangre periférica de la vena caudal y de riñón anterior, disgregado empleando tamices 100 y 40 µm de diámetro sucesivamente. Después, las células se incubaron con el compuesto SYTO RNASelect para teñir de manera fluorescente los ácidos ribonucleicos con el objetivo de poder distinguir las distintas poblaciones celulares presentes en los tejidos en función de su cantidad de RNA. El resultado del aislamiento de célula única fue validado mediante microscopía óptica. El flujo general de trabajo para realizar esta técnica se indica en la Figura 8.

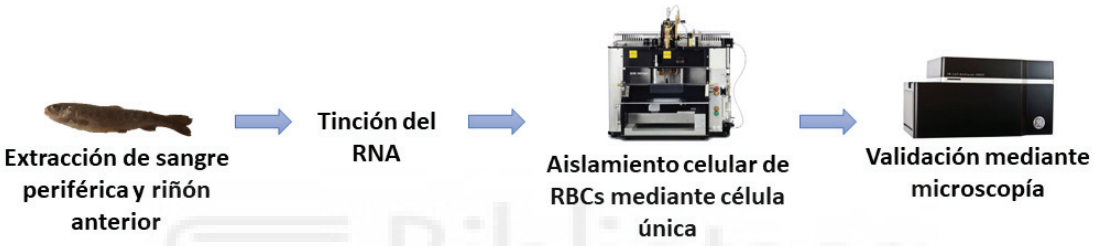


Figura 8. Representación gráfica del flujo de trabajo empleado para la purificación de RBCs desde sangre periférica y riñón anterior para la realización del análisis *in vivo.*

4. RESULTADOS Y DISCUSIÓN

4.1. Resultados obtenidos de la Publicación 1.

Publicación 1. Nombela, I., Puente-Marin, S., Chico, V., Villena, A. J., Carracedo, B., Ciordia, S., ... & Estepa, A. (2017). Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus. *F1000Research*, *6*.

La infección por VHSV es arrestada en RBCs

Para analizar la capacidad de replicación del VHSV en RBCs, primero se purificaron RBCs de sangre periférica de ejemplares de trucha arcoíris. Posteriormente, los RBCs fueron expuestos a VHSV a distintos tiempos para monitorizar su replicación mediante análisis de la expresión del gen N de VHSV (*NVHSV*) mediante qPCR. Los resultados de la Figura 1A P1 muestran un aumento significativo de la expresión del gen *NVHSV* a 3 horas post-exposición (hpe) pero dicho incremento se detiene tras 6 hpe, mientras que en la línea celular RTG-2, susceptible de ser infectada por VHSV, el incremento de los transcritos del gen NVHSV es exponencial. Paralelamente, la ratio de expresión de los genes *NVHSV* y G de VHSV (*GVHSV*) fue evaluada en RBCs y la línea celular RTG-2 (Figura 1B P1). En la línea celular RTG-2, la ratio *NVHSV* / *GVHSV* presentaba un valor de aproximadamente 8 mientras que en RBCs dicha ratio correspondía a 2. Además, la titulación de los sobrenadantes de RBCs expuestos a VHSV a varias multiplicidades de infección mostró un descenso del título vírico de aproximadamente 4 logaritmos en base 10 respecto al título empleado para infectar (Figura 1C P1). No obstante, a tiempos más largos después de la exposición (6 días post-exposición, dpe) se observó un incremento en el título vírico de aproximadamente el doble (Figura 1D P1), demostrando que el RBC es capaz de mantener un título vírico bajo varios días tras la exposición. Para descartar que dicho título vírico no procedía de VHSV residual adherido a la membrana celular, se utilizó un tratamiento con cloruro de amonio, capaz de bloquear la acidificación de endosoma, y por tanto, inhibir la entrada del VHSV en la célula [119]. El resultado mostró un descenso del título vírico detectado en RBCs tratados con cloruro de amonio (Figura 1D P1). Los RBCs también fueron tratados con neuroaminidasa, una enzima capaz de eliminar el ácido siálico de la membrana de los RBCs, que previamente se ha visto que puede favorecer la interacción entre rhabdovirus y membrana celular [120]. El resultado mostró un incremento de transcritos *NVHSV* en RBCs tratados con neuroaminidasa hasta 10 veces superior tras 3 hpe. Sin embargo, los transcritos de *NVHSV* volvieron a decaer a las 72 hpe (Figura 1E P1). Paralelamente, la proteína NVHSV pudo ser

detectada mediante tinción intracelular (Figura 1F P1) y citometría de flujo (Figura 1G P1) tras 24 hpe, pero no a 72 hpe.

Los resultados aquí mostrados demuestran que los RBCs de trucha arcoíris no son permisivos a la infección por VHSV, en contraste con otros tejidos como estroma o riñón anterior donde existe infección productiva de VHSV [121, 122]. De este modo, en RBCs la infección por VHSV sería arrestada o abortada. Este tipo de infección ocurre cuando un virus es capaz de entrar en la célula, sintetizar parte de sus componentes pero no es capaz de liberar viriones ensamblados [123]. Nuestros resultados muestran unión e internalización de VHSV en RBCs seguida de transcripción de genes de VHSV a tiempos cortos tras la exposición y posterior eliminación casi completa del VHSV internalizado. La inhibición del ciclo de replicación de VHSV ocurre tras la entrada del VHSV, ya que la ratio entre los genes *NVHSV* y *GVHSV* es de 2, indicando que el genoma vírico solamente pudo replicarse o transcribirse una vez. No obstante, se pudo recuperar VHSV de sobrenadantes de RBCs expuestos a VHSV a tiempos largos post-exposición, hasta 40 días después de la exposición. El motivo por el cual una infección acaba siendo abortada suele ser desconocido en la mayor parte de los casos. Diversos estudios han hipotetizado diversas causas para explicar infecciones abortivas, como puede ser la alta expresión constitutiva de genes de carácter antiviral [124] o expresión de IFN y genes relacionados [125, 126].

Evaluación de la regulación de la respuesta de IFN en RBCs expuestos a VHSV

Posteriormente se investigó si los RBCs expuestos a VHSV eran capaces de generar una respuesta inmune examinando la expresión de diversos genes característicos de la respuesta antivírica de teleósteos. En un ensayo de monitorización de la expresión de los genes inducibles por IFN1, *mx1-3* y *pkr*, a distintos tiempos post-exposición, se observó que en RBCs expuestos a VHSV sólo se producía regulación negativa de *mx1-3* (Figura 2A P1). Sin embargo, no se observaron cambios en la expresión de *pkr* (Figura 2B P1). A 3 hpe, la expresión de *ifn1* también se observó regulada negativamente. Complementariamente, se evaluó la expresión de IFN1 y Mx en RBCs de trucha arcoíris expuestos a VHSV mediante citometría de flujo. Los resultados no mostraron cambios en la regulación a nivel proteico (Figura 3A P1)

Estos resultados indican que la inhibición de la infección de VHSV en RBCs de trucha arcoíris no parece estar relacionada con IFN1 o genes inducibles por IFN1, ya que la expresión de *ifn1*, *mx* y *pkr* se encontraba escasamente modulada durante la exposición a VHSV. Sin embargo, la expresión de IFN1 y genes relacionados con IFN1 aumenta

significativamente en RBCs durante la infección de PRV [33] o ISAV [30], cuyas infecciones son productivas en RBCs. Aunque generalmente se piensa que los PAMPs víricos son los encargados de activar la producción de IFN, existen diversas excepciones donde los PAMPs víricos no han sido capaces de activar dicha producción [127]. Se ha apuntado a que partículas víricas defectivas serían las encargadas de realizar la inducción de IFN [127]. Alternativamente, también se ha observado que los virus han desarrollado estrategias evolutivas para bloquear la señalización mediada por IFN [128].

Comunicación cruzada entre RBCs y TSS

El bazo de trucha arcoíris es un órgano que está formado por varios tipos celulares, como RBCs, linfocitos o estroma [129]. Se ha descrito que los factores solubles producidos por las células de estroma son necesarios para el desarrollo de RBCs en riñón anterior o bazo [130]. Para evaluar una posible comunicación paracrina entre RBCs y TSS, se estimularon RBCs con VHSV inactivado mediante radiación ultravioleta y se cocultivaron con la línea celular TSS en un sistema Transwell. Los resultados mostraron una correlación significativa entre la expresión de *ifn1* y la expresión del gen inducido por VHSV 1 (*vig1*) e *il15* en RBCs (Figura 4A P1). La expresión de *ifn1* entre RBCs y TSS también se encontraba significativamente correlacionada (Figura 4B P1). En TSS, se observó una correlación significativa entre *ifn1* con *mx*, *vig1* e *il15* (Figura 4C P1). Por otro lado, se observó un incremento en la expresión de *ifn1* y *mx* en RBCs tratados con medio de cultivo condicionado procedente de células RTG-2 estimuladas con VHSV inactivado mediante ultravioleta (Figura 4D P1).

Estos resultados confirman una comunicación paracrina entre RBCs y la línea celular TSS mediada por IFN. Ya que los resultados muestran la regulación conjunta de *ifn1* en ambos tipos celulares, así como la regulación correlativa del ISG *mx* en la línea celular TSS, junto con *il15*, una interleucina capaz de activar respuestas antivirales mediante un mecanismo dependiente de IFN [131]; y *vig1*, un gen inducido por VHSV así como IFN [132]. Por tanto, estas observaciones demuestran que los RBCs de trucha arcoíris pueden establecer comunicación paracrina con otras células mediada por moléculas de señalización relacionada con IFN.

VHSV induce la expresión de IL8 y BD1 en RBCs en condiciones *ex vivo*

Se analizó la expresión de IL8, β-defensina 1 (BD1), IL1β, el péptido antimicrobiano hepcidina, IFNγ y el factor estimulador de células NK (NKEF) con el objetivo de buscar moléculas relacionadas con la actividad antiviral en RBCs expuestos a VHSV. De todas las proteínas mencionadas, solamente se observó incremento en la expresión para la IL8 (Figura 3B/E P1) y BD1 (Figura 3C/F P1). Las citoquinas IL1β e IFNγ (Figura 3B P1) junto con hepcidina (Figura 3C P1) y NKEF (Figura 3D P1) no mostraron cambios en su expresión a 72 hpe. No obstante, posibles implicaciones de la implicación de IL8 o BD1 en arrestar la infección de VHSV deberán ser evaluadas en futuros estudios. Previamente se ha descrito que la inducción de IL8 podría estar relacionada con la infección abortiva del virus sincitial respiratorio en macrófagos alveolares [133].

Perfil de proteínas mediante iTRAQ de RBCs expuestos a VHSV

El análisis proteómico de RBCs expuestos a VHSV, mediante iTRAQ, mostró que únicamente 64 proteínas se encontraban reguladas significativamente tras 72 hpe (Figura 5 P1). En concreto, 59 proteínas estaban reguladas negativamente mientras que sólo 5 de ellas se encontraban reguladas positivamente. El análisis de enriquecimiento de procesos celulares diferencialmente representados en los RBCs expuestos a VHSV mostró una alta representación de los procesos de estabilidad del RNA mensajero, proteasoma, procesos víricos y procesos catabólicos celulares (Figura 6 P1). El análisis de interacción de proteínas de las 59 proteínas reguladas negativamente identificó la interacción de proteínas pertenecientes a procesos del espliceosoma y proteasoma. También se encontró que 17 proteínas estaban catalogadas en procesos víricos y que gran parte de ellas interaccionaban entre sí (Figura 7 P1).

En resumen, estos análisis mostraron la regulación de dos mecanismos típicos de subversión vírica: secuestro del espliceosoma y apagado celular a nivel hospedador [134, 135]. Típicamente, estas estrategias suelen conducir hacia la muerte celular del hospedador y la replicación vírica aumentada [134], sin embargo este efecto no se observa en los RBCs expuestos a VHSV. Por lo tanto, es posible que en los RBCs se establezca un bloqueo de la traducción que impida la traducción de los mensajeros tanto celulares como víricos [136], algo que se pasará a investigar a continuación.

eIF2α se encuentra fosforilado en RBCs expuestos a VHSV

La fosforilación de la subunidad α del factor de iniciación de la traducción 2 (eIF2α) es un reconocido mecanismo de inhibición de la iniciación de la traducción [137]. Por ello, se evaluó dicha fosforilación en RBCs expuestos a VHSV en comparación con RBCs no expuestos mediante western blot. Los resultados mostraron un incremento de dicha fosforilación en RBCs expuestos a VHSV (Figura 8A/B P1). Es sabido que cuatro quinasas fosforilan a este factor [138]. Entre ellas, destaca la quinasa eIF2α hemo-regulada (HRI), cuyo grupo hemo regula la transcripción de genes de las α y β globinas en RBCs por unión al factor de transcripción Bach1 [139]. Por ello, se exploró la expresión del gen de β-globina durante el curso de una exposición a VHSV y los resultados mostraron que este gen se encontraba regulado negativamente a 6 hpe (Figura 9 P1), sugiriendo una posible activación de HRI y consecuente fosforilación de eIF2α e inhibición de la síntesis de proteínas.

En definitiva, la fosforilación de eIF2α podría ser el mecanismo subyacente de la inhibición de la traducción y consecuente regulación negativa del proteoma en los RBCs expuestos a VHSV.

VHSV incrementa el estrés oxidativo y la expresión de genes de enzimas antioxidantes en RBCs

El estrés oxidativo puede ser inducido como consecuencia de una infección vírica mediante alteración de los mecanismos que controlan los procesos de reducción y oxidación [140]. Además, el estrés oxidativo puede activar HRI [141]. Se observó la producción de las especies reactivas de oxígeno (ROS) intracelular mediante intensidad de fluorescencia de DCFDA en RBCs expuestos a VHSV (Figura 10A P1). Los resultados mostraron un incremento significativo en la intensidad de fluorescencia a 72 hpe. Además, para determinar la capacidad de respuesta de los RBCs a este estrés oxidativo, se evaluó la expresión génica de las enzimas antioxidantes ferritina (*fth*), glutatión S-transferasa P (*gstp1*), *nkef*, superoxido dismutasa (*sod1*) y tiorredoxina (*trx*). Los resultados mostraron un incremento en la expresión de todos los genes mencionados, excepto *sod1* (Figura 10B P1), demostrando de esta manera la capacidad de los RBCs de trucha arcoíris para contrarrestar el estrés oxidativo.

Considerando que las ROS se encuentran incrementadas en los RBCs expuestos a VHSV, este resultado podría estar sugiriendo un efecto antimicrobiano en estas [142], aunque por otro lado pueden ejercer efectos citotóxicos [143]. Este mismo efecto se

observa en el contexto infecciones víricas permisivas en virus de distintas familias tal como virus de la inmunodeficiencia humana, virus de la gripe o Epstein Barr virus, entre otros [144]. Por otro lado, el aumento de ROS parece estar compensado por un aumento de la expresión génica de enzimas antioxidantes, como ya se ha descrito previamente [143].

4.2. Resultados obtenidos de la Publicación 2.

Nombela, I., Carrion, A., Puente-Marin, S., Chico, V., Mercado, L., Perez, L., ... & del Mar Ortega-Villaizan, M. (2017). Infectious pancreatic necrosis virus triggers antiviral immune response in rainbow trout red blood cells, despite not being infective. *F1000Research*, *6*.

IPNV no replica en RBCs en condiciones *ex vivo*

A diferencia de VHSV, IPNV es un virus con un genoma de RNA de cadena doble y carente de envoltura por lo que su ciclo de replicación podría ser distinto al de VHSV en los RBCs. En primer lugar, se analizó la replicación de IPNV en RBCs. Los resultados mostraron que los transcritos del segmento A de IPNV presentaban niveles semejantes desde 3 hpe hasta 72 hpe, al contrario que en CHSE-214 (Figura 1A P2), donde aumentan exponencialmente. Por otro lado, el título vírico de los sobrenadantes procedentes de RBCs expuestos a IPNV mostró un título de aproximadamente 5 logaritmos menor que el título empleado para infectar (Figura 1B P2). Tampoco se detectó presencia intracelular de IPNV mediante citometría de flujo en RBCs expuestos a IPNV (Figura 1C/D P2).

Por tanto, estos resultados concluyen que IPNV no infecta a RBCs de trucha arcoíris. IPNV tiene como diana células de páncreas e hígado [145] aunque también se ha visto que es capaz de infectar a células de origen hematopoyético [145]. Similarmente, se ha visto que IPNV es capaz de entrar en células de mamíferos pero sin ser capaz de replicar en ellas [146], sugiriendo una entrada mediada por receptor. Nuestros resultados además muestran una escasa presencia de IPNV tras 72 hpe en sobrenadante de RBCs expuestos a IPNV y dicha persistencia podría indicar una leve replicación de IPNV en RBCs.

La exposición a IPNV incrementa la expresión de genes y proteínas inducibles por IFN1 en RBCs

Para determinar si IPNV era capaz de inducir una respuesta inmune relacionada con IFN1 en RBCs, se analizó la expresión de genes y proteínas inducibles por IFN1 mediante qPCR, citometría de flujo y microscopía de fluorescencia. Los resultados mostraron un incremento significativo en la expresión de *mx* y *pkr* a 72 hpe (Figura 2A P2). Por otra parte, *ifn1* presentaba su pico de expresión a 24 hpe (Figura 2A P2). Además, la expresión del gen *tlr3* presentó una tendencia a estar sobreexpresado a 24

hpe mientras *irf7* estaba sobreexpresado a 72 hpe (Figura 2A P2). Más aún, se analizó la expresión de la proteína Mx mediante microscopía de fluorescencia y citometría de flujo. Los resultados mostraron un incremento en la expresión de Mx a 6 dpe (Figura 2B/D P2).

La respuesta inmune observada en RBCs expuestos a IPNV sigue la línea de la respuesta inmune observada en RBCs frente a virus cuya infección es permisiva en RBCs como PRV [33] e ISAV [30]. Aunque no se pudo detectar IPNV intracelularmente, se observó un incremento en la expresión del gen *tlr3*, receptor de tipo Toll cuyo PAMP es el RNA vírico de doble cadena, que podría estar involucrado en la activación de la cascada de señalización de IFN1. La producción de IFN1 conllevaría a la síntesis de la proteína antiviral Mx. La expresión de Mx en RBCs expuestos IPNV podría explicar los bajos títulos víricos observados [147]. Aún más, la alta expresión basal de Mx determinada en RBCs no expuestos podría ser la causa de la desaparición temprana de IPNV dentro del RBC.

El medio condicionado de RBCs expuestos a IPNV protege a la línea celular CHSE-214 frente a la infección de IPNV

Para analizar si los RBCs expuestos a IPNV eran capaces de secretar factores que fueran capaces de proteger a otros tipos celulares de teleósteos frente a una infección de IPNV, se trataron células CHSE-214 con medio condicionado de RBCs no expuestos y expuestos a IPNV, y a continuación se infectaron con IPNV. Los resultados mostraron un descenso significativo en el título de IPNV cuando se realizó un pretratamiento con el medio condicionado de RBCs expuestos a IPNV a una dilución 1/5 (Figura 3A P2) sin afectar a la viabilidad de la línea celular CHSE-214 (Figura 3B P2).

Este hecho indicaría que los RBCs serían capaces de secretar factores que podrían estar ejerciendo una actividad antiviral. La actividad de protección frente a infecciones víricas inducida por los sobrenadantes condicionados ya ha sido previamente reportada para otros tipos celulares, como las líneas celulares RTS11 y RTG-2 expuestas a poli(I:C) o infectadas con reovirus de salmón chum [148].

IPNV no induce la expresión de citoquinas pro-inflamatorias en RBCs

RBCs fueron expuestos a IPNV y se analizó la expresión de IL8, IL1β y TNFα a nivel de proteína mediante citometría de flujo. Los resultados mostraron un ligero descenso en la expresión proteica de estas citoquinas (Figura 4A P2).

La expresión de estas citoquinas se ha determinado en tejidos de origen hematopoyético en presencia de IPNV [149-151]. También se ha observado la inducción de la expresión IL8 en RBCs en respuesta a VHSV, como parte de la Publicación 1 de esta tesis. No obstante, IL8, IL1β y TNFα no parecen estar implicadas en la respuesta frente a IPNV en RBCs.

La exposición de RBCs a IPNV no produce la fosforilación de eIF2α

La fosforilación de eIF2α es un mecanismo global de inhibición de la traducción proteica [152], además se ha descrito que este proceso ocurre en la infección de la línea celular CHSE por IPNV [153] y, como se indicó en la Publicación 1 de esta tesis, en RBCs expuestos a VHSV. Además, se ha descrito que IPNV es capaz de realizar un apagado celular en la línea celular K (AGK) de mero (*Epinephelus coioides*) sin embargo la implicación de eIF2α fosforilado es desconocida [154]. También, se ha demostrado que la transfección con la proteína VP2 del virus de la enfermedad bursitis infecciosa en células HeLa es capaz de inducir la fosforilación de eIF2α [155]. Teniendo en cuenta que en RBCs expuestos a IPNV se detectó una leve regulación negativa en la expresión de diversas citoquinas, se decidió investigar este mecanismo en RBCs expuestos a IPNV. Los resultados no mostraron fosforilación de eIF2α (Figura 4B P2).

El hecho de que eIF2α no se encuentre fosforilado estaría relacionado con el hecho de que los RBCs aumentan la expresión de la proteína Mx en respuesta a IPNV. Del mismo modo, Mx se trata de una proteína producida en respuesta a IFN1. Se ha descrito que IFN1 es capaz de inhibir la expresión de IL1β [156], lo que explicaría los resultados obtenidos en el apartado anterior y confirmaría que existe una activación de la cascada de señalización de IFN1.

4.3. Resultados obtenidos en la Publicación 3.

Nombela, I., Requena-Platek, R., Morales-Lange, B., Chico, V., Puente-Marin, S., Ciordia, S., ... & Ortega-Villaizan, M. D. M. (2019). 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*, *8*(5), 386.

Análisis transcriptómico de RBCs expuestos a VHSV: regulación de la presentación de antígenos

Se realizó un análisis de transcriptómica con el objetivo de identificar los procesos mayoritariamente representados en RBCs expuestos a VHSV tras 4 y 72 hpe. Los resultados mostraron la identificación de diversos genes pertenecientes a los términos de Gene Ontology (GO) de ubiquitinación y degradación por proteasoma junto con presentación de antígenos vía MHCI tras 4 hpe (Figura 1 P3). Entre ellos destaca la regulación positiva de culina 3 (*cul3*) y proteína 1 semejante a KELCH asociada a ECH (*keap1),* dos componentes de la ubiquitina E3 ligasa; las subunidades del proteasoma α6 y β5 (*psma6* y *psmb5*); y la calnexina (*canx*), implicada en el ensamblaje del complejo de MHCI [157]. También se identificaron diversos genes implicados en el proceso de autofagia como *ulk1*, *becn1* y *atg9a*. La validación mediante qPCR mostró regulación positiva en genes exclusivamente a 4 hpe, mientras que a 72 hpe se observa una regulación negativa general (Figura S4 P3), al igual que muestran los resultados de transcritptómica a 72 hpe.

Análisis de proteómica de RBCs expuestos a VHSV *ex vivo*

El análisis proteómico mediante LC-MS/MS de RBCs expuestos a VHSV mostraron sobreexpresados procesos relacionados con la presentación de antígenos vía MHCII, procesos catabólicos dependientes de ubiquitinación mediados por el proteasoma, componentes del proteasoma, procesado y presentación de antígenos de péptidos exógenos, señalización por receptores citoplasmáticos de reconocimiento de patrones y activación de leucocitos, mientras que el proceso de presentación de antígenos mediante MHCI se encontró regulado negativamente (Figura 2A/B P3).

VHSV induce ubiquitinación, pero altera la degradación proteasómica en RBCs expuestos a VHSV *ex vivo*

La implicación del sistema ubiquitina-proteasoma (UPS) en la infección no permisiva de VHSV en RBCs se evaluó en primer lugar mediante un ensayo de cinética de expresión de los genes *cul3* y *keap1*. Los resultados mostraron un incremento en la expresión de *cul3* a 3 hpe mientras que *keap1* incrementó a 24 hpe (Figura 3A P3). A continuación, se midió la actividad de la unidad del proteasoma 20S empleando un kit comercial y se observó un descenso en su actividad cuando los RBCs se exponían a VHSV (Figura 3B P3). Posteriormente, se realizó un western blot empleando un anticuerpo específico frente a ubiquitina en RBCs no expuestos y expuestos a VHSV con o sin el inhibidor de proteasoma MG132. La ubiquitinación de proteínas fue mayor en RBCs expuestos a VHSV (Figura 3C/D P3). Para evaluar si UPS estaba implicado directamente en la degradación de VHSV en el interior celular, se realizó una tinción intracelular de la proteína NVHSV en RBCs expuestos a VHSV y tratadas con el inhibidor de la actividad del proteasoma MG132, previamente usado para analizar la implicación del UPS en el ciclo de replicación de otro rhabdovirus [158]. Los resultados de citometría de flujo no mostraron cambios en el VHSV intracelular (Figura 3E P3).

El aumento de la ubiquitinación de proteínas en RBCs expuestos a VHSV se correlacionó con los procesos detectados mediante el análisis de proteómica y transcriptómica. Se ha visto que diversos virus han sido capaces de inducir ubiquitinación de proteínas, incluidas proteínas víricas [159-161]. No obstante, nuestros resultados demuestran que existe un descenso en la actividad del proteasoma como consecuencia a la exposición a VHSV, y que probablemente esto sea la causa de la acumulación de proteínas ubiquitinadas. La actividad del proteasoma se ha visto implicada en efectos tanto provirales [162] como antivirales [163], aunque según nuestros resultados la actividad del proteasoma no estaría implicada en la degradación de VHSV.

VHSV induce autofagia en RBCs

El análisis de transcriptómica de RBCs expuestos a VHSV detectó sobreexpresados genes relacionados con el proceso de autofagia a 4 hpe (Tabla 4 P3). A continuación, mediante microscopía electrónica de transmisión de RBCs expuestos a VHSV a 24 hpe, se comprobó la presencia de vesículas semejantes autofagosomas en el citoplasma de dichos RBCs (Figura 4A P3), de aproximadamente 0.5 µm de longitud, con doble membrana y material en su interior. Visualmente, se contaron estas vesículas en RBCs

y se detectó un incremento en su número cuando los RBCs se exponían a VHSV, en comparación con aquellos que no estaban expuestos. El cambio en la expresión de la proteína LC3, proteína que conforma y estabiliza la membrana de los autofagosomas, también se evaluó mediante tinción intracelular. La expresión de LC3 incrementó en RBCs expuestos a VHSV de manera dependiente de la multiplicidad de infección, a una multiplicidad de infección mayor se observó mayor expresión de LC3 (Figura 4C P3). Mediante microscopía de fluorescencia se detectó la presencia de puntos de LC3 en el citoplasma de RBCs expuestos a VHSV (Figura 4D P3). Más aun, la implicación de secuestrosoma 1 o p62, molécula adaptadora que interviene en el marcaje para degradación por autofagia de proteínas ubiquitinadas [164], también se evaluó mediante citometría de flujo. Los resultados mostraron un descenso de p62 a 6 hpe en RBCs expuestos a VHSV (Figura 4E/F P3). A partir de las 24 hpe, los niveles de p62 se recuperaron.

Por lo tanto, nuestros resultados demostraron actividad autofágica en RBCs expuestos a VHSV. Considerando que la infección de RBCs por VHSV es arrestada a las pocas horas post-exposición, la autofagia inducida por VHSV tendría un carácter antiviral en los RBCs, como previamente se ha descrito para la autofagia inducida por rhabdovirus [165]. Tanto la expresión temprana de los genes relacionados con la autofagia como la degradación de p62, que confirmaría la activación de este mecanismo, ocurren en la escala espacial donde el ciclo de replicación de VHSV quedaría inhibido, es decir, alrededor de las 6 hpe. No obstante, no se ha podido determinar si existe interacción entre p62 y VHSV, hecho que establecería a p62 como mediador entre las proteínas ubiquitinadas acumuladas y su degradación en el autofagosoma. Aun así, existen otros mediadores semejantes a p62, como el gen 1 vecino de BRCA1 (NRB1), proteína 52 de punto nuclear (NDP52) y optineurina [166], cuya implicación en la infección de VHSV en RBCs es desconocida de momento.

Inhibición de la degradación del autofagosoma incrementa los niveles de p62 y VHSV intracelular en RBCs

La niclosamida es una droga antihelmíntica que es capaz de bloquear la degradación del autofagosoma evitando su fusión con el lisosoma [167]. La niclosamida se ha usado previamente en el contexto de infecciones víricas [168]. En este estudio, después de exponer RBCs a VHSV, los RBCs se trataron con niclosamida a las concentraciones de 10 y 20 μM. A continuación, se realizó una tinción intracelular empleando anticuerpos específicos frente a la proteína NVHSV y p62. Los resultados mostraron que los RBCs

expuestos a VHSV y tratados con niclosamida a ambas concentraciones revelaban una mayor presencia de p62 y NVHSV intracelular en comparación con aquellos RBCs no tratados con niclosamida (Figura 5A/B P3) tras 72 hpe.

Este resultado corroboró lo afirmado en el apartado anterior sobre el carácter antiviral de la autofagia inducida por VHSV en RBCs. La niclosamida previene la fusión del autofagosoma con el lisosoma, por lo tanto, estaría implicada en inhibir el flujo de la autofagia en su último paso. Nuestros resultados mostraron una acumulación en el interior de los RBCs de NVHSV y p62 cuando se bloquea el flujo de autofagia con la niclosamida. La acumulación de VHSV confirmó que el virus es degradado por autofagia en RBCs, mientras que la acumulación p62 indicó que los autofagosomas no estarían fusionándose con los lisosomas, ya que p62 se degrada junto al autofagosoma tras la fusión con lisosoma.

RBCs expuestos a VHSV regulan positivamente MHCI, MHCII, CD86 y CD83

Los procesos de presentación y procesado de antígenos vía MHCI y MHCII resultaron sobrerrepresentados en los análisis de transcriptómica y proteómica. Se ha reportado que los RBCs expresan MHCI [69, 70] y MHCII [114]. En este estudio investigamos si los RBCs modulaban la expresión de MHCI y MHCII en presencia de VHSV, así como la de otros marcadores característicos de células presentadoras de antígenos como CD86 y CD83. Primero, se realizó una PCR semicuantitativa para valorar la expresión de transcritos de RNA de *mhcI*, *mhcII*, *cd86* y *cd83*. Una mezcla de tejidos procedentes de branquia, riñón anterior y bazo se empleó como control positivo de expresión. El resultado mostró expresión de *mhcI*, *mhcII* y *cd83* en RBCs, mientras que no se observó expresión de *cd86* (Figura 6A P3). A continuación, se evaluó como los RBCs modulaban la expresión de dichos genes mediante qPCR. Los resultados mostraron un ligero incremento en *mhcI*, mientras que dicho incremento fue notablemente superior en *mhcII*, *cd86* y *cd83* a 4 hpe (Figura 6B P3). A 72 hpe, sólo se observó un incremento en *cd86* (Figura 6B P3). Esta regulación positiva se confirmó a nivel de proteína en RBCs expuestos a VHSV tras 24 hpe mediante citometría de flujo y microscopía de fluorescencia (Figura 6C/D/E P3).

Estos resultados demostraron que los RBCs pueden modular la expresión de MHCI, MHCII, CD86 y CD83 cuando son expuestos a VHSV. Previamente, se ha demostrado regulación positiva de MHCI durante las infecciones de ISAV [71] y PRV [39]. CD86 y CD83 son moléculas coestimuladoras del proceso de presentación de antígenos, además de marcadores de maduración de APCs. Estos dos marcadores, junto con la

presencia de MHCII en RBCs, sugieren que los RBCs presentan un perfil de APC. Recientemente, se ha propuesto la existencia de células APC atípicas [77], es decir, células capaces de expresar las moléculas de una APC pero cuya capacidad de primar y activar linfocitos T *naïve* no está demostrada. Los RBCs de teleósteos podrían clasificarse en esta categoría, a falta de establecer ensayos futuros que revelen su capacidad de activar de linfocitos T *naïve*.

Inducción de genes de autofagia y presentación de antígenos en RBCs de individuos desafiados con VHSV

Los RBCs purificados extraídos de sangre periférica de individuos desafiados con VHSV mostraron expresión de transcritos de *NVHSV* por qPCR, pero esta fue menor que en otros tejidos analizados tras 2 días post-desafío (dpd) (Figura 7A P3). Se evaluó la ubiquitinación de proteínas en RBCs provenientes de individuos desafiados con VHSV y no se detectaron cambios en la ubiquitinación tras 2 dpd (Figura 7B/C P3). La expresión de genes relacionados con autofagia, ubiquitina ligasa E3 y presentación de antígenos fue analizada a 1 y 2 dpd. En relación con los genes pertenecientes al proceso de autofagia, tras 1 dpd se observó regulación positiva significativa de *gabarap* y la subunidad catalítica 3 de PIK3 (*pik3c3*), y sólo regulación positiva significativa de *pik3c3* tras 2 dpd. Sin embargo, a 1 y 2 dpd se observó regulación negativa de *atg4b* y *becn1*. Los componentes de la ubiquitina ligasa E3, *cul3* y *keap1,* se observaron significativamente sobreexpresados a 1 y 2 dpd, respectivamente. Por último, los genes marcadores de células presentadoras de antígenos *mhcI* y *cd83* se observaron significativamente sobreexpresados tanto a 1 como 2 dpd (Figura 7D P3).

Los resultados del estudio *in vivo* confirmaron parcialmente los resultados observados en el estudio *ex vivo*. En resumen, se observó que los RBCs de individuos desafiados con VHSV presentaban una baja carga vírica de VHSV, tal y cómo se observa en condiciones *ex vivo*. Por el contrario, no se observa ubiquitinación en RBCs procedentes de individuos desafiados con VHSV, en comparación con los ensayos *ex vivo*. La expresión de genes relacionados con autofagia mostró cinéticas similares a las observadas *ex vivo*. Más aún, los genes de los componentes de la ubiquitina ligasa E3, *cul3* y *keap1,* muestran la misma cinética de expresión tanto *in vivo* como *ex vivo*. En relación a los genes involucrados en presentación de antígenos, sobretodo destaca la sobreexpresión de *mhcI* y *cd83* en RBCs de individuos desafiados con VHSV, en comparación con la sobreexpresión de *mhcI*, *mhcII*, *cd83* y *cd86* observada *ex vivo*.

La proteína G de VHSV colocaliza con MHC I & II en RBCs expuestos a VHSV

Para establecer una correlación entre la presencia de péptidos de VHSV provenientes de la degradación de VHSV desde la autofagia y la sobreexpresión de MHC I & II, se realizó un ensayo de ligación por proximidad (PLA). Tras 24 hpe, los RBCs no expuestos o expuestos a VHSV fueron incubados con un anticuerpo policlonal de conejo frente a la proteína de G de VHSV y un anticuerpo monoclonal de ratón frente a MHCI o MHCII. Para determinar la presencia de fondo, se sustituyó el anticuerpo proveniente de conejo con suero de conejo, que contiene anticuerpos inespecíficos. Los resultados mostraron un incremento en el porcentaje de células positivas para la interacción GVHSV – MHCI y GVHSV – MHCII cuando los RBCs se encontraban expuestos a VHSV (Figura 8A/B P3).

Este resultado demostró que la presentación de antígenos vía MHCI y MHCII podría ser potencialmente funcional en RBCs, ya que péptidos de GVHSV se encuentran colocalizados con MHCI y MHCII. Sin embargo, para clasificar a los RBCs como una APC típica sería necesario verificar la polarización de linfocitos T *naïve* por RBCs con VHSV.

4.4 Resultados obtenidos de la Publicación 4.

Nombela, I., Lopez-Lorigados, M., Salvador-Mira, M.E., Puente-Marín, S., Chico, V., Ciordia, S., … & del Mar Ortega-Villaizan, M. (2019) Integrated transcriptomic and proteomic analysis of red blood cells from rainbow trout challenged with VHSV points towards novel immunomodulant targets. En revisión en *Vaccines*.

Análisis transcriptómico de RBCs de sangre periférica y riñón anterior de individuos desafiados con VHSV

Individuos de trucha arcoíris se desafiaron con VHSV mediante inyección intramuscular (como control negativo se inyectó RPMI 2% FBS a otro grupo de individuos). Tras 2 dpd, los RBCs se purificaron de sangre periférica (PB-RBCs) y de riñón anterior (HK-RBCs) como se explica en la Figura 1 P4 de la sección Materiales y métodos de la Publicación 4 y en el apartado 3.13 de los Materiales y Métodos de esta tesis (Figura 8). El análisis de enriquecimiento funcional de rutas, utilizando la base de datos de ontología génica (GO) de procesos de sistema inmune, de los genes diferencialmente expresados (DEGs) de PB-RBCs mostró una regulación positiva de procesos relacionados con: i) la activación del sistema del complemento ii) la respuesta humoral mediada por inmunoglobulinas, iii) activación de granulocitos, iv) quimiotaxis de neutrófilos v) diferenciación de eritrocitos, vi) señalización del receptor de células B, vii) vía de señalización del receptor de lectinas tipo C y viii) señalización por receptor de reconocimiento de patrones citoplasmáticos (Figura 2A/B P4). Como procesos secundarios también se encontraron sobrerrepresentados el procesado y presentación de péptidos de antígenos exógenos y la vía de señalización de TLR dependiente de TRIF (adaptador inductor de IFNβ con dominios Toll/IL-1). Por otro lado, el análisis de enriquecimiento funcional de rutas de los DEGs de HK-RBCs, utilizando la base de datos GO de procesos de sistema inmune, mostró regulación positiva de genes implicados en las categorías de señalización de IFN1 y procesado y presentación de péptidos de antígenos endógenos vía MHCI (Figura 2C).

Desde un punto de vista comparativo entre PB-RBCs y HK-RBCs, ambos compartieron un 13.9% del total de DEGs (Figura 3A P4). Cabe destacar que los genes con mayor expresión en PB-RBCs presentaron menor expresión en HK-RBCs y viceversa, mostrando de este modo un perfil de expresión opuesto entre ambos tejidos (Figura 3B P4). Los DEGs comunes a RBCs de ambos órganos se agruparon principalmente en las categorías de las vías de señalización de IFN1 y del receptor de lectinas tipo C. Por otro lado, el análisis de DEGs exclusivamente detectados en PB-RBCs mostró regulación

positiva de los procesos de: i) procesado y presentación de péptidos de antígenos exógenos vía MHCI dependiente de TAP, ii) vía de señalización del receptor de lectinas tipo C, iii) respuesta inmune mediada por inmunoglobulinas y iv) vía de señalización del receptor de reconocimiento de patrones citoplasmáticos (Figura 4 P4).

Los resultados observados en este estudio no siguen la línea de la respuesta inmune desarrollada por el RBC en respuesta a VHSV *ex vivo* descrita en la Publicación 1. En la Publicación 1 se mostró una respuesta antiviral moderada y regulación negativa de IFN1 a tiempos cortos tras la exposición a VHSV junto con un apagado celular a tiempos largos tras dicha exposición. Por el contrario, los resultados del estudio de transcriptómica que conforman esta Publicación muestran principalmente sobrerrepresentación de procesos relacionados con IFN1 y procesado y presentación de antígenos. Este último resultado ya se observó en los resultados de transcriptómica de la Publicación 3 a tiempos cortos post-exposición al virus, donde se muestra activación de la autofagia, procesado de antígenos y regulación positiva de los marcadores de presentación de antígenos MHCI y MHCII. Previamente, otro estudio de proteómica de RBCs de besugo de roca (*Oplegnathus fasciatus*) de individuos desafiados con el iridovirus del besugo de roca RBIV mostró regulación positiva del proceso de presentación de antígenos vía MHCI [169]. Del mismo modo, la presentación de antígenos vía MHCI fue un proceso positivamente regulado en RBCs de salmón del Atlántico desafiados con PRV [39].

Los genes regulados positivamente de procesos sobrerrepresentados se analizaron por qPCR

Genes de distintos procesos regulados positivamente se seleccionaron para la analizar mediante qPCR en PB-RBCs de individuos desafiados con VHSV, en comparación con individuos inyectados con RPMI 2% FBS. En relación con el proceso de activación del sistema del complemento se seleccionaron *c4bpa*, *cd55* y *cd59*. De la señalización de receptores de tipo Toll mediada por TRIF se seleccionó el factor asociado con el receptor de TNF3 (*traf3*). Para validar el proceso de vía de señalización del receptor de reconocimiento de patrones citoplasmáticos se seleccionaron *dhx58*, también conocido como LGP2, *irak1*, *mavs*, el miembro de la familia NLR X1 (*nlrx1*) y el dominio de oligomerización de unión a nucleótidos 2 (*nod2*). De los genes relacionados con la señalización de IFN1 se seleccionaron *gbp1*, *ifi35*, el receptor de tipo NOD 5 con dominio CARD (*nlrc5*), *stat1* y la helicasa C 1 inducida por IFN (*ifih1*). Se detectó regulación positiva significativa en RBCs de individuos desafiados con VHSV en todos los genes

citados anteriormente excepto para *cd55*, *gbp1*, *nlrx1* y *mavs* (Figura 5 P4). El alto número de genes que se encontraron regulados positivamente por qPCR ratificó el resultado mostrado por el análisis de transcriptómica.

Análisis de proteómica de PB-RBCs de individuos desafiados con VHSV

Individuos de trucha arcoíris se desafiaron con VHSV mediante inyección intramuscular (como control negativo se inyectó RPMI 2% FBS a otro grupo de individuos). Tras 2 dpd, se purificaron PB-RBCs para analizar su proteoma. El análisis de enriquecimiento funcional de rutas de las proteínas diferencialmente expresadas (DEPs), utilizando la base de datos GO de procesos de sistema inmune, mostró sobrerrepresentación de procesos relacionados con la transcripción y expresión génica vírica (Figura 6A P4). Por otro lado, los procesos de señalización de Wnt, biosíntesis de carbohidratos y la cascada de las proteínas quinasas activadas por mitógenos (MAPK) activada por estrés se encontraron regulados negativamente (Figura 6A P4). Otros procesos como estabilidad del RNA mensajero y procesado y presentación de antígenos vía MHCI aparecieron sobrerrepresentados pero regulados inespecíficamente. Además, entre el listado de DEPs identificadas en PB-RBCs de individuos desafiados con VHSV cabe destacar la presencia de proteínas con funciones inmunológicas e antivirales como NLRC3, GBP1, IFIT5, IFI35, RSAD2 y la GTPasa grande inducible por interferón (GVINP1).

El análisis proteómico identificó sobrerrepresentadas proteínas relacionadas con los mecanismos de inmunidad innata y con la transcripción y replicación del RNA vírico. Previamente en la Publicación 1 se mostró que VHSV no puede replicar en RBCs de trucha arcoíris en condiciones *ex vivo*. Sin embargo, en este trabajo detectamos proteínas ribosomales L (RPL) y S (RPS), las cuales han sido previamente implicadas en contextos de infecciones víricas [170]. No obstante, previamente en la Publicación 1 se mostró que la replicación de VHSV en RBCs de trucha arcoíris es arrestada a tiempos cortos tras la infección. Además, los transcritos de *NVHSV* muestran niveles muy bajos en PB-RBCs de individuos desafiados con VHSV. Por tanto, debido a que los mecanismos de replicación vírica esta regulados positivamente, otros mecanismos activados en los RBCs podrían estar interrumpiendo la replicación vírica. Entre ellos se podrían encontrar la autofagia, cuyo carácter antiviral se discutió en la Publicación 3, o el alto número de ISGs que se han encontrado regulados positivamente en esta Publicación. El análisis integrado de las “ómicas” reveló que, en respuesta a VHSV, los PB-RBCs regulan proteínas y genes que pueden interferir con el virus a distintas etapas de su ciclo de replicación. Entre los efectores antivirales que se han identificado en este

trabajo se encuentran IFIT5, MX, GBP1, GVINP1, RSAD2, IFITM3, IFI35 y diversas proteínas TRIM, que serán discutidas en el siguiente apartado. Algunos de estos efectores antivirales han sido previamente implicados en la respuesta de RBCs de salmón del Atlántico a PRV [39], pero es necesario tener en consideración que PRV tiene como célula diana a los RBCs y es capaz de replicar en ellos, mientras que VHSV no es capaz de realizar dicha replicación, como se demuestra en las Publicaciones 1 y 3.

Regulación positiva de efectores antivirales en RBCs de individuos desafiados con VHSV

Distintos efectores antivirales como *gvinp1*, *mx*, *ifi35*, *rsad2* o viperina, *ifit5*, *ifitm3* o proteínas con motivos tripartitos (*trim*), colesterol hidroxilasa 25 (ch25h) y desoxinucleósido trifosfato trifosfohidrolasa 1 con dominios SAM y HD (*samhd1*) fueron regulados positivamente en el análisis de transcriptómica de PB-RBCs y HK-RBCs de individuos desafiados con VHSV. En la Tabla 2 P4 se muestra un resumen de dichos genes con las veces de cambio en su expresión. Del mismo modo, se encontró también regulada positivamente la expresión de genes específicos de teleósteos de carácter antiviral como el gen inducido por VHSV (*vig2*) en PB-RBCs y el gen inducido por el reovirus de carpa china (*gigh2*) tanto en PB-RBCs como HK-RBCs.

Entre los distintos efectores antivirales que se han destacado en esta Publicación nos encontramos con las proteínas IFIT. Recientemente nuestro grupo de investigación mostró que la proteína IFIT5 de trucha arcoíris, que es capaz de unir RNA vírico, es regulada positivamente para prevenir la replicación de VHSV en un escenario *ex vivo*. El presente estudio demuestra que dicha regulación positiva de su expresión también ocurre en un escenario *in vivo* tras un periodo de tiempo corto tras el desafío por VHSV. También destacamos GVINP1 y GBP1, proteínas inducibles por IFN con mecanismos de inhibición vírica semejante a MX debido a su actividad GTPasa [171], aunque algunas de las isoformas de las GBP se ha visto que poseen actividades distintas a antivirales [172]. RSAD2, también conocida como viperina, se ha visto involucrada en la inhibición del ciclo de replicación del virus de la influenza y del virus de la hepatitis C durante el ensamblaje [173]. En cambio, las IFITM se han asociado al bloqueo de la entrada vírica debido a su localización en la membrana celular [174]. Del mismo modo, CH25H inhibe el proceso de infección vírica a nivel de la entrada del virus [175] Las proteínas TRIM intervienen en la defensa del hospedador frente a virus mediante antagonismo de distintos estadios del ciclo de replicación vírico, autofagia y regulación de la señalización

de sensores de respuesta inmune innata involucrados en la modulación de citoquinas [176]. Otro mecanismo de inhibición de la replicación vírica es llevado a cabo por SAMHD1. Este mecanismo consiste en la hidrólisis de los desoxirribonucleótidos trifosfato intracelulares con el objetivo de bajar sus niveles hasta alcanzar una concentración crítica que no permite la replicación del virus [177].

Además, en este trabajo se analizó la expresión de las proteínas antivirales Mx, BD1 e IFIT5 mediante western blot. Los resultados mostraron que a 1 dpd, la expresión de las proteínas Mx3 (Figura 7A P4) e IFIT5 (Figura 7B P4) se incrementó en RBCs provenientes de individuos desafiados en comparación con el control negativo. Mx3 y BD1 son dos proteínas antivirales cuya expresión se ha asociado a IFN1 [178, 179]. En este estudio, nosotros demostramos como los RBCs de individuos desafiados con VHSV tienen mayor expresión de Mx3. Este resultado también es corroborado por el análisis de transcriptómica, que muestra una sobreexpresión del gen de *mx* en individuos desafiados. El carácter antiviral de las proteínas Mx ha sido objeto de estudio en profundidad [180] y su implicación en la infección por VHSV ya se ha determinado a varios niveles: desde respuesta inmune *in vivo* [181] hasta vacunación [182]. En RBCs, los resultados de la Publicación 1 mostraron que estos no inducen IFN1 en respuesta a VHSV. Sin embargo, en un contexto *in vivo*, el estímulo necesario para producir Mx mediante activación de la cascada de IFN1 en RBCs podría provenir del IFN1 producido por células adyacentes. En relación a BD1, se ha visto que su expresión puede ser inducida por IFN1 [178] o por diversos virus [183]. Nuestros resultados muestran que, en RBCs de individuos desafiados con VHSV, la expresión de BD1 no varía en la expresión monomérica de BD1, pero sí que se detectan cambios en la expresión de BD1 en la banda de aproximadamente 28 kDa, a 2 y 7 dpd (Figura 7C P4). Este tamaño correspondería con un tetrámero de BD1, teniendo en cuenta que la forma monomérica posee un peso molecular de 7.1 kDa. Previamente, se ha sugerido que la BD1 podría necesitar formar un oligomero para ejercer su actividad antimicrobiana [184].

4.5 Resumen de la Publicación 5.

Nombela, I., & del Mar Ortega-Villaizan, M. (2018). Nucleated red blood cells: Immune cell mediators of the antiviral response. *PLoS pathogens*, *14*(4), e1006910.

La Publicación 5 corresponde a un artículo de revisión en el que se ofrece una actualización de la respuesta inmune mediada por RBCs nucleados en infecciones víricas, incluyendo los resultados y conclusiones obtenidos en las Publicaciones 1 y 2. A continuación, se ofrece un resumen de los principales puntos revisados en esta publicación:

- **Los PAMPs son capaces de inducir señalización mediada por receptores que reconocen patrones moleculares en RBCs nucleados**.
  Estudios previos han indicado la expresión de los receptores TLR3, TLR9 y gen inducible por ácido retinoico (RIG-I), capaces de reconocer RNA vírico de doble cadena, en RBCs de trucha arcoíris y salmón del Atlántico (*Salmo salar*). La activación de estos receptores conlleva a la expresión de IFN1 y de genes relacionados con IFN1, como *mx*, *ccl4* o *il8*. Los RBCs nucleados expresan además otros tipos de receptores de la familia RLR cuyo rol todavía es desconocido. También se desconoce si los RBCs nucleados son capaces de expresar TLR7 o TRL8, capaces de reconocer RNA vírico de cadena simple.
- **Los RBCs podrían ser capaces de inducir una respuesta inmune adaptativa**.
  Previamente se ha reportado la expresión de MHCI en RBCs de diversas especies como trucha arcoíris, salmón del Atlántico, rana africana de uñas (*Xenopus laevis*) y gallo (*Gallus gallus domesticus*) y cómo la estimulación con poli(I:C) induce una activación de genes relacionados con los procesos de procesado y presentación de antígenos en RBCs de trucha arcoíris. También se destaca la presencia de moléculas relacionadas con procesos de respuesta inmune adaptativa, como el inmunoreceptor con motivos basados en tirosina (ITAM) o el receptor acoplado al Epstein Barr virus 2 (EBI2), las cuales juegan un papel esencial en la regulación de la respuesta inmune mediada por células T.
- **Los RBCs nucleados activan diversos mecanismos de respuesta frente agresiones víricas**.
  En este punto se compara la respuesta de los RBCs a virus cuya infección es productiva en RBCs (PRV e ISAV) frente a los dos virus estudiados en esta tesis, cuya infección no es productiva en RBCs (VHSV e ISAV) (Figura 1 P5). A nivel de respuesta inmune, se observa como PRV e ISAV son capaces de inducir una

respuesta inmune basada en la expresión de IFN y genes estimulados por IFN. Del mismo modo, IPNV, a pesar de no ser capaz de infectar RBCs, es capaz de producir el mismo tipo de respuesta. Por el contrario, esta respuesta no es observada en el caso de la infección no productiva por VHSV *ex vivo*.

- **Los RBCs nucleados pueden desarrollar respuestas inmunes frente a patógenos no víricos**.

  En este punto se destaca la escasez de estudios centrados en determinar la respuesta de RBCs nucleados a pesar de la diversa gama de patógenos protistas y procariotas que tienen como diana a estas células. Previamente se ha descrito que el lipopolisacárido bacteriano es capaz de estimular la expresión del receptor de TNF y MHCI. Además, se ha detectado que la hemoglobina posee actividad antibacteriana y diversos estudios han demostrado la capacidad fagocítica de RBCs frente al hongo *Candida albicans*.

- **Los RBCs nucleados son una futura diana para el desarrollo de nuevas vacunas**.

  Los RBCs no nucleados de humanos han sido empleados en estudios para evaluar el transporte de diversas drogas o antígenos por la sangre. En el caso de RBCs nucleados, debido a su capacidad de generar y modular la respuesta inmune, desarrollar una nueva generación de vacunas capaces de activar respuestas antivirales de los RBCs nucleados es un interesante campo de estudio que está siendo desarrollado por nuestro grupo. En este sentido, se ha la respuesta de RBCs de trucha arcoíris a la vacuna DNA de GVHSV.

5. CONCLUSIONES

Las conclusiones generales de esta tesis son:

1. VHSV e IPNV no replican eficientemente en los RBCs de trucha arcoíris, aun así, desencadenan una respuesta inmune en estas células.
2. VHSV induce activación de los mecanismos de autofagia, degradación y presentación antigénica en RBCs de trucha arcoíris expuestos a VHSV *ex vivo*.
3. VHSV induce la activación de genes de respuesta a estrés oxidativo en RBCs *ex vivo*.
4. A tiempos largos post-exposición a VHSV el RBC tiende a sufrir un apagado celular por la fosforilación del factor eIF2α que produce una regulación negativa del proteoma y transcriptoma de los RBCs.
5. La respuesta *in vivo* de RBCs al desafío por VHSV se basa en la expresión de genes de respuesta a IFN1 en RBCs de riñón anterior y componentes del sistema del complemento en RBCs de sangre periférica, procesado y presentación de antígenos en ambos, así como la producción de diversos efectores antivirales, entre ellos Mx, BD1 e IFIT5.
6. La respuesta de los RBCs de trucha arcoíris frente a IPNV *ex vivo* se basa en la sobreexpresión de IFN1 junto con otros genes expresados en respuesta a IFN1, como Mx.

Estas conclusiones quedan recogidas en forma de resumen gráfico en las Figuras 9 y 10.

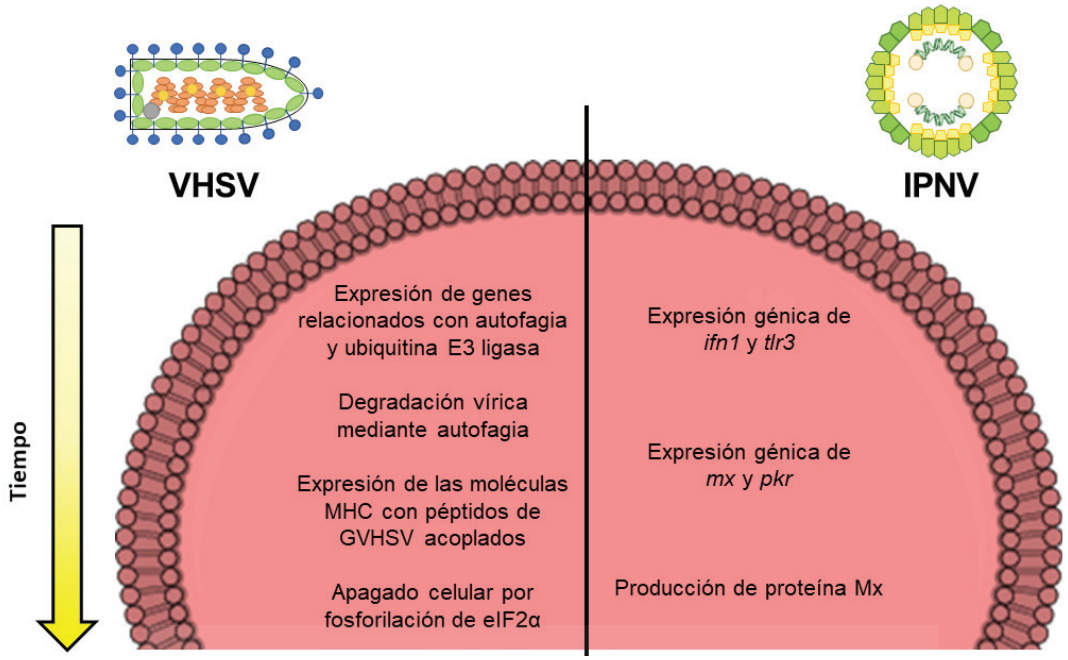


Figura 9. Esquema representativo de la respuesta inmune *ex vivo* desarrollada por RBCs frente a VHSV e IPNV, considerando los distintos eventos en escala temporal

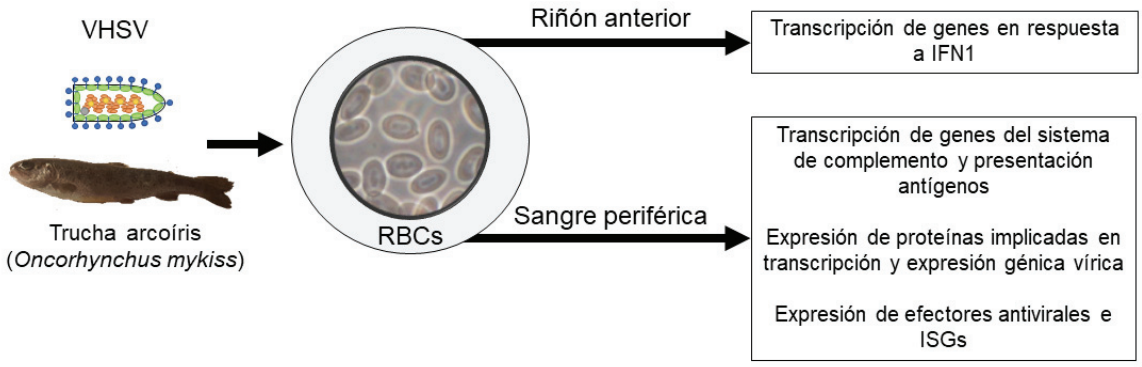


Figura 10. Esquema representativo de la respuesta inmune *in vivo* desarrollada por RBCs de riñón anterior y sangre periférica procedentes de truchas arcoíris desafiadas con VHSV.

6. REFERENCIAS

1. Bengten, E., et al., *Channel catfish immunoglobulins: repertoire and expression.* Dev Comp Immunol, 2006. **30**(1-2): p. 77-92.

2. Du Pasquier, L., *Antibody diversity in lower vertebrates--why is it so restricted?* Nature, 1982. **296**(5855): p. 311-3.

3. Sunyer, J.O., *Fishing for mammalian paradigms in the teleost immune system.* Nat Immunol, 2013. **14**(4): p. 320-6.

4. Secombes, C.J. y T. Wang, *1 - The innate and adaptive immune system of fish*, in *Infectious Disease in Aquaculture*, B. Austin, Editor. 2012, Woodhead Publishing. p. 3-68.

5. Robertsen, B., *The interferon system of teleost fish.* Fish Shellfish Immunol, 2006. **20**(2): p. 172-91.

6. Samuel, C.E., *Antiviral actions of interferons.* Clin Microbiol Rev, 2001. **14**(4): p. 778-809, table of contents.

7. Zou, J., et al., *Identification of a second group of type I IFNs in fish sheds light on IFN evolution in vertebrates.* J Immunol, 2007. **179**(6): p. 3859-71.

8. Rokenes, T.P., R. Larsen, y B. Robertsen, *Atlantic salmon ISG15: Expression and conjugation to cellular proteins in response to interferon, double-stranded RNA and virus infections.* Mol Immunol, 2007. **44**(5): p. 950-9.

9. Uribe, C.F., H.; Enriquez, R.; Moran, G., *Innate and adaptive immunity in teleost fish: a review.* Veterinarni Medicina, 2011. **56**: p. 486-503.

10. Zou, J. y C.J. Secombes, *Teleost fish interferons and their role in immunity.* Dev Comp Immunol, 2011. **35**(12): p. 1376-87.

11. Claver, J.A. y A.I.E. Quaglia, *Comparative Morphology, Development, and Function of Blood Cells in Nonmammalian Vertebrates.* Journal of Exotic Pet Medicine, 2009. **18**(2): p. 87-97.

12. Enns, T., *Gas transport and the red cell.* Fed Proc, 1967. **26**(6): p. 1802-4.

13. Davies, H.G., *Structure in nucleated erythrocytes.* J Biophys Biochem Cytol, 1961. **9**: p. 671-87.

14. Fawcett, W. y F. Witebsky, *Observations on the Ultrastructure of Nucleated Erythrocytes and Thrombocytes, with Particular Reference to the Structural Basis of Their Discoidal Shape.* Z Zellforsch Mikrosk Anat, 1964. **62**: p. 785-806.

15. Fischer, U., M. Ototake, y T. Nakanishi, *Life span of circulating blood cells in ginbuna crucian carp (Carassius auratus langsdorfii).* Fish & Shellfish Immunology, 1998. **8**(5): p. 339-349.

16. Lund, S.G., et al., *The effects of cell ageing on protein synthesis in rainbow trout (Oncorhynchus mykiss) red blood cells.* J Exp Biol, 2000. **203**(Pt 14): p. 2219-28.

17. Keerthivasan, G., A. Wickrema, y J.D. Crispino, *Erythroblast enucleation.* Stem Cells Int, 2011. **2011**: p. 139851.

18. Singh, A.K., *Chapter 12 - Erythropoiesis: The Roles of Erythropoietin and Iron*, in *Textbook of Nephro-Endocrinology (Second Edition)*, A.K. Singh and G.H. Williams, Editors. 2018, Academic Press. p. 207-215.

19. Davidson, A.J. y L.I. Zon, *The 'definitive' (and 'primitive') guide to zebrafish hematopoiesis.* Oncogene, 2004. **23**: p. 7233.

20. Evans, C.J., V. Hartenstein, y U. Banerjee, *Thicker Than Blood.* Developmental Cell, 2003. **5**(5): p. 673-690.

21. Pesquero, J., et al., *Glucose metabolism by trout (Salmo trutta) red blood cells.* J Comp Physiol B, 1992. **162**(5): p. 448-54.

22. Orlov, S.N., S.L. Aksentsev, y S.V. Kotelevtsev, *Extracellular calcium is required for the maintenance of plasma membrane integrity in nucleated cells.* Cell Calcium, 2005. **38**(1): p. 53-57.

23. Jensen, F.B., *The role of nitrite in nitric oxide homeostasis: a comparative perspective.* Biochim Biophys Acta, 2009. **1787**(7): p. 841-8.

24. Morera, D. y S.A. MacKenzie, *Is there a direct role for erythrocytes in the immune response?* Vet Res, 2011. **42**: p. 89.

25. Pereiro, P., et al., *Nucleated Teleost Erythrocytes Play an Nk-Lysin- and Autophagy-Dependent Role in Antiviral Immunity.* Front Immunol, 2017. **8**: p. 1458.

26. Morera, D., et al., *RNA-Seq reveals an integrated immune response in nucleated erythrocytes.* PLoS One, 2011. **6**(10): p. e26998.

27. Dahle, M.K. y J.B. Jorgensen, *Antiviral defense in salmonids - Mission made possible?* Fish Shellfish Immunol, 2019. **87**: p. 421-437.

28. Davies, A.J. y M.R.L. Johnston, *The biology of some intraerythrocytic parasites of fishes, amphibia and reptiles.* J Advances in parasitology, 2000. **45**: p. 1-107.

29. Walker, R. y S.W. Sherburne, *Piscine Erythrocytic Necrosis Virus in Atlantic Cod, Gadus morhua, and Other Fish: Ultrastructure and Distribution.* Journal of the Fisheries Research Board of Canada, 1977. **34**(8): p. 1188-1195.

30. Workenhe, S.T., et al., *Infectious salmon anaemia virus replication and induction of alpha interferon in Atlantic salmon erythrocytes.* Virol J, 2008. **5**: p. 36.

31. Finstad, O.W., et al., *Piscine orthoreovirus (PRV) infects Atlantic salmon erythrocytes.* Vet Res, 2014. **45**: p. 35.

32. Appy, R.G., M.D.B. Burt, y T.J. Morris, *Viral Nature of Piscine Erythrocytic Necrosis (PEN) in the Blood of Atlantic Cod (Gadus morhua).* Journal of the Fisheries Research Board of Canada, 1976. **33**(6): p. 1380-1385.

33. Wessel, O., et al., *Piscine orthoreovirus (PRV) replicates in Atlantic salmon (Salmo salar L.) erythrocytes ex vivo.* Vet Res, 2015. **46**: p. 26.

34. Rauta, P.R., et al., *Toll-like receptors (TLRs) in aquatic animals: signaling pathways, expressions and immune responses.* Immunol Lett, 2014. **158**(1-2): p. 14-24.

35. Bricknell, I. y R.A. Dalmo, *The use of immunostimulants in fish larval aquaculture.* Fish & Shellfish Immunology, 2005. **19**(5): p. 457-472.

36. Passantino, L., et al., *Antigenically activated avian erythrocytes release cytokine-like factors: a conserved phylogenetic function discovered in fish.* Immunopharmacol Immunotoxicol, 2007. **29**(1): p. 141-52.

37. Passantino, L., et al., *Maturation of fish erythrocytes coincides with changes in their morphology, enhanced ability to interact with Candida albicans and release of cytokine-like factors active upon autologous macrophages.* Immunopharmacol Immunotoxicol, 2004. **26**(4): p. 573-85.

38. Schoenborn, J.R. y C.B. Wilson, *Regulation of interferon-gamma during innate and adaptive immune responses.* Adv Immunol, 2007. **96**: p. 41-101.

39. Dahle, M.K., et al., *Transcriptome analyses of Atlantic salmon (Salmo salar L.) erythrocytes infected with piscine orthoreovirus (PRV).* Fish Shellfish Immunol, 2015. **45**(2): p. 780-90.

40. Rosales, C. y E. Uribe-Querol, *Phagocytosis: A Fundamental Process in Immunity.* Biomed Res Int, 2017. **2017**: p. 9042851.

41. Zhu, Q., et al., *Human B cells have an active phagocytic capability and undergo immune activation upon phagocytosis of Mycobacterium tuberculosis.* Immunobiology, 2016. **221**(4): p. 558-567.

42. Neumann, N.F., et al., *Antimicrobial mechanisms of fish phagocytes and their role in host defense.* Dev Comp Immunol, 2001. **25**(8-9): p. 807-25.

43. Passantino, L., et al., *Fish immunology. I. Binding and engulfment of Candida albicans by erythrocytes of rainbow trout (Salmo gairdneri Richardson).* Immunopharmacol Immunotoxicol, 2002. **24**(4): p. 665-78.

44. Grosso, R., C.M. Fader, y M.I. Colombo, *Autophagy: A necessary event during erythropoiesis.* Blood Rev, 2017. **31**(5): p. 300-305.

45. Parzych, K.R. y D.J. Klionsky, *An overview of autophagy: morphology, mechanism, and regulation.* Antioxid Redox Signal, 2014. **20**(3): p. 460-73.

46. Alers, S., et al., *Role of AMPK-mTOR-Ulk1/2 in the regulation of autophagy: cross talk, shortcuts, and feedbacks.* Mol Cell Biol, 2012. **32**(1): p. 2-11.

47. Wu, L., et al., *Rapamycin upregulates autophagy by inhibiting the mTOR-ULK1 pathway, resulting in reduced podocyte injury.* PLoS One, 2013. **8**(5): p. e63799.

48. Karakaş, H.E. y Gözüaçik, D. *Autophagy and cancer.* Turkish Journal of Biology, 2014. **38**(6): p. 720 - 739.

49. Mizushima, N., *The role of the Atg1/ULK1 complex in autophagy regulation.* Curr Opin Cell Biol, 2010. **22**(2): p. 132-9.

50. Funderburk, S.F., Q.J. Wang, y Z. Yue, *The Beclin 1-VPS34 complex--at the crossroads of autophagy and beyond.* Trends Cell Biol, 2010. **20**(6): p. 355-62.

51. Walczak, M. y S. Martens, *Dissecting the role of the Atg12-Atg5-Atg16 complex during autophagosome formation.* Autophagy, 2013. **9**(3): p. 424-5.

52. Weidberg, H., et al., *LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis.* EMBO J, 2010. **29**(11): p. 1792-802.

53. Harnett, M.M., et al., *From Christian de Duve to Yoshinori Ohsumi: More to autophagy than just dining at home.* Biomed J, 2017. **40**(1): p. 9-22.

54. Chiramel, A.I., N.R. Brady, and R. Bartenschlager, *Divergent roles of autophagy in virus infection.* Cells, 2013. **2**(1): p. 83-104.

55. Miller, S. y J. Krijnse-Locker, *Modification of intracellular membrane structures for virus replication.* Nat Rev Microbiol, 2008. **6**(5): p. 363-74.

56. Fliss, P.M., et al., *Viral mediated redirection of NEMO/IKKgamma to autophagosomes curtails the inflammatory cascade.* PLoS Pathog, 2012. **8**(2): p. e1002517.

57. Kawai, T. y S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. **11**(5): p. 373-84.

58. Xagorari, A. y K. Chlichlia, *Toll-like receptors and viruses: induction of innate antiviral immune responses.* Open Microbiol J, 2008. **2**: p. 49-59.

59. Vadlamudi, R.K., et al., *p62, a phosphotyrosine-independent ligand of the SH2 domain of p56lck, belongs to a new class of ubiquitin-binding proteins.* J Biol Chem, 1996. **271**(34): p. 20235-7.

60. Orvedahl, A., et al., *Autophagy protects against Sindbis virus infection of the central nervous system.* Cell Host Microbe, 2010. **7**(2): p. 115-27.

61. English, L., et al., *Autophagy enhances the presentation of endogenous viral antigens on MHC class I molecules during HSV-1 infection.* Nat Immunol, 2009. **10**(5): p. 480-7.

62. Leung, C.S. y G.S. Taylor, *Nuclear shelter: the influence of subcellular location on the processing of antigens by macroautophagy.* Autophagy, 2010. **6**(4): p. 560-1.

63. Hancock, R.E. y G. Diamond, *The role of cationic antimicrobial peptides in innate host defences.* Trends Microbiol, 2000. **8**(9): p. 402-10.

64. Zasloff, M., *Antimicrobial peptides of multicellular organisms.* Nature, 2002. **415**(6870): p. 389-95.

65. Kamysz, W., M. Okroj, y J. Lukasiak, *Novel properties of antimicrobial peptides.* Acta Biochim Pol, 2003. **50**(2): p. 461-9.

66. Fernandes, J.M. y V.J. Smith, *Partial purification of antibacterial proteinaceous factors from erythrocytes of Oncorhynchus mykiss.* Fish Shellfish Immunol, 2004. **16**(1): p. 1-9.

67. Ullal, A.J., R.W. Litaker, y E.J. Noga, *Antimicrobial peptides derived from hemoglobin are expressed in epithelium of channel catfish (Ictalurus punctatus, Rafinesque).* Dev Comp Immunol, 2008. **32**(11): p. 1301-12.

68. Dijkstra, J.M., et al., *The rainbow trout classical MHC class I molecule Onmy-UBA*501 is expressed in similar cell types as mammalian classical MHC class I molecules.* Fish Shellfish Immunol, 2003. **14**(1): p. 1-23.

69. Fischer, U., et al., *The ontogeny of MHC class I expression in rainbow trout (Oncorhynchus mykiss).* Fish Shellfish Immunol, 2005. **18**(1): p. 49-60.

70. Sarder, M.R., et al., *The MHC class I linkage group is a major determinant in the in vivo rejection of allogeneic erythrocytes in rainbow trout (Oncorhynchus mykiss).* Immunogenetics, 2003. **55**(5): p. 315-24.

71. Jorgensen, S.M., et al., *Expression of MHC class I pathway genes in response to infectious salmon anaemia virus in Atlantic salmon (Salmo salar L.) cells.* Fish Shellfish Immunol, 2006. **21**(5): p. 548-60.

72. St Paul, M., et al., *Chicken erythrocytes respond to Toll-like receptor ligands by up-regulating cytokine transcripts.* Res Vet Sci, 2013. **95**(1): p. 87-91.

73. Wieczorek, M., et al., *Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation.* Front Immunol, 2017. **8**: p. 292.

74. O'Rourke, A.M. y M.F. Mescher, *The roles of CD8 in cytotoxic T lymphocyte function.* Immunology Today, 1993. **14**(4): p. 177-183.

75. Holling, T.M., E. Schooten, y P.J. van Den Elsen, *Function and regulation of MHC class II molecules in T-lymphocytes: of mice and men.* Human Immunology, 2004. **65**(4): p. 282-290.

76. Fehres, C.M., et al., *Understanding the biology of antigen cross-presentation for the design of vaccines against cancer.* Front Immunol, 2014. **5**: p. 149.

77. Kambayashi, T. y T.M. Laufer, *Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell?* Nat Rev Immunol, 2014. **14**(11): p. 719-30.

78. Lewis, K.L., N. Del Cid, y D. Traver, *Perspectives on antigen presenting cells in zebrafish.* Dev Comp Immunol, 2014. **46**(1): p. 63-73.

79. Boshra, H., J. Li, y J.O. Sunyer, *Recent advances on the complement system of teleost fish.* Fish Shellfish Immunol, 2006. **20**(2): p. 239-62.

80. Zarkadis, I.K., D. Mastellos, y J.D. Lambris, *Phylogenetic aspects of the complement system.* Dev Comp Immunol, 2001. **25**(8-9): p. 745-62.

81. Rochowiak, A. y Z.I. Niemir, *[The role of CR1 complement receptor in pathology].* Pol Merkur Lekarski, 2010. **28**(163): p. 84-8.

82. Schraml, B., M.A. Baker, y B.D. Reilly, *A complement receptor for opsonized immune complexes on erythrocytes from Oncorhynchus mykiss but not Ictalarus punctatus.* Mol Immunol, 2006. **43**(10): p. 1595-603.

83. Nelson, R.A., Jr., *The immune-adherence phenomenon; an immunologically specific reaction between microorganisms and erythrocytes leading to enhanced phagocytosis.* Science, 1953. **118**(3077): p. 733-7.

84. Erickson, C.L., *An artificial landscape-scale fishery in the Bolivian Amazon.* Nature, 2000. **408**(6809): p. 190-3.

85. FAO, *Fishstat Plus, Food and Agricultural Organisation of the United Nations, Rome.* 2009.

86. FAO, *State of World Fisheries and Aquaculture (SOFIA), Food and Agricultural Organisation of the United Nations, Rome.* 2008.

87. Walker, P.J. y J.R. Winton, *Emerging viral diseases of fish and shrimp.* Vet Res, 2010. **41**(6): p. 51.

88. Ammayappan, A. y V.N. Vakharia, *Molecular characterization of the Great Lakes viral hemorrhagic septicemia virus (VHSV) isolate from USA.* Virol J, 2009. **6**: p. 171.

89. OIE. Organización Mundial de Sanidad Animal. *Septicemia Hemorrágica Viral. Manual de las Pruebas de Diagnóstico para los Animales Acuáticos.* 2018.

90. Crane, M. y A. Hyatt, *Viruses of fish: an overview of significant pathogens.* Viruses, 2011. **3**(11): p. 2025-46.

91. Bearzotti, M., et al., *Identification of a cellular receptor for fish rhabdoviruses.* Vet Res, 1995. **26**(5-6): p. 544-5.

92. Schlegel, R., et al., *Inhibition of VSV binding and infectivity by phosphatidylserine: is phosphatidylserine a VSV-binding site?* Cell, 1983. **32**(2): p. 639-46.

93. Superti, F., M. Derer, y H. Tsiang, *Mechanism of rabies virus entry into CER cells.* J Gen Virol, 1984. **65 (Pt 4)**: p. 781-9.

94. Lentz, T.L., et al., *Is the acetylcholine receptor a rabies virus receptor?* Science, 1982. **215**(4529): p. 182-4.

95. Li, J.Z., Y., *Messenger RNA Cap Methylation in Vesicular Stomatitis Virus, a Prototype of Non-Segmented Negative-Sense RNA Virus, Methylation - From DNA, RNA and Histones to Diseases and Treatment, Anica Dricu.* IntechOpen, 2012.

96. Wolf, K., et al., *Virus nature of infectious pancreatic necrosis in trout.* Proc Soc Exp Biol Med, 1960. **104**: p. 105-8.

97. Zhu, L., et al., *Outbreak of infectious pancreatic necrosis virus (IPNV) in farmed rainbow trout in China.* Acta Trop, 2017. **170**: p. 63-69.

98. Dobos, P. y T.E. Roberts, *The molecular biology of infectious pancreatic necrosis virus: a review.* Can J Microbiol, 1983. **29**(4): p. 377-84.

99. Dobos, P., et al., *Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes.* J Virol, 1979. **32**(2): p. 593-605.

100. Bang Jensen, B. y A.B. Kristoffersen, *Risk factors for outbreaks of infectious pancreatic necrosis (IPN) and associated mortality in Norwegian salmonid farming.* Dis Aquat Organ, 2015. **114**(3): p. 177-87.

101. Snieszko, S.F., *Your fishes' health: Diseases of fishes.* Trop. Fish Hobbyist, 1981. **3**(78).

102. Dixon, P.F., et al., *Proposal for a fourth aquabirnavirus serogroup.* Arch Virol, 2008. **153**(10): p. 1937-41.

103. Ramstad, A. y P.J. Midtlyng, *Strong genetic influence on IPN vaccination-and-challenge trials in Atlantic salmon, Salmo salar L.* J Fish Dis, 2008. **31**(8): p. 567-78.

104. Xu, L., et al., *Bivalent DNA vaccine induces significant immune responses against infectious hematopoietic necrosis virus and infectious pancreatic necrosis virus in rainbow trout.* Sci Rep, 2017. **7**(1): p. 5700.

105. Jørgensen, P.E.V., *Egtved virus: temperature-dependent immune response of trout to infection with low-virulence virus.* Journal of Fish Diseases, 1982. **5**(1): p. 47-55.

106. de Kinkelin, P., et al., *Eighteen years of vaccination against viral haemorrhagic septicaemia in France.* Vet Res, 1995. **26**(5-6): p. 379-87.

107. Isshiki, T., et al., *Protective Efficacy of a Formalin-inactivated Vaccine against Viral Hemorrhagic Septicemia in Japanese Flounder Paralichthys olivaceus.* Fish Pathology, 2012. **47**(4): p. 121-128.

108. Standish, I.F., et al., *A DNA vaccine encoding the viral hemorrhagic septicemia virus genotype IVb glycoprotein confers protection in muskellunge (Esox masquinongy), rainbow trout (Oncorhynchus mykiss), brown trout (Salmo trutta), and lake trout (Salvelinus namaycush).* Virol J, 2016. **13**(1): p. 203.

109. Martinez-Lopez, A., et al., *Increasing versatility of the DNA vaccines through modification of the subcellular location of plasmid-encoded antigen expression in the in vivo transfected cells.* PLoS One, 2013. **8**(10): p. e77426.

110. Chico, V., et al., *The immunogenicity of viral haemorragic septicaemia rhabdovirus (VHSV) DNA vaccines can depend on plasmid regulatory sequences.* Vaccine, 2009. **27**(13): p. 1938-48.

111. LeBerre, M., P. De Kinkelin, y A. Metzger, *Identification sérologique des rhabdovirus des salmonidés.* Bull Off Int Epizoot, 1977. **87**: p. 391–3.

112. Hill, B.J. y K. Way, *Serological classification of infectious pancreatic necrosis (IPN) virus and other aquatic birnaviruses.* Annual Review of Fish Diseases, 1995. **5**: p. 55-77.

113. Livak, K.J. y T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.

114. Puente-Marin, S., et al., *In Silico Functional Networks Identified in Fish Nucleated Red Blood Cells by Means of Transcriptomic and Proteomic Profiling.* Genes (Basel), 2018. **9**(4).

115. Shannon, P., et al., *Cytoscape: a software environment for integrated models of biomolecular interaction networks.* Genome Res, 2003. **13**(11): p. 2498-504.

116. Bindea, G., et al., *ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks.* Bioinformatics, 2009. **25**(8): p. 1091-3.

117. Bindea, G., J. Galon, y B. Mlecnik, *CluePedia Cytoscape plugin: pathway insights using integrated experimental and in silico data.* Bioinformatics, 2013. **29**(5): p. 661-3.

118. Szklarczyk, D., et al., *STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets.* Nucleic Acids Res, 2019. **47**(D1): p. D607-D613.

119. Mas, V., et al., *Salmonid viral haemorrhagic septicaemia virus: fusion-related enhancement of virus infectivity by peptides derived from viral glycoprotein G or a combinatorial library.* J Gen Virol, 2002. **83**(Pt 11): p. 2671-81.

120. Schloemer, R.H. y R.R. Wagner, *Cellular adsorption function of the sialoglycoprotein of vesicular stomatitis virus and its neuraminic acid.* J Virol, 1975. **15**(4): p. 882-93.

121. Tafalla, C., A. Figueras, y B. Novoa, *Role of nitric oxide on the replication of viral haemorrhagic septicemia virus (VHSV), a fish rhabdovirus.* Vet Immunol Immunopathol, 1999. **72**(3-4): p. 249-56.

122. Diago, M.L., et al., *The in vitro infection of the hematopoietic stroma of trout kidney by hemorrhagic septicemia rhabdovirus.* Viral Immunol, 1993. **6**(3): p. 185-91.

123. Boukhvalova, M.S., G.A. Prince, y J.C. Blanco, *Respiratory syncytial virus infects and abortively replicates in the lungs in spite of preexisting immunity.* J Virol, 2007. **81**(17): p. 9443-50.

124. Pham, P.H., et al., *Differential effects of viral hemorrhagic septicaemia virus (VHSV) genotypes IVa and IVb on gill epithelial and spleen macrophage cell lines from rainbow trout (Oncorhynchus mykiss).* Fish Shellfish Immunol, 2013. **34**(2): p. 632-40.

125. Wang, W., et al., *Abortive infection of snakehead fish vesiculovirus in ZF4 cells was associated with the RLRs pathway activation by viral replicative intermediates.* Int J Mol Sci, 2015. **16**(3): p. 6235-50.

126. Pfefferkorn, C., et al., *Abortively Infected Astrocytes Appear To Represent the Main Source of Interferon Beta in the Virus-Infected Brain.* J Virol, 2016. **90**(4): p. 2031-8.

127. Killip, M.J., et al., *Failure to activate the IFN-beta promoter by a paramyxovirus lacking an interferon antagonist.* Virology, 2011. **415**(1): p. 39-46.

128. Komatsu, T., et al., *Sendai virus blocks alpha interferon signaling to signal transducers and activators of transcription.* J Virol, 2000. **74**(5): p. 2477-80.

129. Fange, R. y S. Nilsson, *The fish spleen: structure and function.* Experientia, 1985. **41**(2): p. 152-8.

130. Ganassin, R.C. y N.C. Bols, *A stromal cell line from rainbow trout spleen, RTS34ST, that supports the growth of rainbow trout macrophages and produces conditioned medium with mitogenic effects on leukocytes.* In Vitro Cell Dev Biol Anim, 1999. **35**(2): p. 80-6.

131. Anthony, S.M., et al., *Soluble interleukin-15 complexes are generated in vivo by type I interferon dependent and independent pathways.* PLoS One, 2015. **10**(3): p. e0120274.

132. Boudinot, P., et al., *vig-1, a new fish gene induced by the rhabdovirus glycoprotein, has a virus-induced homologue in humans and shares conserved motifs with the MoaA family.* J Virol, 1999. **73**(3): p. 1846-52.

133. Becker, S., J. Quay, y J. Soukup, *Cytokine (tumor necrosis factor, IL-6, and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages.* J Immunol, 1991. **147**(12): p. 4307-12.

134. Mohr, I., *Closing in on the causes of host shutoff.* Elife, 2016. **5**.

135. Meyer, F., *Chapter Eight - Viral interactions with components of the splicing machinery*, in *Progress in Molecular Biology and Translational Science*, M. San Francisco and B. San Francisco, Editors. 2016, Academic Press. p. 241-268.

136. Connor, J.H. y D.S. Lyles, *Inhibition of host and viral translation during vesicular stomatitis virus infection. eIF2 is responsible for the inhibition of viral but not host translation.* J Biol Chem, 2005. **280**(14): p. 13512-9.

137. Kimball, S.R., *Eukaryotic initiation factor eIF2.* The International Journal of Biochemistry & Cell Biology, 1999. **31**(1): p. 25-29.

138. Donnelly, N.G., A.M.; Gupta, S. y Samali, A., *The eIF2α kinases: their structures and functions.* Cell. Mol. Life Sci. , 2013. **70**(19): p. 3493–3511.

139. Chen, J.J., *Regulation of protein synthesis by the heme-regulated eIF2alpha kinase: relevance to anemias.* Blood, 2007. **109**(7): p. 2693-9.

140. Camini, F.C., et al., *Implications of oxidative stress on viral pathogenesis.* Arch Virol, 2017. **162**(4): p. 907-917.

141. Lu, L., A.P. Han, y J.J. Chen, *Translation initiation control by heme-regulated eukaryotic initiation factor 2alpha kinase in erythroid cells under cytoplasmic stresses.* Mol Cell Biol, 2001. **21**(23): p. 7971-80.

142. Fang, F.C., *Antimicrobial actions of reactive oxygen species.* MBio, 2011. **2**(5).

143. Birben, E., et al., *Oxidative stress and antioxidant defense.* World Allergy Organ J, 2012. **5**(1): p. 9-19.

144. Ivanov, A.V., B. Bartosch, y M.G. Isaguliants, *Oxidative Stress in Infection and Consequent Disease.* Oxidative medicine and cellular longevity, 2017. **2017**: p. 3496043-3496043.

145. Ellis, A.E., et al., *Histology, immunocytochemistry and qRT-PCR analysis of Atlantic salmon, Salmo salar L., post-smolts following infection with infectious pancreatic necrosis virus (IPNV).* J Fish Dis, 2010. **33**(10): p. 803-18.

146. Orpetveit, I., et al., *Infectious pancreatic necrosis virus (IPNV) from salmonid fish enters, but does not replicate in, mammalian cells.* Virol J, 2012. **9**: p. 228.

147. Verhelst, J., P. Hulpiau, y X. Saelens, *Mx proteins: antiviral gatekeepers that restrain the uninvited.* Microbiol Mol Biol Rev, 2013. **77**(4): p. 551-66.

148. DeWitte-Orr, S.J., J.A. Leong, y N.C. Bols, *Induction of antiviral genes, Mx and vig-1, by dsRNA and Chum salmon reovirus in rainbow trout monocyte/macrophage and fibroblast cell lines.* Fish Shellfish Immunol, 2007. **23**(3): p. 670-82.

149. Ingerslev, H.C., et al., *Differential expression of immune genes in Atlantic salmon (Salmo salar L.) challenged intraperitoneally or by cohabitation with IPNV.* Scand J Immunol, 2009. **69**(2): p. 90-8.

150. Reyes-Cerpa, S., et al., *Induction of anti-inflammatory cytokine expression by IPNV in persistent infection.* Fish Shellfish Immunol, 2014. **41**(2): p. 172-82.

151. Wang, W.L., et al., *Activation of cytokine expression occurs through the TNFalpha/NF-kappaB-mediated pathway in birnavirus-infected cells.* Fish Shellfish Immunol, 2011. **31**(1): p. 10-21.

152. Levin, D. y I.M. London, *Regulation of protein synthesis: activation by double-stranded RNA of a protein kinase that phosphorylates eukaryotic initiation factor 2.* Proc Natl Acad Sci U S A, 1978. **75**(3): p. 1121-5.

153. Gamil, A.A., S. Mutoloki, y O. Evensen, *A piscine birnavirus induces inhibition of protein synthesis in CHSE-214 cells primarily through the induction of eIF2alpha phosphorylation.* Viruses, 2015. **7**(4): p. 1987-2005.

154. Chen, L., O. Evensen, y S. Mutoloki, *Delayed protein shut down and cytopathic changes lead to high yields of infectious pancreatic necrosis virus cultured in Asian Grouper cells.* J Virol Methods, 2014. **195**: p. 228-35.

155. Busnadiego, I., et al., *The infectious bursal disease virus RNA-binding VP3 polypeptide inhibits PKR-mediated apoptosis.* PLoS One, 2012. **7**(10): p. e46768.

156. Guarda, G., et al., *Type I interferon inhibits interleukin-1 production and inflammasome activation.* Immunity, 2011. **34**(2): p. 213-23.

157. Diedrich, G., et al., *A role for calnexin in the assembly of the MHC class I loading complex in the endoplasmic reticulum.* J Immunol, 2001. **166**(3): p. 1703-9.

158. Harty, R.N., et al., *Rhabdoviruses and the cellular ubiquitin-proteasome system: a budding interaction.* J Virol, 2001. **75**(22): p. 10623-9.

159. Ko, A., et al., *MKRN1 induces degradation of West Nile virus capsid protein by functioning as an E3 ligase.* J Virol, 2010. **84**(1): p. 426-36.

160. Suzuki, R., et al., *Proteasomal turnover of hepatitis C virus core protein is regulated by two distinct mechanisms: a ubiquitin-dependent mechanism and a ubiquitin-independent but PA28gamma-dependent mechanism.* J Virol, 2009. **83**(5): p. 2389-92.

161. Drugeon, G. y I. Jupin, *Stability in vitro of the 69K movement protein of Turnip yellow mosaic virus is regulated by the ubiquitin-mediated proteasome pathway.* J Gen Virol, 2002. **83**(Pt 12): p. 3187-97.

162. Gilfoy, F., R. Fayzulin, y P.W. Mason, *West Nile virus genome amplification requires the functional activities of the proteasome.* Virology, 2009. **385**(1): p. 74-84.

163. Reichel, C. y R.N. Beachy, *Degradation of tobacco mosaic virus movement protein by the 26S proteasome.* J Virol, 2000. **74**(7): p. 3330-7.

164. Liu, W.J., et al., *p62 links the autophagy pathway and the ubiqutin-proteasome system upon ubiquitinated protein degradation.* Cell Mol Biol Lett, 2016. **21**: p. 29.

165. Garcia-Valtanen, P., et al., *Autophagy-inducing peptides from mammalian VSV and fish VHSV rhabdoviral G glycoproteins (G) as models for the development of new therapeutic molecules.* Autophagy, 2014. **10**(9): p. 1666-80.

166. Lippai, M. y P. Low, *The role of the selective adaptor p62 and ubiquitin-like proteins in autophagy.* Biomed Res Int, 2014. **2014**: p. 832704.

167. Li, M., et al., *Suppression of lysosome function induces autophagy via a feedback down-regulation of MTOR complex 1 (MTORC1) activity.* J Biol Chem, 2013. **288**(50): p. 35769-80.

168. Kao, J.C., et al., *The antiparasitic drug niclosamide inhibits dengue virus infection by interfering with endosomal acidification independent of mTOR.* PLoS Negl Trop Dis, 2018. **12**(8): p. e0006715.

169. Jung, M.-H., et al., *The Megalocytivirus RBIV Induces Apoptosis and MHC Class I Presentation in Rock Bream (Oplegnathus fasciatus) Red Blood Cells.* Frontiers in Immunology, 2019. **10**(160).

170. Mizuno, C.M., et al., *Numerous cultivated and uncultivated viruses encode ribosomal proteins.* Nat Commun, 2019. **10**(1): p. 752.

171. Kim, B.H., et al., *IFN-inducible GTPases in host cell defense.* Cell Host Microbe, 2012. **12**(4): p. 432-44.

172. Vestal, D.J. y J.A. Jeyaratnam, *The guanylate-binding proteins: emerging insights into the biochemical properties and functions of this family of large interferon-induced guanosine triphosphatase.* Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research, 2011. **31**(1): p. 89-97.

173. Fitzgerald, K.A., *The interferon inducible gene: Viperin.* Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research, 2011. **31**(1): p. 131-135.

174. Narayana, S.K., et al., *The Interferon-induced Transmembrane Proteins, IFITM1, IFITM2, and IFITM3 Inhibit Hepatitis C Virus Entry.* J Biol Chem, 2015. **290**(43): p. 25946-59.

175. Liu, S.Y., et al., *Interferon-inducible cholesterol-25-hydroxylase broadly inhibits viral entry by production of 25-hydroxycholesterol.* Immunity, 2013. **38**(1): p. 92-105.

176. van Gent, M., K.M.J. Sparrer, y M.U. Gack, *TRIM Proteins and Their Roles in Antiviral Host Defenses.* Annual Review of Virology, 2018. **5**(1): p. 385-405.

177. Li, M., et al., *Roles of SAMHD1 in antiviral defense, autoimmunity and cancer.* Rev Med Virol, 2017. **27**(4).

178. Corleis, B., et al., *Early type I Interferon response induces upregulation of human beta-defensin 1 during acute HIV-1 infection.* PLoS One, 2017. **12**(3): p. e0173161.

179. Haller, O. y G. Kochs, *Interferon-induced mx proteins: dynamin-like GTPases with antiviral activity.* Traffic, 2002. **3**(10): p. 710-7.

180. Haller, O., P. Staeheli, y G. Kochs, *Interferon-induced Mx proteins in antiviral host defense.* Biochimie, 2007. **89**(6-7): p. 812-8.

181. Cano, I., et al., *In vivo virulence of viral haemorrhagic septicaemia virus (VHSV) in rainbow trout Oncorhynchus mykiss correlates inversely with in vitro Mx gene expression.* Vet Microbiol, 2016. **187**: p. 31-40.

182. McLauchlan, P.E., et al., *DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injection and*

duration of protection-early protection correlates with Mx expression. Fish Shellfish Immunol, 2003. **15**(1): p. 39-50.

183. Ryan, L.K. y G. Diamond, *Modulation of Human beta-Defensin-1 Production by Viruses.* Viruses, 2017. **9**(6).

184. Bauer, F., et al., *Structure determination of human and murine beta-defensins reveals structural conservation in the absence of significant sequence similarity.* Protein Sci, 2001. **10**(12): p. 2470-9.

PUBLICACIÓN 1

TÍTULO: Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus

AUTORES: Ivan Nombela, Sara Puente-Marin, Veronica Chico, Alberto J. Villena, Begoña Carracedo, Sergio Ciordia, Maria Carmen Mena, Luis Mercado, Luis Pérez, Julio Coll, Amparo Estepa y Maria del Mar Ortega-Villaizan

REVISTA: *F1000Research*

DOI: 10.12688/f1000research.12985.2

RESEARCH ARTICLE

REVISED Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus [version 2; referees: 2 approved, 1 approved with reservations]

Ivan Nombela [1], Sara Puente-Marin[1], Veronica Chico[1], Alberto J. Villena[2], Begoña Carracedo[2], Sergio Ciordia[3], Maria Carmen Mena[3], Luis Mercado[4], Luis Perez [1], Julio Coll[5], Amparo Estepa[1+], Maria del Mar Ortega-Villaizan [1]

[1]Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, Elche, Spain
[2]Área de Biología Celular, Departamento de Biología Molecular, Universidad de León, León, Spain
[3]Unidad de Proteómica, Centro Nacional de Biotecnología, Madrid, Spain
[4]Instituto de Biología, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile
[5]INIA-SIGT–Biotecnología, Madrid, Spain

[+] Deceased author

v2 **First published:** 06 Nov 2017, **6**:1958 (doi: 10.12688/f1000research.12985.1)
Latest published: 09 Feb 2018, **6**:1958 (doi: 10.12688/f1000research.12985.2)

Abstract

Background: It has been described that fish nucleated red blood cells (RBCs) generate a wide variety of immune-related gene transcripts when viruses highly replicate inside them and are their main target cell. The immune response and mechanisms of fish RBCs against viruses targeting other cells or tissues has not yet been explored and is the objective of our study.
Methods: Rainbow trout RBCs were obtained from peripheral blood, ficoll purified and exposed to *Viral Haemorrhagic Septicaemia virus* (VHSV). Immune response was evaluated by means of RT-qPCR, flow cytometry, immunofluorescence and isobaric tag for relative and absolute quantification (iTRAQ) protein profiling.
Results: VHSV N gene transcripts incremented early postexposure and were drastically decreased after 6 hours postexposure (hpe). The expression of type I interferon (*ifn1*) gene was significantly downregulated at early postexposure (3 hpe), together with a gradual downregulation of interferon-inducible *mx* and *pkr* genes until 72 hpe. Type I IFN protein was downregulated and interferon-inducible Mx protein was maintained at basal levels. Co-culture assays of RBCs, previously exposed to UV-inactivated VHSV, and TSS (stromal cell line from spleen) revealed IFN crosstalk between both cell types. On the other hand, anti-microbial peptide β-defensin 1 and neutrophil chemotactic factor interleukin 8 were slightly upregulated in VHSV-exposed RBCs. iTRAQ profiling revealed that VHSV exposure can induce a global protein downregulation in rainbow trout RBCs, mainly related to RNA stability and proteasome pathways. Antioxidant/antiviral response is also suggested to be involved in the response of rainbow trout RBCs to VHSV.
Conclusions: A variety of mechanisms are proposed to be implicated in the

Open Peer Review

Referee Status: ✓ ✓ ?

	Invited Referees 1	2	3
REVISED version 2 published 09 Feb 2018	✓ report	✓ report	
version 1 published 06 Nov 2017	? report	? report	? report

1 **Aleksei Krasnov**, Nofima AS, Norway

2 **Johannes M. Dijkstra**, Fujita Health University, Japan

3 **Uwe Fischer**, Friedrich Loeffler Institute, Germany

Discuss this article

Comments (0)

antiviral response of rainbow trout RBCs against VHSV halted infection.
Ongoing research is focused on understanding the mechanisms in detail.

Corresponding author: Maria del Mar Ortega-Villaizan (mortega-villaizan@umh.es)

Author roles: Nombela I: Formal Analysis, Investigation, Methodology, Validation, Writing – Review & Editing; **Puente-Marin S**: Formal Analysis, Investigation, Writing – Review & Editing; **Chico V**: Formal Analysis, Investigation, Methodology, Writing – Review & Editing; **Villena AJ**: Resources; **Carracedo B**: Resources; **Ciordia S**: Investigation, Methodology; **Mena MC**: Investigation, Methodology; **Mercado L**: Resources; **Perez L**: Writing – Review & Editing; **Coll J**: Writing – Review & Editing; **Estepa A**: Conceptualization; **Ortega-Villaizan MdM**: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

How to cite this article: Nombela I, Puente-Marin S, Chico V *et al.* **Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus [version 2; referees: 2 approved, 1 approved with reservations]** *F1000Research* 2018, **6**:1958 (doi: 10.12688/f1000research.12985.2)

Grant information: This work was supported by the European Research Council (ERC starting grant 2014 GA639249).
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

First published: 06 Nov 2017, **6**:1958 (doi: 10.12688/f1000research.12985.1)

REVISED **Amendments from Version 1**

In this new version of the manuscript we have included the corrections and suggestions of Dr. Krasnov, Dr. Dijkstra and Dr. Fischer, in order to improve manuscript comprehension. The storyline of the manuscript has not changed, but manuscript understanding has been improved. Besides, new experiments/figures have been included in order to support the results presented, which are reflected in Figure S1, Figure S2, Figure S3 and Figure S4. We have clarified some unclear points in the manuscript, eliminating some confusing citations or extending the discussion, as suggested by the reviewers. Therefore, some references have been eliminated and others have been added. Mistyping and linguistic errors have also been corrected. As requested by reviewers Figure 4 and Figure 8 have been modified. Also, minor changes have been introduced in Figure 1, Figure 2, Figure 9 and Figure 10. Modifications are also translated to the datasets, with corrections in Dataset 1, Dataset 3 and Dataset 6.

See referee reports

Introduction

Fish are the most primitive vertebrates possessing many of the immune system cells (lymphocytes, NK cells, macrophages, etc) and molecules (interleukins, chemokins, receptors, etc) found in higher vertebrates. In contrast to higher vertebrates, however, fish lack bone marrow, lymph nodes, IgG-switch, and have tetrameric rather than pentameric IgM, with a more limited binding repertoire than mammals[1]. Fish red blood cells (RBCs), the most abundant cell type in the blood, have receptors capable of recognizing pathogen associated molecular patterns and respond to them with differentially expressed cytokine transcripts[2,3] and cytokine-like factors[4]. Fish RBCs generate a wide variety of immune-related gene transcripts when viruses highly replicate inside them[5–7], while their mammalian counterparts are unable to do this. In light of this evidence, an outstanding question is whether fish RBCs are able to respond to viral infections that are well known to replicate in other cells or tissues, and if they could further contribute with compensatory immune responses in order to physiologically combat viral infections that do not target RBCs.

To explore *in vitro* the above mentioned question, we used rainbow trout (*Oncorhynchus mykiss*), an important aquacultured species, together with *Viral Haemorrhagic Septicemia virus* (VHSV), a rhabdovirus also called the 'fish ebola', which causes important losses of high economic impact on worldwide salmonid aquaculture[8]. VHSV viruses are bullet-shaped enveloped virions with single-stranded negative-sense RNA with a genome of 11.2 kbp[8–10]. In rainbow trout, kidney and spleen endothelial cells are the first targets of VHSV. Afterwards, hematopoietic elements of kidney and spleen undergo necrosis and degeneration, most specifically at melanomacrophage centers (reviewed in Kim and Faisal[11]). However, there are no references for VHSV targeting specifically RBCs, therefore represent a good model to investigate the immune response of RBCs to viruses targeting other cells or tissues. VHSV cell entry has been described to be mediated by binding initially to fibronectin, an abundant glycoprotein of the extracellular matrix, allowing then VHSV to bind to the cells via integrin receptors and enter by fusion or endocytosis[12].

In this study, we describe how *in vitro* cultures of rainbow trout RBCs upregulated the expression of some immune proteins as part of their antiviral immune response against VHSV, whose infection appeared to be halted in rainbow trout RBCs. Simultaneously, interferon-inducible *mx* and *pkr* genes showed a downregulation tendency during VHSV early replication, after 6 hpe. In addition, protein levels corresponding to BD1 (β-defensin 1 – an anti-microbial peptide known to be involved in antiviral innate immunity[13,14]– and IL8 (Interleukin 8 – a neutrophil chemotactic factor–), are shown, to our knowledge, for the first time, as characteristic of rainbow trout RBCs antiviral immune protein responses. Further, iTRAQ-based protein profiling of VHSV-exposed RBCs showed a global protein downregulation, mainly related to RNA stability and proteasome pathways. Related to this fact, phosphorylation of the α-subunit of translational initiation factor 2 (eIF2α) and protein synthesis inhibition could be implicated in the inhibition of VHSV replication and RBCs proteome shut-off. Also, antioxidant related antiviral response is also suggested as involved in the response of rainbow trout RBCs to VHSV halted infection. In summary, we suggest a wide range of mechanisms implicated in the antiviral response of rainbow trout RBCs against VHSV halted infection.

Methods

Animals

Rainbow trout (*Oncorhynchus mykiss*) individuals of approximately 5–6 cm were obtained from a VHSV-free commercial farm (PISZOLLA S.L., CIMBALLA FISH FARM, Zaragoza, Spain), and maintained at University Miguel Hernandez (UMH) facilities at 14°C, with a re-circulating dechlorinated-water system, at a stocking density of 1fish/3L, and fed daily with a commercial diet (SKRETTING, Burgos, Spain). Prior to experiments, fish were acclimatized to laboratory conditions over 2 weeks. The number of individuals used is indicated by an "n" in each experiment.

Antibodies

Rabbit polyclonal antibodies against rainbow trout β-defensin (BD1) (RRID: AB_2716268) (unpublished, Figure S1) and rainbow trout Mx3 (RRID: AB_2716267)[15–17] were produced at the laboratory of Dr. Amparo Estepa. Mouse polyclonal antibodies against rainbow trout IL1β (RRID: AB_2716269)[18,19], IL8 (RRID: AB_2716272)[20], TNFα (RRID: AB_2716270)[21], Hepcidin (RRID: AB_2716273)[22], NKEF (RRID: AB_2716271)[23,24], IFNγ (RRID: AB_2716275) (unpublished, Figure S2A) and IFN1 (RRID: AB_2716274) (unpublished, Figure S2B) were produced at the laboratory of Dr. Luis Mercado. Rabbit polyclonal antibody against human NF-κβ p65 antibody (Cat#ab7970, RRID: AB_306184) was purchased from AbCam (Cambridge, UK). This p65 antibody epitope corresponds to the C-terminal region of the p65 protein, similarly to other p65 antibodies used for teleost species[25–27]. To label VHSV, we used the mouse monoclonal 2C9 antibody (RRID: AB_2716276)[28] against the N protein of VHSV (N_{VHSV}) produced at Dr. Coll's laboratory. Anti-Rabbit

IgG (H+L) CF™ 488 antibody produced in goat and Anti-Mouse IgG (H+L) CF™ 488 antibody produced in goat were used as secondary antibodies (Sigma-Aldrich, Madrid, Spain). Rabbit polyclonal antibody against human eIF2α-P (Cat# E2152, RRID: AB_259283) and rabbit polyclonal antibody against human α-Actin (Cat#2066, RRID:AB_476693) were purchased from Sigma-Aldrich and used for western blotting.

Cell cultures and virus

Rainbow trout RBCs were obtained from peripheral blood of fish sacrificed by overexposure to tricaine (tricaine methanesulfonate, Sigma-Aldrich; 0.2 g/l). Peripheral blood was sampled from the caudal vein using insulin syringes (NIPRO, Bridgewater, NJ). Blood samples were placed in a 2 ml eppendorf with RPMI-1640 medium (Dutch modification) (Gibco, Thermo Fischer Scientific Inc., Carlsbad, CA) supplemented with 10% FBS (fetal bovine serum) gamma irradiated (Cultek, Madrid, Spain), 1 mM pyruvate (Gibco), 2 mM L-glutamine (Gibco), 50 µg/mL gentamicin (Gibco) and 2 µg/mL fungizone (Gibco), 100 U ml−1 penicillin and 100 µg ml−1 streptomycin (Sigma-Aldrich). Then, RBCs were purified by two consecutive density gradient centrifugations (7206g, Ficoll 1.007; Sigma-Aldrich). Purified RBCs were cultured in the above indicated medium at a density of $5{\cdot}10^5$ cells/ml in 24-well cell culture plates at 14°C.

The fish cell lines TSS, RTG-2 and EPC, were also used in this work. TSS (Trout Stroma from Spleen)[29] was donated by the laboratory of Dr. AJ Villena. TSS cells were maintained at 21°C in RPMI medium containing 20% FBS, 1 mM pyruvate, 2 mM L-glutamine, 50 µg/mL gentamicin and 2 µg/mL fungizone. RTG-2 (Rainbow Trout Gonad-2) cell line was purchased from the American Type Culture Collection (ATCC, 50643). RTG-2 cells were maintained at 21°C in MEM medium (Sigma-Aldrich) containing 10% FBS, 1 mM pyruvate, 2 mM L-glutamine, 50 µg/mL gentamicin and 2 µg/mL fungizone. EPC (*Epithelioma Papulosum Cyprini*)[30] cell line was purchased from the ATCC (CRL-2872). Cells were maintained at 28°C, in RPMI-1640 10% FBS, 1 mM pyruvate, 2 mM L-glutamine, 50 µg/mL gentamicin and 2 µg/mL fungizone.

Viral haemorrhagic septicaemia virus (VHSV-07.71)[31], isolated in France from rainbow trout, was purchased from the American Type Culture Collection (ATCC, VR-1388) and propagated in EPC cells at 14°C, as previously reported[32]. Supernatants from VHSV-infected EPC cell monolayers were clarified by centrifugation at 4000 x g during 30 min and kept at -80 °C. The virus stock was titrated in 96-well plates using an immunostaining focus assay[33]. Clarified supernatants were used for the experiments at the indicated dilutions.

Viral exposure assays

RBCs and RTG-2 cells were infected with VHSV at different multiplicities of infection (MOI), at 14°C. After 3 hours of incubation for RBCs and 1.5 hours for RTG-2, cells were washed with cold RPMI, then RPMI 2% FBS was added and infection incubated at 14°C, at the different times indicated for each assay. In the case of the time-course assay, the virus was not removed.

Virus titers present in VHSV-exposed RBCs supernatants were determined by plaque assays. Briefly, different dilutions of the supernatants (from 10^{-1} to 10^{-4}) were added to EPC cell monolayers, grown in 24-well plates, at 14°C for 90 minutes. Then, culture media were removed and infected cell monolayers covered with a solution of RPMI-1640 cell culture medium with 2% FBS and a 2% aqueous solution of methyl cellulose (Sigma-Aldrich). Cell plates were incubated at 14°C for 5 days and then media with methyl cellulose were removed. Finally, EPC cell monolayers were stained with crystal violet-formalin to count plaques. Virus titers were expressed as plaque forming units (PFU) per ml.

Separately, N_{VHSV} RT-qPCR was also used to quantify viral RNA inside VHSV-exposed RBCs.

Blocking of endosome acidification by NH_4Cl

To block endosomal low-pH, NH_4Cl (Sigma-Aldrich) at 7 mM was added to RBCs during VHSV exposure, which was carried out as described in the previous section. No significant cell death was observed in RBCs treated with NH_4Cl, since the concentration used is known as non-cytotoxic in EPC[33] and RTG-2[17] cells, but effective for reducing VHSV infectivity by 40%[33]. After incubation period, viral titer in supernatants was calculated as described in the previous section.

Neuraminidase treatment assay

Ficoll purified RBCs were pre-treated with 50 and 100 mU/ml of neuraminidase from *Vibrio cholerae* (Sigma-Aldrich), at 21°C for 30 minutes, before virus inoculation. After treatment, RBCs were washed once with PBS in order to completely remove the enzyme. After that, pre-treated cells were inoculated with VHSV at MOI 1. RBCs inoculated with UV-inactivated VHSV were used as control. UV-inactivated VHSV was generated by exposure to UV-B at 1 J/cm2 using a Bio-Link Crosslinker BLX E312 (Vilber Lourmat, BLX-E312), as previously described[34]. Infection was monitored by RT-qPCR of N_{VHSV} gene 3 at 72 hpe.

Co-culture assay

One day prior to co-culture, RBCs, extracted and seeded as indicated before, were stimulated using UV-inactivated VHSV over 24 hours. Subsequently, RBCs were washed once with cold RPMI and added to Corning® Transwell® polyester membrane cell culture inserts of 0.4 µm pore size (Corning, Sigma-Aldrich) on 24 well plates with previously cultured confluent TSS cells in RPMI 20% FBS. Co-culture was maintained for 24 hours at 14°C in RPMI 2% FBS. After that, cells were washed and stored in the indicated buffer and conditions for RNA extraction.

Separately, RTG-2 cells were treated with UV-inactivated VHSV, MOI 1, during 24 hours, at 14°C, in RPMI 2% FBS. After that, RTG-2 cell monolayers were washed once with cold

PBS and cultured for 24h in RPMI 2%FBS fresh medium. This conditioned medium was used to stimulate rainbow trout RBCs, during 24h. After that, RBCs were washed and stored in the indicated buffer and conditions for RNA extraction.

RNA isolation and cDNA synthesis

E.Z.N.A. ® Total RNA Kit (Omega Bio-Tek, Inc., Norcross, GA) was used for total RNA extraction in accordance with manufacturer's instructions. Isolated RNAs were stored at −80°C until used. DNAse treatment was done in order to eliminate residual genomic DNA using TURBO™ DNase (Ambion, Thermo Fischer Scientific Inc.), following manufacturer's instructions. RNA was quantified with a NanoDrop® Spectrophotometer (Nanodrop Technologies, Wilmington, DE). M-MLV reverse transcriptase (Invitrogen, Thermo Fischer Scientific Inc.) was used to obtain cDNA, as previously described[35]. For viral messenger RNA (mRNA) quantitation, cDNA was obtained as described in 36.

RT-qPCR and gene expression

Real-Time Quantitative PCR (RT-qPCR) was performed using the ABI PRISM 7300 System (Applied Biosystems, Thermo Fischer Scientific Inc.). Reactions were performed in a total volume of 20 µl comprising 12 ng of cDNA, 900 nM of each primer, 10 µl of TaqMan universal PCR master mix (Applied Biosystems, Thermo Fischer Scientific Inc.) with 300 nM of probe or 10 µl of SYBR green PCR master mix (Applied Biosystems, Thermo Fischer Scientific Inc.). Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Primers and probes used are listed in Table 1.

Gene expression was analyzed by the 2-ΔCt or 2−ΔΔCt method[37] where 18S rRNA or *ef1α* gene (Applied Biosystems, Thermo Fischer Scientific Inc.) were used as endogenous control.

Intracellular stain and flow cytometry

RBCs were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) in RPMI medium for 20 minutes. Permeabilization of RBCs was done in a 0.05% saponin (Sigma-Aldrich) buffer for 15 minutes. Primary antibodies were diluted in permeabilization buffer at recommended dilutions and incubated for 60 minutes at RT. Secondary antibodies were incubated for 30 minutes at RT. After every antibody incubation, RBCs were washed with permeabilization buffer. Finally, RBCs were kept in PFA 1% in PBS. For nuclear staining, RBCs were stained with 1 µg/mL of 4′-6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 5 minutes. RBCs were analyzed by flow cytometry (FC) in a BD FACSCanto™ (BD Biosciences) flow cytometer. Immunofluorescence (IF) images were performed in an IN Cell Analyzer 6000 Cell Imaging system (GE Healthcare, Little Chalfont, UK).

Table 1. Primer and probe sequences.

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')	Probe (5' – 3')	Reference or accession number
ef1α	ACCCTCCTCTTGGTCGTTTC	TGATGACACCAACAGCAACA	GCTGTGCGTGACATGAGGCA	97
tlr3	ACTCGGTGGTGCTGGTCTTC	GAGGAGGCAATTTGGACGAA	CAAGTTGTCCCGCTGTCTGCTCCTG	NM_001124578.1
irf7	CCCAGGGTTCAGCTCCACTA	GGTCTGGCAACCCGTCAGT	TCGAGCCAAACACCAGCCCCT	AJ829673
ifn1	ACCAGATGGGAGGAGATATCACA	GTCCTCAAACTCAGCATCATCTATGT	AATGCCCCAGTCCTTTTCCCAAATC	AM489418.1
mx1-3	TGAAGCCCAGGATGAAATGG	TGGCAGGTCGATGAGTGTGA	ACCTCATCAGCCTAGAGATTGGCTCCCC	98
pkr	GACACCGCGTACCGATGTG	GGACGAACTGCTGCCTGAAT	CACCACCTCTGAGAGCGACACCACTTC	NM_001145891.1
il15	TACTATCCACACCAGCGTCTGAAC	TTTCAGCAGCACCAGCAATG	TTCATAATATTGAGCTGCCTGAGTGCCACC	XM_021575070.1
vig1	CTACAATCAAGGTGGTGAACAATGT	GTGGAAACAAAAACCGCACTTATA	TCTCAAGCTTCGGCAACTCCAAGCA	XM_021582972.1
hepcidin	TCCCGGAGCATTTCAGGTT	GCCCTTGTTGTGACAGCAGTT	AGCCACCTCTCCCTGTGCCGTTG	AF281354.1
β-globin	CAACATCTTGGCCACATACAAGTC	TTGTCAGGGTCGACGAAGAGT		NM_001160555.2
fth	GGCGTATTACTTCGATCGTGATG	CCCTCCCCTCTGGTTCTGA		EU302524.1
gstp1	CCCCTCCCTGAAGAGTTTTGT	GCAGTTTCTTGTAGGCGTCAGA		BT048561.1
nkef	CGCTGGACTTCACCTTTGTGT	ACCTCACAACCGATCTTCCTAAAC		U27125.1
sod1	GCCGGACCCCACTTCAAC	CATTGTCAGCTCCTGCAGTCA		AF469663.1
trx	AGACTTCACAGCCTCCTGGT	ACGTCCACCTTGAGGAAAAC		XM_021614924.1
N_{VHSV}	GACTCAACGGGACAGGAATGA	GGGCAATGCCCAAGTTGTT	TGGGTTGTTCACCCAGGCCGC	35

Protein digestion and tagging with iTRAQ 4plex™ reagent

Two pools of eight samples (two control: C1 and C2, and two VHSV-exposed (MOI 1, 14°C, 72 hpe): V1 and V2), with $8{\cdot}10^6$ cells per sample, were used for iTRAQ 4plex protein profiling.

Pools, containing $6.4{\cdot}10^7$ cells, were pelletized by centrifugation (5 min, 700 × g). Supernatant was carefully removed and RBC pellets (~70–100 µL) were mixed with 250 µL of deionized water and frozen at – 80°C for 3 h. After thawing the lysate, it was centrifuged at 17000 × g for 20 min at 4°C to separate cytosolic supernatant and pelleted membrane fractions, as described in Puente-Marin *et al.* (unpublished report, Puente-Marin S, Nombela I, Ciordia S, Mena MC, Chico V, Coll J, and Ortega-Villaizan M). Subsequently, a new proteomic analysis method was carried out that combines fractionation into cytosolic and membrane fractions, haemoglobin removal of cytosolic fraction, protein digestion, pH reversed-phase peptide fractionation and finally LC ESI-MS/MS analysis of each fraction, as described in Puente-Marin *et al*. (unpublished report, as before). Briefly, haemoglobin of cytosolic fraction was removed using HemoVoid™ kit (Biotech Support Group, Monmouth Junction, NJ), following manufacturer instructions[38]. For protein digestion of each fraction, 120 µg from haemoglobin-depleted cytosolic fraction were digested in chaotropic buffer, and 40 µg of membrane fraction was precipitated by methanol/chloroform method and re-suspended in 20 µl of chaotropic buffer. Digested samples (membrane and cytosol separately) were subsequently labelled using iTRAQ-4plex Isobaric Mass Tagging Kit (SCIEX), according to manufacturer's instructions as follows: 114, C1 (Pool control 1); 115, V1 (Pool VHSV-exposed 1); 116, C2 (Pool control 2); 117, V2 (Pool VHSV-exposed 2). Then, offline high pH reversed-phase peptide fractionation of the peptides from cytosolic fraction was performed on a SmartLine (Knauer, Berlin, Germany) HPLC system using an XBridge C18 column (100 × 2.1 mm, 5 µm particle; Waters, Milford, MA). Thirty fractions were collected and then pooled alternatively into 5 fractions. After labelling, samples were pooled, evaporated to dryness and stored at -20°C until LC–MS analysis.

Liquid chromatography and mass spectrometry analysis (LC-MS)

A 1 µg aliquot of labelled mixture was subjected to 1D-nano LC ESI-MSMS (Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric) analysis using a nano liquid chromatography system (Eksigent Technologies nanoLC Ultra 1D plus, SCIEX,) coupled to high speed Triple TOF 5600 mass spectrometer (SCIEX) with a Nanospray III source. The analytical column used was a silica-based reversed phase Acquity UPLC® M-Class Peptide BEH C18 Column, 75 µm × 150 mm, 1.7 µm particle size and 130 Å pore size (Waters Corporation, Milford, MA). The trap column was a C18 Acclaim PepMap™ 100 (Thermo Fischer Scientific), 100 µm × 2 cm, 5 µm particle diameter, 100 Å pore size, switched on-line with the analytical column. The loading pump delivered a solution of 0.1% formic acid in water at 2 µl/min. The nano-pump provided a flow-rate of 250 nl/min and was operated under gradient elution conditions. Peptides were separated using a 250 minutes gradient ranging from 2% to 90% mobile phase B (mobile phase A: 2% acetonitrile, 0.1% formic acid; mobile phase B: 100% acetonitrile, 0.1% formic acid). Injection volume was 5 µl.

Data acquisition was performed with a TripleTOF 5600 System (SCIEX). Data was acquired using an ionspray voltage floating, 2300 V; curtain gas, 35; interface heater temperature, 150; ion source gas 1, 25; declustering potential, 150 V. All data was acquired using information-dependent acquisition (IDA) mode with Analyst TF 1.7 software (RRID: SCR_015785) (SCIEX). For IDA parameters, 0.25 s MS survey scan in the mass range of 350–1250 Da were followed by 30 MS/MS scans of 150ms in the mass range of 100–1800. Switching criteria were set to ions greater than mass to charge ratio (m/z) 350 and smaller than m/z 1250 with charge state of 2–5 and an abundance threshold of more than 90 counts (cps). Former target ions were excluded for 20 s. IDA rolling collision energy (CE) parameters script was used for automatically controlling the CE.

Proteomics data analysis and sequence search

MS/MS spectra were exported to MGF format using Peak View v1.2.0.3 (RRID: SCR_015786)(SCIEX) and searched using Mascot Server v2.5.1 (RRID:SCR_014322)(Matrix Science, London, UK), OMSSA v2.1.9[39], X!TANDEM 2013.02.01.1[40], and Myrimatch v2.2.140[41] against a composite target/decoy database built from the Oncorhynchus mykiss sequences at Uniprot/Swissprot Knowledgebase (available here, last update: 2017/01/26, 50.125 sequences), together with commonly occurring contaminants. Search engines were configured to match potential peptide candidates with mass error tolerance of 25 ppm and fragment ion tolerance of 0.02D, allowing for up to two missed tryptic cleavage sites and a maximum isotope error (^{13}C) of 1, considering fixed methyl methanethiosulfonate modification of cysteine and variable oxidation of methionine, pyroglutamic acid from glutamine or glutamic acid at the peptide N-terminus, acetylation of the protein N-terminus, and modification of lysine, tyrosine and peptide N-terminus with iTRAQ 4-plex reagents. Score distribution models were used to compute peptide-spectrum match *P*-values[42], and spectra recovered by a FDR (False Discovery Rate) ≤ 0.01 (peptide-level) filter were selected for quantitative analysis. Approximately 1% of the signals with lowest quality were removed prior to further analysis. Differential regulation was measured using linear models[43], and statistical significance was measured using *q*-values (FDR). All analyses were conducted using Proteobotics software (Isobaric Tagging Analysis Workflow v.1.0, RRID:SCR_015787; Madrid, Spain). The cutoff for differentially regulated proteins was established at FDR *q*-value 5%.

Pathway enrichment analysis

In order to evaluate the functionally grouped Gene Ontology (GO) and pathway annotation networks of the differentially expressed proteins, pathway enrichment analysis was performed using ClueGO (RRID:SCR_005748)[44] and CluePedia (RRID: SCR_015784)[45] Cytoscape plugins (Cytoscape v3.4.0, RRID: SCR_003032,[46]). GO Biological process, GO Immunological process, KEGG (Kyoto Encyclopedia of Genes and Genomes),

Wikipathways and Reactome functional pathway databases were used. A *P*-value ≤0.05 and Kappa score of 0.4 were considered as threshold values.

Western blot assays

Control and VHSV-exposed RBCs cell pellets were resuspended in 30 µl of PBS with a cocktail of protease inhibitors (Sigma-Aldrich). Cells were then frozen/thawed 3 times and protein concentration adjusted before loading. Samples were loaded in Tris–Glycine sodium dodecyl sulfate 17% polyacrylamide gels under reducing conditions. Electrophoresis was performed at 100 V for 90 min. For blotting, proteins in the gel were transferred for 75 min at 100 V in transfer buffer (2.5 mM Tris, 9 mM glycine, 20% methanol) to nitrocellulose membranes (BioRad, Madrid, Spain). Membranes were then blocked with 8% dry milk, 1% Tween-20 in PBS and incubated with rabbit polyclonal antibody against human eIF2α-P (36.1 KDa) or rabbit polyclonal antibody against human α-Actin (42 KDa,) in PBS containing 0.5% dry milk, and 0.5% Tween-20 (PMT buffer), overnight at 4°C. Membranes were then washed 3 times with PMT buffer for 15 min before incubation with GAR-Po (Sigma-Aldrich) in PMT buffer for 45 min. Finally, membranes were washed 3 times with PBS containing 0.5% Tween-20. Peroxidase activity was detected using ECL chemiluminescence reagents (Amersham Biosciences, Buckinghamshire, UK) and revealed by exposure to X-ray. Protein bands were analyzed by densitometry using the Scion Image 4.0.2 Software (RRID: SCR_008673) (www.scionorg.com).

ROS measurement

The intracellular ROS level was assessed in VHSV-exposed RBCs using the cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (DCFDA, Sigma-Aldrich). RBCs were exposed to VHSV at MOI 1, during 72 h, at 14°C. After that, RBCs were washed with RPMI and incubated with 20 µM DCFDA in RPMI, for 30 min at RT. Fluorescence intensity of 2′,7′-dichlorofluorescin was measured using POLARstar Omega microplate reader (BMG LABTECH, USA) at excitation 480 nm and emission 530 nm.

Software and statistics

Graphpad Prism 6 (RRID:SCR_002798, www.graphpad.com) was used for graphic representation and statistics calculation. Statistic tests and *P*-values associated with graphics are indicated in each assay. Flow cytometry data was processed and analyzed using *Flowing Software 2.5.1* (www.flowingsoftware.com/) (RRID: SCR_015781).

Ethics statement

All experimental protocols and methods of the experimental animals were reviewed and approved by the Animal Welfare Body and the Research Ethics Committee at the University Miguel Hernandez (approval number 2014.205.E.OEP; 2016.221.E.OEP) and by the competent authority of the Regional Ministry of Presidency and Agriculture, Fisheries, Food and Water supply (approval number 2014/VSC/PEA/00205). All methods were carried out in accordance with the Spanish Royal Decree RD 53/2013 and EU Directive 2010/63/EU for the protection of animals used for research experimentation and other scientific purposes.

Results

VHSV course of replication in rainbow trout RBCs

For this analysis we first purified RBCs (oval nucleated cells) to 99.9% (as evaluated by optical microscopy) and then exposed the purified RBCs to VHSV, for different times, to monitor replication of VHSV in rainbow trout RBCs. For that, time course expression of N gene of VHSV (N_{VHSV}) was measured by RT-qPCR with cDNA performed with random hexamer primers (to target total RNA). Expression of N_{VHSV} gene was significantly upregulated at 3 hpe. However, it drastically decreased from 6 to 72 hpe, indicating that VHSV could replicate at early times postexposure, at the same levels as VHSV susceptible rainbow trout cell line RTG-2. However, viral replication was halted in RBCs at later stages of infection, in contrast to RTG-2 (Figure 1A). Besides, cDNA synthesis was also performed with oligo(dT) primers to target N_{VHSV} mRNA expression in VHSV-exposed RBCs and the result was consistent with total RNA expression (Figure S3). On the other hand, after VHSV enters the cell, the first gene that starts to transcribe is N_{VHSV} gene, since it is the closest to the 3' transcriptional start, and the more distal, excluding the polymerase, is the G glycoprotein gene (G_{VHSV}) gene. Therefore, under a normal transcription scenario a high ratio between the N_{VHSV} and G_{VHSV} viral genes transcripts is to be expected, taking into account the attenuation phenomenon found in rhabdoviruses[47,48]. However, a ratio of 2 was observed in RBCs, compared to the ratio of 8 found in RTG-2 cells, at 1 and 3 hpe (Figure 1B).

Also, RBCs were exposed to different VHSV multiplicities of infection (MOI). Initial VHSV inoculum titer declined ~4-logs after 3 days of incubation at the indicated MOI assayed (1, 10 or 100, respectively corresponding to inoculum virus titers $2{\cdot}10^6$, $2{\cdot}10^7$, $2{\cdot}10^8$ PFU/ml) (Figure 1C), in contrast to the usual 1-log titer increment in RTG-2 cells infected in the same conditions (Figure 1H). Later on, RBCs showed only a minor and statistically non-significant ~1-fold increment of VHSV titer as time of infection increased from 3 to 6 days (Figure 1D), showing that low VHSV titers are maintained in the cell after 72 hpe. These low VHSV titers were due to true VHSV internalization and not to residual VHSV binding, since they were diminished with NH_4Cl treatment, a characteristic of rhabdovirus infections (Figure 1D). NH_4Cl acts as a lysosomotropic drug, blocking endosomal acidification and inhibiting rhabdoviral cytoplasmic entrance steps including those of VHSV[33].

In order to increase the amount of VHSV inside rainbow trout RBCs, RBCs were pre-treated with neuraminidase (NA) and then exposed to UV-inactivated or live VHSV. NA has been shown to enhance rhabdovirus infection in NA pre-treated cells by favoring interaction with cellular membranes[49]. As a result, VHSV RNA inside RBCs was increased about ten times at 3 hpe in live VHSV-exposed RBCs, in comparison with UV-inactivated VHSV-exposed RBCs. However, seventy-two hpe the VHSV RNA drastically decreased to almost disappear, as indicated by N_{VHSV} RT-qPCR (Figure 1E).

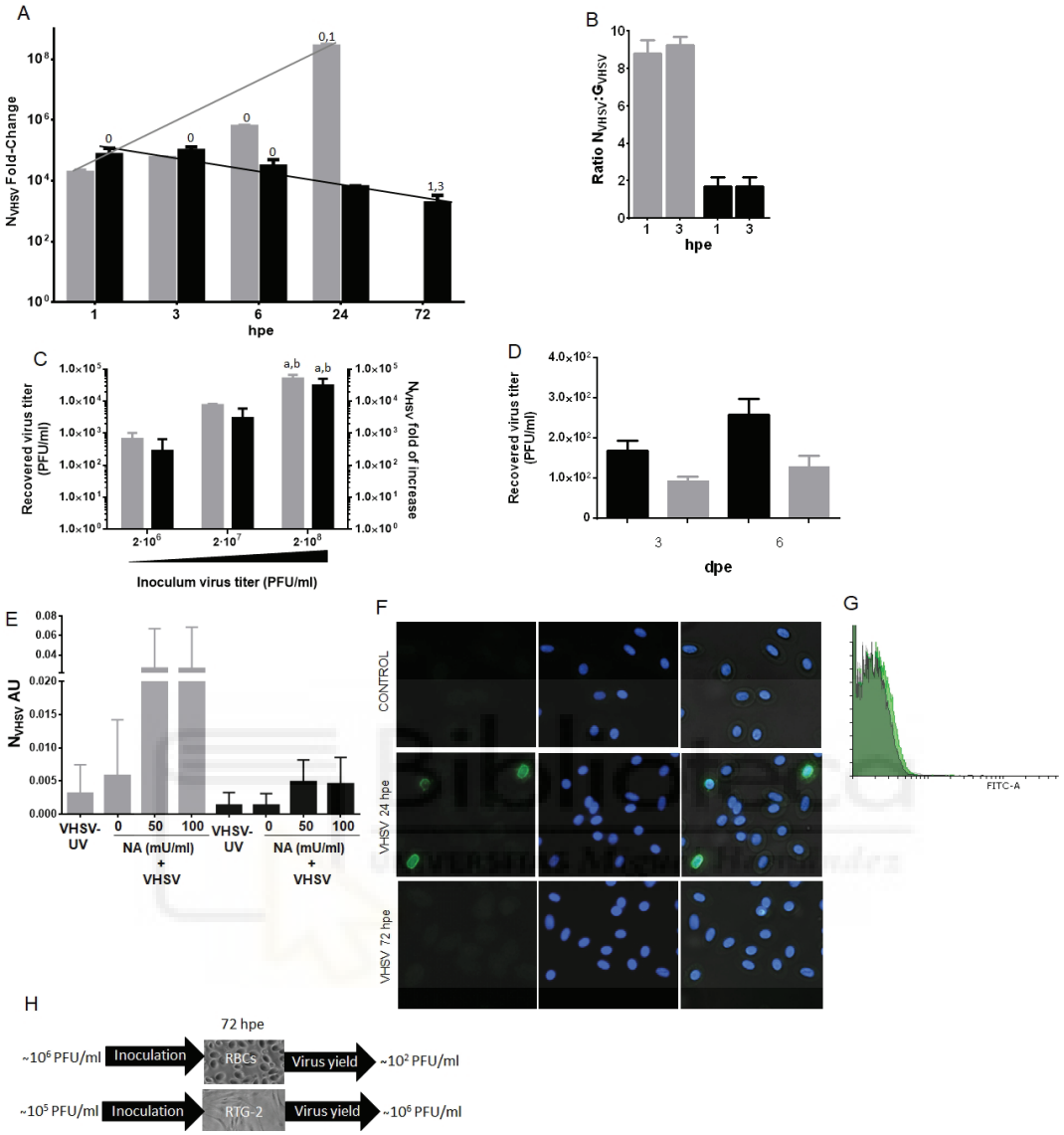


Figure 1. VHSV exposure and replication in rainbow trout RBCs. (**A**) Time course of VHSV gene replication in rainbow trout RBCs and RTG-2 cell line. N gene of VHSV (N_{VHSV}) expression profile was quantified by RT-qPCR at time 0, 1, 3, 6, 24 and 72 hours postexposure (hpe) to VHSV, in RBCs (black bars) and RTG-2 (grey bars), with a multiplicity of infection (MOI) of 1 at 14°C. Gene expression was normalized against eukaryotic 18S rRNA and *ef1α*, respectively for RBCs and RTG-2 cells, and relativized to control cells (non-exposed, time 0) (fold-change). Data represent the mean ± SD (n = 4 for RBCs and n=2 for RTG-2). (**B**) Ratio of N_{VHSV} and G_{VHSV} genes expression by RT-qPCR at time 0, 1, and 3 hpe, relative to control cells (non-exposed, time 0), in RBCs (black bars) and RTG-2 (grey bars). Ratio was calculated as $2^{-\Delta\Delta Ct}$ N_{VHSV}: $2^{-\Delta\Delta Ct}$ G_{VHSV}. Gene expression was normalized against *ef1α*. Data represent the mean ± SD (n = 3 for RBCs and n=2 for RTG-2). (**C**) Viral yield in VHSV-exposed rainbow trout RBCs. Viral titer (grey bars) (plaque forming units per millilitre, PFU/ml) and N_{VHSV} gene expression by RT-qPCR (black bars) of VHSV-exposed RBCs, with MOI 1, 10 and 100, respectively corresponding to inoculum virus titers $2{\cdot}10^6$ (**a**), $2{\cdot}10^7$ (**b**) and $2{\cdot}10^8$ (**c**) PFU/ml, 72 hpe, at 14°C. Gene expression was normalized against *ef1α*. Data represent the mean ± SD (n = 3 for viral titer and n=4 for N_{VHSV} gene expression). (**D**) VHSV titers diminished in rainbow trout RBCs after NH_4Cl treatment. VHSV titers obtained in VHSV-exposed RBCs at MOI 1, at 3 and 6 days postexposure (dpe), at 14°C, in the absence (black bars) or in the presence (grey bars) of NH_4Cl. Data represent the mean ± SD (n = 4). (**E**) Pre-treatment of RBCs with neuraminidase enhances early replication of VHSV. RBCs were inoculated with UV-inactivated or live VHSV, with a MOI of 1, at 14°C. Before infection, cells were pretreated with neuraminidase (NA) at 50 or 100 mU/ml during 30 minutes at 14°C. VHSV infectivity was quantified by N_{VHSV} gene expression analysis by RT-qPCR at 3 hpe (grey bars) and 72 hpe (black bars). Gene expression was normalized against 18S rRNA gene and represented as arbitrary units (AU). Data represent the mean ± SD (n = 4). (**F**) Representative immunofluorescence of VHSV labelling in RBCs exposed to VHSV (MOI 100, 24 and 72 hpe, 14°C) stained from left to right with anti-N_{VHSV} 2C9 (FITC), DAPI for nuclei stained and merged (IF representative of 32 images). (**G**) Representative flow cytometry overlay histograms showing untreated RBCs (grey filled histogram), VHSV-exposed RBCs with a MOI 100, at 14°C, 24 hpe (green filled histogram) and 72 hpe (black filled histogram). (**H**) Schematic representation of the VHSV infectivity in RBCs and RTG-2 cells, indicating the virus inoculation titer and recovered virus yield after 72 hpe in each cell line. Kruskal-Wallis Test with Dunn´s Multiple Comparison post-hoc test was performed for statistical analysis among all conditions. Values over the bars denote pairwise significant differences with the value-indicated time point or condition (*P*-value < 0.05).

Besides, N_{VHSV} protein (2C9 antibody) was detected in RBCs exposed to VHSV MOI 100, at 24 hpe, but not at 72 hpe. IF images (Figure 1F) showed an intracellular stain, mainly in nuclear and perinuclear regions. FC histogram (Figure 1G) showed a slight increment of VHSV N protein in VHSV-exposed RBCs, at 24 hpe, but not at 72 hpe. VHSV could not be detected by IF or FC in RBCs exposed to lower MOIs.

Antiviral transcriptional immune responses in rainbow trout RBCs exposed to VHSV *in vitro*

We next investigated whether rainbow trout RBCs exposed to VHSV could be capable of generating immune responses *in vitro*, by means of examining the differential expression profile of some genes characteristic of fish antiviral response. First, a time course monitoring of the expression of interferon-inducible *mx* and *pkr* genes was carried out at different time postexposure. The results showed that *mx* and *pkr* genes exhibited the same increment peak at 3 hpe and a tendency to downregulation from 6 to 72 hpe, in parallel to N_{VHSV} gene transcription levels tendency (Figure 2A and B, and Figure 1A). The expression of *mx* and *pkr* genes did not change over the time-course in control cells (Figure S4). On the other hand, at 3 hpe, *ifn1* gene expression already exhibited a statistically significant downregulation (Figure 2C), and a slight downregulation for *tlr3* and *irf7* genes.

Antiviral immune protein responses in RBCs exposed to VHSV *in vitro*

Changes in RBCs immune protein response induced by VHSV exposure were assessed using specific antibodies. VHSV-exposed RBCs showed only an increment in protein levels of chemokine IL8 (Figure 3B and E, Figure S5A) and antimicrobial peptide BD1 (Figure 3C and F, Figure S5B), verified by means of FC and IF. Mx and IFN1 protein levels, according to the RT-qPCR results, did not change or downregulate, respectively (Figure 3A). Cytokines IL1β, IFNγ (Figure 3B), antimicrobial peptide Hepcidin (Figure 3C) and natural killer enhancing factor (NKEF) (Figure 3D) did not show regulation at 72 hpe.

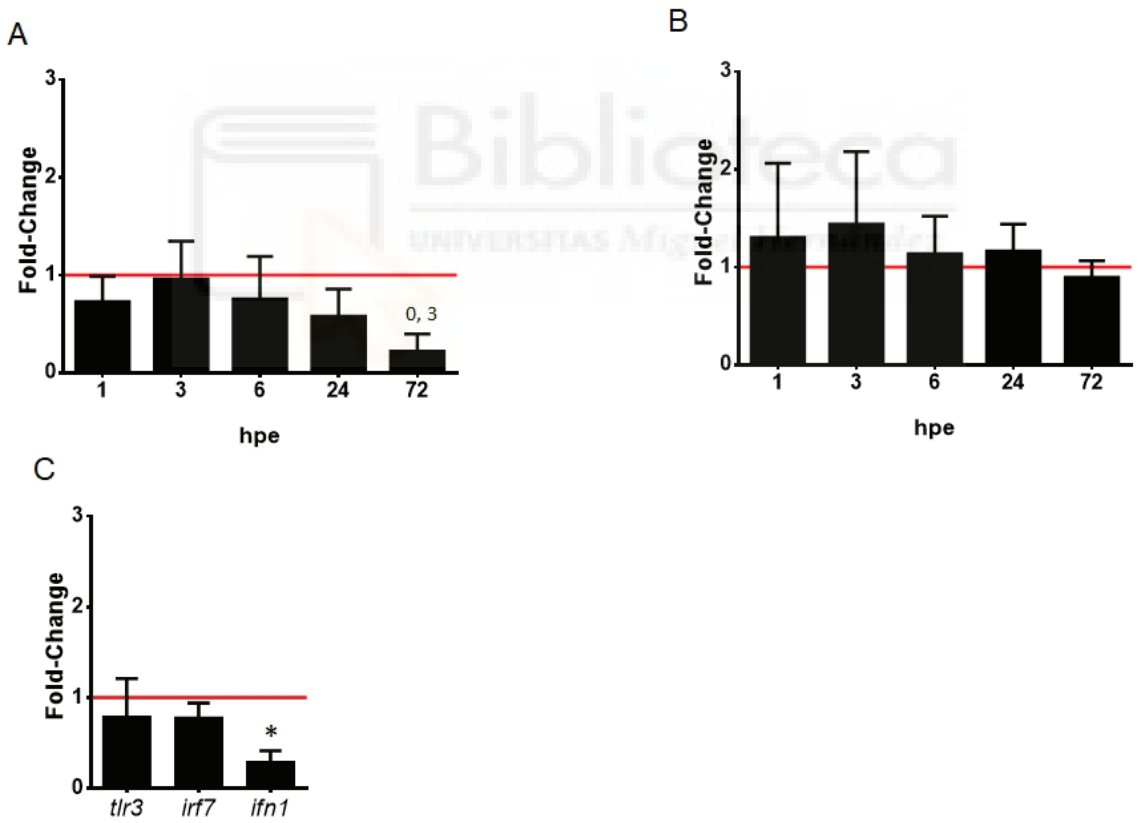


Figure 2. Interferon signaling in VHSV-exposed rainbow trout RBCs. Time course of interferon-inducible antiviral genes *mx* (**A**) and *pkr* (**B**). RBCs were exposed to VHSV with a multiplicity of infection (MOI) of 1 at 14°C, and *mx1-3* and *pkr* genes expression was quantified by RT-qPCR at time 0, 1, 3, 6, 24, 72 hours postexposure (hpe). Data is displayed as mean ± SD (n = 3). Kruskal-Wallis Test with Dunn´s Multiple Comparison post-hoc test was performed among all conditions. (**C**) Interferon signaling at early time postexposure. RBCs were exposed to VHSV with a MOI of 1 at 14°C, and *tlr3*, *irf7* and *ifn1* gene expression profiles were quantified by RT-qPCR at time 0, and 3 hpe. Data is displayed mean ± SD (n = 3). Mann Whitney Test was performed for statistical analysis between the VHSV-exposed and control cells (non-exposed, time 0, red line). Gene expression was normalized against eukaryotic 18S rRNA for *mx*, *tlr3*, *irf7* and *ifn1* genes and *ef1α* for *pkr* gene, and relativized to control cells (fold-change). Asterisk denote statistically significant differences between VHSV-exposed and control cells (*P*-value < 0.05).

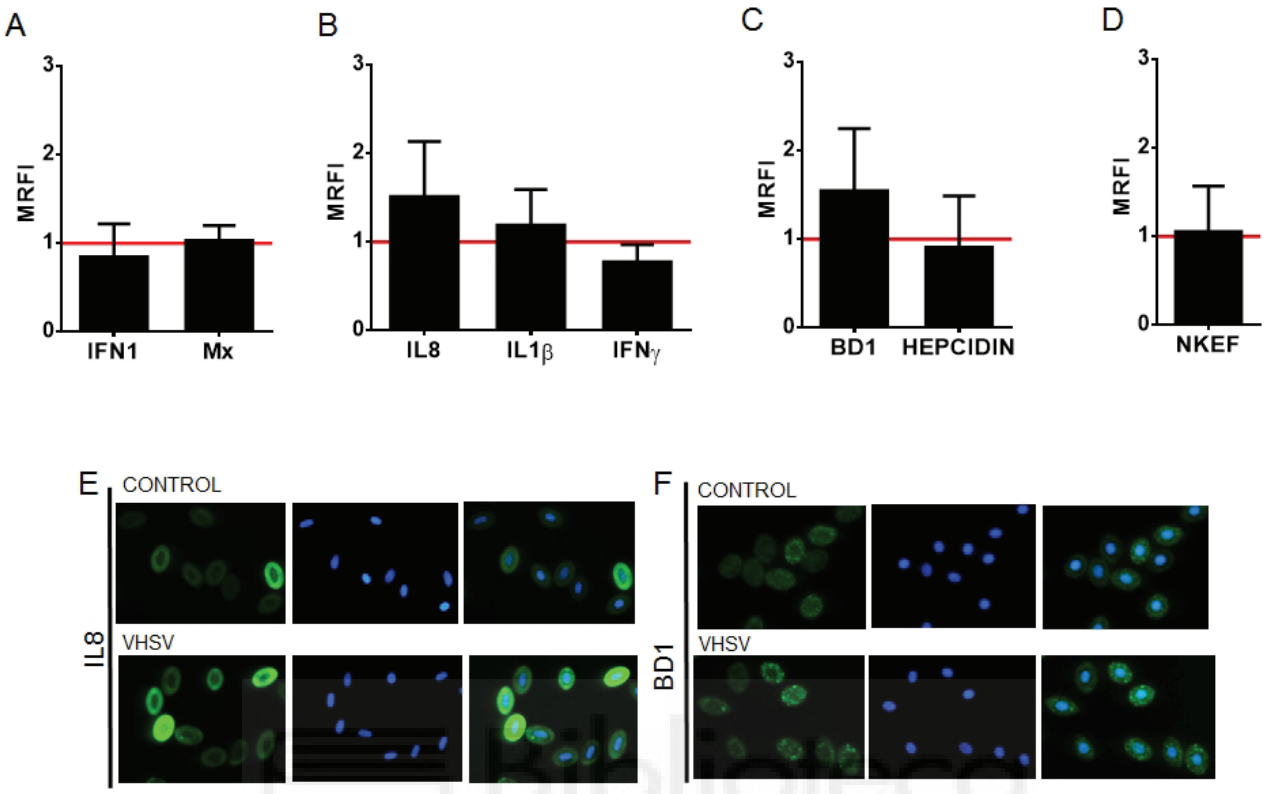


Figure 3. Immune protein responses of VHSV-exposed RBCs. Relative immune protein expression levels, (**A**) interferon pathway related proteins (IFN1 and Mx), (**B**) cytokines (IL8, IL1β and IFNγ), (**C**) antimicrobial peptides (BD1 and Hepcidin) and (**D**) antioxidant protein NKEF, measured by flow cytometry and calculated by the formula MRFI (Mean Relative Fluorescence Intensity) = fluorescence in VHSV-exposed RBCs / fluorescence in non-exposed RBCs, at multiplicity of infection (MOI) 1, 72 hours postexposure (hpe), at 14°C, relative to control cells (non-exposed, red line). Data is displayed as mean ± SD (n=5). Mann Whitney Test was performed for statistical analysis between the VHSV-exposed cells and control cells. Representative immunofluorescences of control and VHSV-exposed RBCs stained with anti-IL8 (IF representative of 44 images) (**E**) and anti-BD1 (IF representative of 46 images) (**F**) (FITC) and DAPI for nuclei stain.

Interferon crosstalk between RBCs and spleen stromal TSS cell line

Rainbow trout spleen is an active hematopoietic organ[50], and it is composed of various cell types, such as red blood cells, leukocytes and reticular or stromal cells[51]. It has been demonstrated that cytokines and soluble factors produced by stromal cells are required for rainbow trout blood cells development in spleen or head kidney[52]. In this regard, we wanted to evaluate the paracrine effects of the cytokines produced by VHSV stimulated RBCs over the stromal cell line from rainbow trout spleen, TSS[29]. For that, rainbow trout RBCs stimulated with VHSV UV-inactivated were co-cultured with TSS cell line, using a Transwell system to test whether a cross-stimulation mediated by soluble molecules was involved. Gene expression profiles for *ifn1*, and interferon stimulated genes (ISGs) *mx*, viral inducible gene *vig1*, and interleukin *il15* genes were examined for each cell line 24 hours post co-culture (Figure 4E). Linear regression analysis of RBCs *ifn1* gene expression with their respective *mx, vig1* and *il15* genes showed a significant correlation between *ifn1* and *vig1* and *il15*, but not with *mx* gene (Figure 4A). *ifn1* gene expression from RBCs and TSS cells also showed a significant correlation (Figure 4B). TSS cells showed significant correlation between *ifn1* and *mx*, *vig1* and *il15* (Figure 4C). The results demonstrated an IFN crosstalk between stimulated RBCs and TSS cells. Besides, this IFN crosstalk was also observed when RBCs were incubated with conditioned medium from RTG-2 cells previously treated with UV-inactivated VHSV, since we could observe an increment in *ifn1* and *mx* genes expression, in contrast to *ifn1* or *mx* downregulation when RBCs were directly exposed to VHSV (Figure 4D).

iTRAQ protein profile of VHSV-exposed RBCs

The iTRAQ data showed a total of 9246 MS/MS Spectra, 2639 unique peptides with peptide-level FDR<0.01 and 872 inferred proteins common in all samples. Significant up/down regulations between samples were determined by a log2FoldChange)>1 with a *q*-value<0.05. In total, 64 proteins were significantly up or down-regulated during VHSV exposure (Figure 5). Specifically, 59 proteins were downregulated and only 5 proteins were upregulated during VHSV exposure. Cytoscape functional annotation was used to investigate underlying biologically functional differences that may be related to VHSV exposure. The results showed four strongly represented networks of interest (mRNA stability, proteasome, viral process and cellular catabolic processes) (Figure 5 and Figure S6).

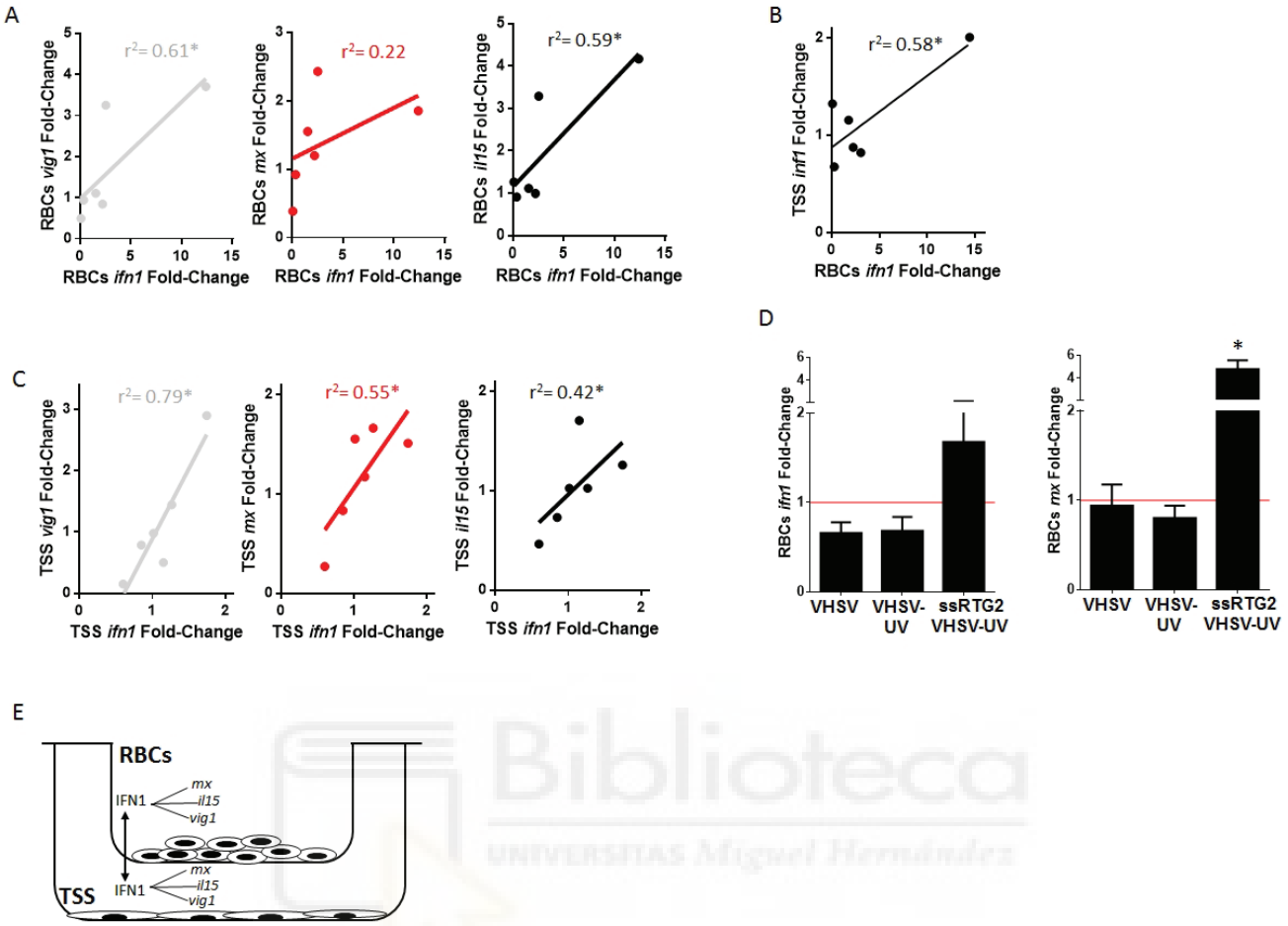


Figure 4. Crosstalk between rainbow trout RBCs and spleen stromal cell line TSS. Rainbow trout RBCs, control (non-exposed) and exposed to UV-inactivated VHSV, with multiplicity of infection (MOI) 1, were posteriorly co-cultured with TSS cell line, at 14°C, and *ifn1*, *mx*, *vig1* and *il15* gene expression profiles were quantified by RT-qPCR at 24 hours postexposure (hpe) for RBCs and TSS. (**A**) Linear regression between *ifn1* and interferon stimulated genes *vig1, mx*, and *il15* gene expression profiles in RBCs. (**B**) Linear regression between RBCs and TSS *ifn1* gene expression profile. (**C**) Linear regression between *ifn1* and *vig1*, *mx*, and *il15* gene expression profiles in TSS. Gene expression was normalized against eukaryotic 18S rRNA and relativized to control cells (red line) (fold-change). Data is displayed as a linear regression line, with individual dots, between indicated cell lines and expressed genes (r^2: coefficient of determination, asterisk denote statistical significance, *P*-value < 0.05) (n = 6). (**D**) Rainbow trout RBCs exposed to VHSV, UV-inactivated VHSV (VHSV-UV) (MOI 1, 14°C, 24h) or treated with conditioned medium from RTG-2 cells pre-treated with VHSV-UV, for 24h at 14°C. RBCs *ifn1* and *mx* gene expression profiles were quantified by RT-qPCR. Gene expression was normalized against eukaryotic 18S rRNA and relativized to control cells (RBCs incubated with conditioned medium from untreated RTG-2 cells, red line) (fold-change). Data represent the mean ± SD (n = 4). Kruskal-Wallis Test with Dunn´s Multiple Comparison post-hoc test was performed among all conditions. Asterisk denote significant differences with the indicated condition and control cells (*P*-value < 0.05). (**E**) Schematic representation of RBCs and TSS co-culture assay and analysis.

Among the 59 down-regulated proteins (Figure 6, Table S1), the top-score network was mRNA stability, being SNRPD3 (Small nuclear ribonucleoprotein D3 polypeptide) the most down-regulated protein with ~ -3 log2FoldChange. This protein is a core component of spliceosomal small nuclear ribonucleoproteins (snRNPs), the building blocks of the spliceosome, and therefore, it plays an important role in the splicing of cellular pre-mRNAs. Other proteins related to splicing processes were also highly downregulated (-2>log2FoldChange>-1), such as SRSF4 (Serine/arginine-rich splicing factor 4), which plays a role in alternative splice site selection during pre-mRNA splicing, RNPS1 (RNA binding protein S1, serine-rich domain), which is part of pre- and post-splicing multiprotein messenger ribonucleoprotein (mRNP) complexes. Apart from that, several heat shock chaperones were also downregulated (-2>log2FoldChange>-1), such as HSPA1L (Heat shock 70kDa protein 1-like) and HSPA5 (Heat shock 70kDa protein 5) both involved in the correct folding of proteins and degradation of misfolded proteins, and HSPA8 (Heat shock 70kDa protein 8), which may have a scaffolding role in the spliceosome assembly. Besides, another protein highly downregulated was NPEPL1 (Aminopeptidase-like 1), a novel protein which has been implicated in HIV replication[53].

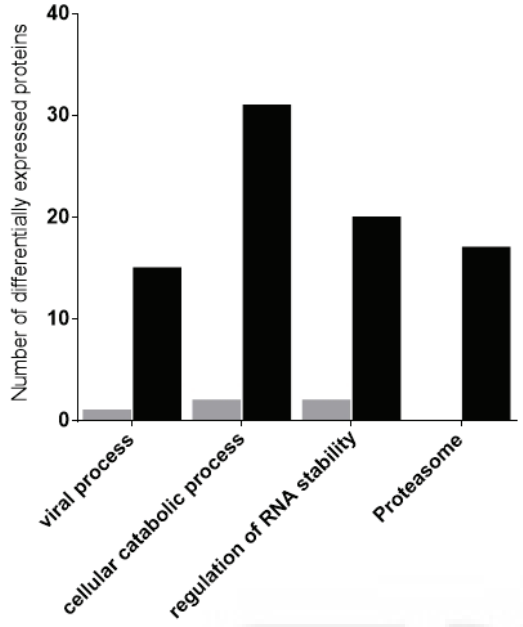


Figure 5. Gene ontology (GO) analysis of iTRAQ-based differentially expressed proteins in VHSV-exposed rainbow trout RBCs. RBCs were exposed to VHSV with a multiplicity of infection (MOI) of 1 at 14°C, and protein quantified at 72 hpe. Proteins were classified into five specific GO-Biological Process categories indicated in the x-axis. The y-axis indicates the number of proteins in each category. Grey bars indicate upregulated proteins and black bars down-regulated proteins.

On the other hand, among the five upregulated proteins (Figure 6, Table S1), BANF1 (Barrier to Autointegration factor 1) has been directly implicated in viral processes and plays fundamental role in nuclear assembly, chromatin organization and gene expression. Besides, HNRNPR (Heterogeneous nuclear ribonucleoprotein R) plays an important role in processing precursor mRNA in the nucleus, and SRSF1 (Serine/arginine-rich splicing factor 1) is also implicated in mRNA splicing, via spliceosome.

The 59 downregulated proteins were analyzed using STRING v10.5 (RRID:SCR_005223, http://string.embl.de/)[54] with a medium confidence score threshold of 0.4. An interactome network was built for these set of proteins to find out protein-protein interaction and predict functional associations. We found that proteins within spliceosome and proteasome networks interacted with each other as well as with their partners. We also found that 17 proteins were involved in viral process category and that most of them interacted with each other as well as with their partners (Figure 7).

Phosphorylation of eIF2α in VHSV-exposed RBCs

Since a global protein downregulation was observed in VHSV-exposed RBCs, we further investigated whether this phenomena could be due to the phosphorylation of the α-subunit of translational initiation factor 2 (eIF2α), a recognized key mechanism of global inhibition of translational initiation. For that, phosphorylation of eIF2α (eIF2α-P) was evaluated in VHSV-exposed RBCs compared to control cells by western blot (Figure 8A and B). The results showed a small upregulation of eIF2α-P in VHSV-exposed RBCs.

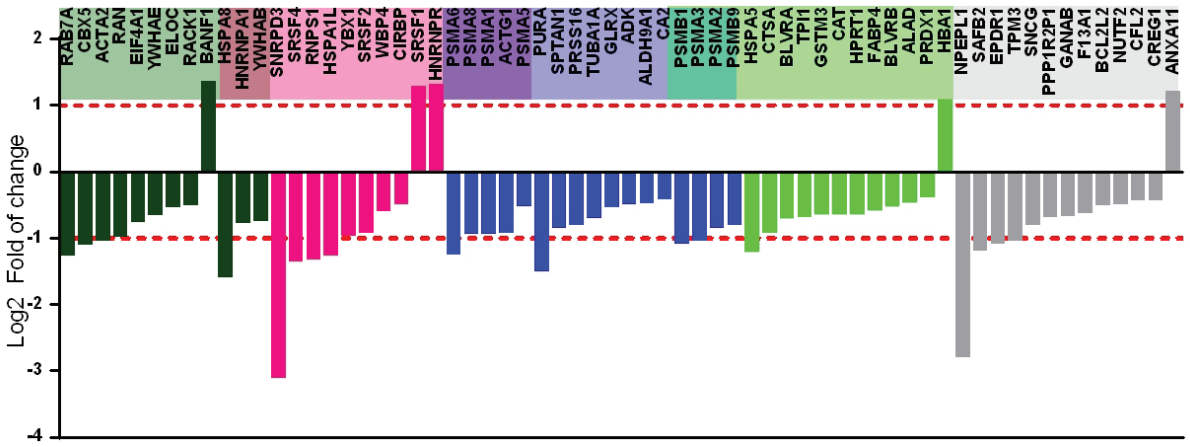


Figure 6. iTRAQ-based quantitative protein expression profile of VHSV-exposed rainbow trout RBCs. Bar plot of statistically significant differentially expressed proteins in VHSV-exposed RBCs (MOI 1, 14°C, 72 hpe) compared to control cells (non-exposed) (*P*-value < 0.05, FDR *q*-value < 0.05). Functional categories are labelled as follows: Blue = proteasome, pink = regulation of RNA stability, light green = cellular catabolic process, dark green= viral process, grey = proteins not associated to any function.

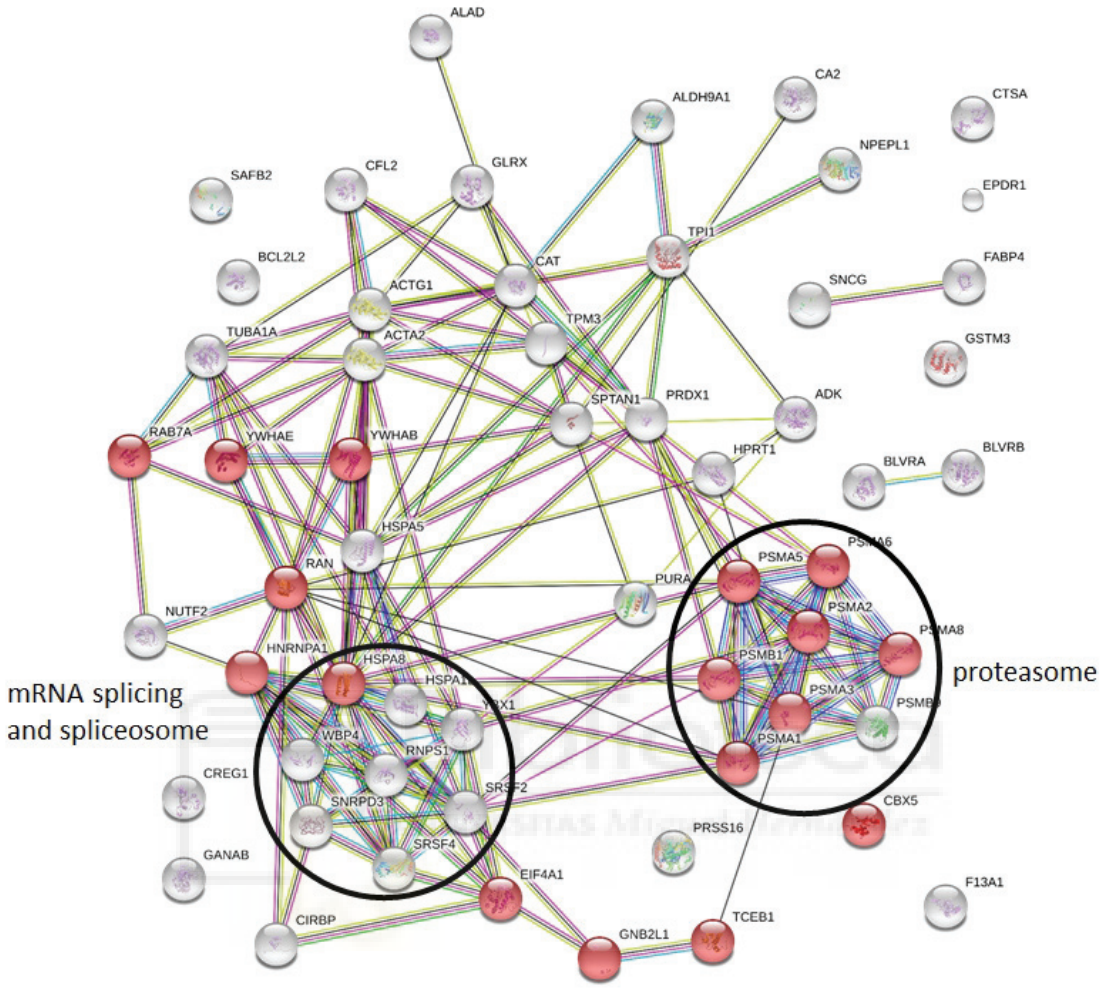


Figure 7. Constructed protein-protein interaction of differentially downregulated proteins (DDPs) predicted using STRING software. Nodes represent DDPs and edges the interactions between two proteins. The colour of the edge indicates the interaction score (edge score). Red nodes highlight DDPs functionally annotated in viral processes.

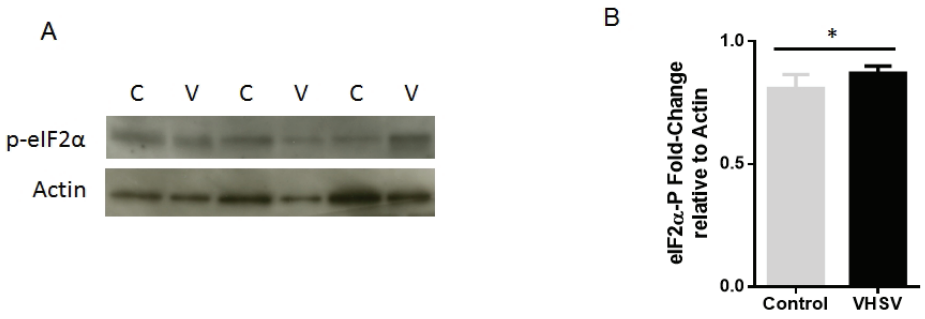


Figure 8. Phosphorylation of translation initiation factor eIF2α in VHSV-exposed rainbow trout RBCs. (**A**) Representative western blot of eIF2α phosphorylation (eIF2α-P) in VHSV-exposed (V) (MOI 1, 14°C, 72 hpe) and control RBCs (C) (non-exposed). (**B**) Bar plot of eIF2α-P protein content of stained bands estimated by densitometry, relative to α-Actin. Mann Whitney Test was performed for statistical analysis between VHSV-exposed cells and control cells. Asterisk denote statistically significant differences (*P*-value < 0.05).

Four eIF2α kinases have been identified to inhibit protein synthesis by phosphorylation of eIF2α: double-stranded RNA-dependent eIF2α kinase (PKR), mammalian orthologue of the yeast GCN2 protein kinase, endoplasmic reticulum (ER) resident kinase (PERK) and heme-regulated eIF2α kinase (HRI)[55]. HRI, which was first discovered in reticulocytes under conditions of iron and heme deficiencies[56,57], was later known to regulate the synthesis of both α- and β-globins in RBCs and erythroid cells by phosphorylation of eIF2α[58], and therefore inhibiting protein synthesis. Besides, heme is also known to regulate the transcription of globin genes through its binding to transcriptional factor Bach1[59]. Taking this fact into account, we explored RBCs *β-globin* gene expression during the course of VHSV exposure and the results showed that *β-globin* gene was downregulated after 6 hpe (Figure 9), therefore suggesting an activation/phosphorylation of HRI and consequent phosphorylation of eIF2α and protein inhibition.

Oxidative stress and antioxidant response in VHSV-exposed RBCs

Oxidative stress is known to be induced by viral infections, being one of the major pathogenic mechanisms by altering the balance of intracellular redox[60]. On the other hand, oxidative stress is known to activate HRI, which in turn phosphorylates eIF2α and inhibits protein translation. In order to evaluate the oxidative stress induced in VHSV-exposed RBCs as possible causative mechanism for the proteome downregulation found in our study, we examined, at 72 hpe, the ROS intracellular production by means of DCFDA (2′,7′-Dichlorofluorescin diacetate) fluorescence intensity. The results showed that VHSV-exposed RBCs significantly augmented DCFDA fluorescent intensity 72 hpe (Figure 10A), therefore VHSV halted infection in RBCs generated oxidative stress in rainbow trout RBCs. Besides, in order to evaluate the capability of RBCs to respond to oxidative stress, antioxidant response of VHSV-exposed RBCs was evaluated examining

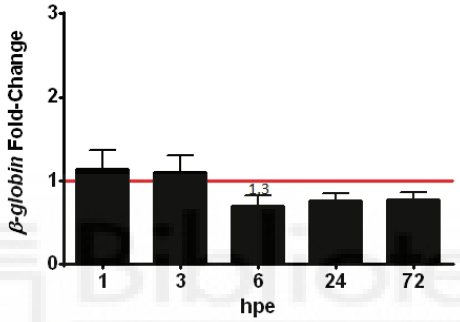


Figure 9. *β-globin* gene expression time-course in VHSV-exposed rainbow trout RBCs. RBCs were exposed to VHSV with a multiplicity of infection (MOI) of 1 at 14°C. Gene expression was quantified by RT-qPCR at time 0, 1, 3, 6, 24, 72 hours postexposure (hpe). Gene expression was normalized against eukaryotic 18S rRNA and relativized to control cells (non-exposed, time 0, red line) (fold-change). Data is displayed as mean ± SD (n = 3). Kruskal-Wallis Test with Dunn´s Multiple Comparison post-hoc test was performed among all conditions. Values denote pairwise significant differences with the value-indicated condition (*P*-value < 0.05).

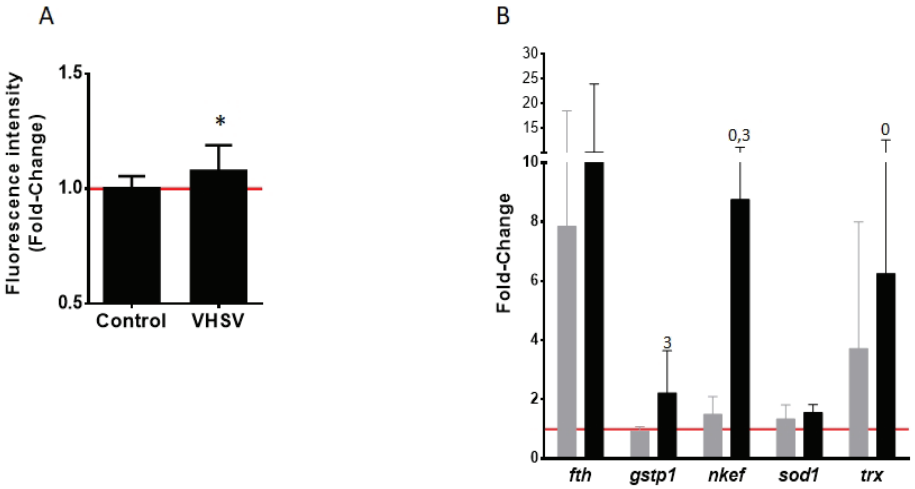


Figure 10. Effect of VHSV on ROS intracellular production and antioxidant enzymes gene expression in rainbow trout RBCs. RBCs were exposed to VHSV with a multiplicity of infection (MOI) of 1 at 14°C, (**A**) DCFDA (2′,7′-Dichlorofluorescin diacetate) fluorescence intensity of VHSV-exposed RBCs relative to control cells (non-exposed), 72 hours postexposure (hpe). (**B**) Antioxidant genes (*fth*: ferritin, *gstp1*: glutathione S-transferase P, *nkef*: natural killer enhancement factor-like protein, *sod1*: superoxide dismutase [Cu-Zn], *trx*: thioredoxin) gene expression quantified by RT-qPCR at 3 hpe (grey bars) and 72 hpe (black bars). Gene expression was normalized against eukaryotic 18S rRNA and relativized to control cells (time 0, red line) (fold-change). Data is displayed as mean ± SD (n = 3). Kruskal-Wallis Test with Dunn´s Multiple Comparison post-hoc test was performed among all conditions. Values denote pairwise significant differences (*P*-value < 0.05) with the value-indicated condition.

transcript levels of antioxidant genes *fth* (ferritin), *gstp1* (glutathione S-transferase P), *nkef* (natural killer enhancement factor-like protein), *sod1* (superoxide dismutase [Cu-Zn]) and *trx* (thioredoxin). The results depicted an increment in transcript levels of *fth*, *gstp1*, *nkef* and *trx* (Figure 10B) as the time of exposure increased from 3 to 72 hours, demonstrating the capability of rainbow trout RBCs to counteract oxidative stress.

Dataset 1. Excel file containing qPCR data. Each sheet contains the raw Ct values for the indicated figure numbers, organized by samples (rows) and genes (columns)

http://dx.doi.org/10.5256/f1000research.12985.d192872

Dataset 2. Excel file containing the virus titration data. Each sheet contains the virus titer (PFU/mL) results of the indicated figure number

http://dx.doi.org/10.5256/f1000research.12985.d182834

Dataset 3. Flow cytometry data. Each folder contains the Flow Cytometry Standard (.fcs) format files. Source data files are organized by figure number, and then by antibody, sample number and condition

http://dx.doi.org/10.5256/f1000research.12985.d192873

Dataset 4. Excel file containing the computed peptide spectrum match (PSM) raw data, and the spectra recovered in the iTRAQ 4-plex analysis

http://dx.doi.org/10.5256/f1000research.12985.d182836

Dataset 5. Excel file containing the iTRAQ 4-plex quantitative analysis raw data

http://dx.doi.org/10.5256/f1000research.12985.d182837

Dataset 6. Excel file containing the densitometry raw data of eIF2α-P and α-Actin western blots. Related uncropped blots are included

http://dx.doi.org/10.5256/f1000research.12985.d192879

Dataset 7. Excel file containing DCFDA absorbance raw data

http://dx.doi.org/10.5256/f1000research.12985.d182839

Discussion

Most viral infections release their progeny to the outside of the cells (productive infections). However, viral infections can be also non-productive in non-permissive cells (also called abortive). Viral abortive infections occur when a virus enters a host-cell, then some or all viral components are synthesized but finally no infective viruses are released[61]. This situation may result from an infection with defective viruses or because the host cell is non-permissive and inhibits replication of a particular virus. Our results are consistent with VHSV binding and internalization in rainbow trout RBCs. Internalization of VHSV occurs via fibronectin-integrin receptors in the host cell[12]. Integrin proteins expression has been found in human red blood cells[62], however, it is unknown whether non-mammalian nucleated RBCs express integrins. VHSV internalization is followed by viral genes transcription at early times of viral exposure and posterior quasi-inhibition inside rainbow trout RBCs. In this sense, rainbow trout RBCs could be classified as a non-permissive cell for VHSV replication, in contrast to other rainbow trout cells or tissues where VHSV is productive, such as RTG-2 cells[63,64], fin cells[65] or stroma[66]. Therefore, from our results, VHSV infection could be classified as halted in rainbow trout RBCs, since it enters the cell, but does not replicate at the levels comparable to the ~100-fold increase in titre of PRV and ISAV infections in salmon RBCs[5,7]. In fact, an apparent inhibition of the early viral genes transcription seemed to occur since N_{VHSV}: G_{VHSV} viral genes transcripts ratio was very low, and therefore did not follow the attenuation phenomenon found in rhabdoviruses[47]. However, strikingly, even though recovered VHSV titer in the RBCs supernatant was very low at 3 and 6 dpe, at 40 dpe almost the same virus titer could be recovered from RBCs supernatant (data not shown), suggesting an *ex vivo* persistence of VHSV inside RBCs.

In the literature, innate immune responses have been associated with viral abortive infections, including rhabdoviruses. Pham *et al.*[67] speculated that the cause of aborted VHSV infection in rainbow trout macrophage cell line (RTS-11) could be the constitutive expression and/or upregulation of *mx* genes. The abortive infection of snakehead fish vesiculovirus (SHVV) in zebrafish embryonic fibroblast cell line (ZF4) was associated with activation of Retinoic acid-Inducible Gene I (RIG-I)-like receptors and interferon pathway by viral replicative intermediates[68]. Similarly, in mammals, Pfefferkorn *et al.*[69] demonstrated that abortive viral infection of astrocytes by rabies virus (RABV) and vesicular stomatitis virus (VSV) triggered a pattern recognition receptor signaling which resulted in the secretion of IFN-β. On the other hand, it has been also described that alveolar macrophages are able to restrict respiratory syncytial virus (RSV) replication even in the absence of type I IFNs (IFN1)[70]. In this sense, VHSV halted infection in rainbow trout RBCs did not seem to be related to IFN1 or IFN1-inducible genes, since *inf1*, *mx* and *pkr* genes as well as Mx and IFN1 proteins appeared poorly modulated or downregulated during VHSV exposure, in contrary to the 8-fold increase in ISAV productive infection in salmon RBCs[7], the 50-fold increases in PRV productive infection in salmon RBCs[5] or the 50-fold increases in IPNV non-productive infection in rainbow trout RBCs[71]. Alternatively, high levels of constitutive Mx protein expression might have prevented its further increase in VHSV-exposed RBCs, like it is the case of the rainbow trout monocyte-macrophage RTS-11 cell line[72]. On the other hand, several cell mechanisms have been reported to suppress IFN1-mediated responses, which include downregulation of cell surface IFNα receptor (IFNAR) expression, induction of negative regulators (such as suppressor of cytokine signalling (SOCS) proteins and ubiquitin carboxy-terminal hydrolase 18 (USP18)), as part of a negative feedback loop to limit the extent and duration of IFN1 responses[73]. Separately, a putative antagonistic effect of VHSV virus on Mx induction has been previously

reported[74,75]. From our results, in VHSV-exposed RBCs, *mx* gene poor induction or slight downregulation could be probably supported by the existence of a VHSV antagonistic effect against RBCs IFN response. To further clarify whether a viral antagonistic effect or a feedback loop of IFN1 and/or IFN1-inducible genes induction is related to or responsible for aborting or halting viral infections in rainbow trout RBCs remains to be studied, and are part of our ongoing research.

Separately, although the IFN levels were low, our results demonstrated the paracrine IFN crosstalk between RBCs, stimulated with UV-inactivated VHSV, and spleen stromal TSS cell line. TSS cell line has been described to resemble the immune responses observed in cultures of head kidney macrophages[76]. Also, it has been demonstrated the ability of TSS to positively respond to conditioned supernatants from head kidney macrophage cultures exposed to poly I:C[76]. As well, after exposure to poly I:C, TSS produced a high upregulation of the Mx-1 gene[77]. Our results showed the correlated *ifn1* regulation in both cell lines, as well as the correlative regulation of interferon-inducible *mx* gene in TSS, the regulation of *il15*, an interleukin that can activate antiviral responses via an interferon-dependent mechanism[78], and the regulation of VHSV-inducible *vig1*, a gene induced by VHSV as well as by interferon[79]. Separately, we observed that conditioned medium from RTG-2 cells previously treated with UV-inactivated VHSV could induce an increment in *ifn1* and *mx* genes expression in RBCs, in contrast to *ifn1* or *mx* observed downregulation when RBCs were directly exposed to VHSV. Therefore, this crosstalk observations demonstrated the capacity of rainbow trout RBCs to exert a paracrine molecular antiviral communication with other cells with capacity to generate an immune response, as it is the case of the TSS cell line[77] or RTG-2 cells[15]. More extended research is need to further identify the molecules involved in this crosstalk.

On the other hand, other immune proteins, such as BD1, IL1β and IL8, known to be involved in antiviral immunity, which were upregulated in VHSV- exposed RBCs, appeared to be part of the antiviral immune response of rainbow trout RBCs and could be implicated in the halted viral replication inside RBCs.

To further investigate the mechanisms implicated in the immune response of rainbow trout RBCs to VHSV, the comprehensive analysis of differentially expressed proteins, obtained by means of iTRAQ proteome profiling, revealed the regulation of two typical mechanisms for viral subversive strategies: regulation of spliceosome, or splicing hijacking, and host-cell shut-off. However, even though these strategies usually lead to viral augmented replication and cell death, in the case of VHSV-exposed RBCs this is not observed. Therefore, how these strategies or another strategies contribute to halting viral replication yet remains elusive. Future research could be directed to investigate the role/implication of small nuclear ribonucleoprotein SNRPD3, aminopeptidase NPEPL1, serine/arginine-rich splicing factor SRSF1 and heterogeneous nuclear ribonucleoprotein HNRNPR, in the response of RBCs against VHSV replication, since these proteins were the more regulated ones and they have been shown to be implicated in HIV replication[53,80–82]).

On the other hand, inhibition of both host and viral translation has been shown during infection with the prototype rhabdovirus vesicular stomatitis virus (VSV)[83]. During VSV infection, there is a rapid inhibition of host mRNA translation early after infection, followed by a later inhibition of viral mRNA translation, which has been associated to eIF2α phosphorylation[84]. Our results showed a slight increment in eIF2α phosphorylation in VHSV-exposed RBCs, indicating that this mechanism could be implicated in the inhibition of VHSV replication in rainbow trout RBCs. In this context, HRI, heme-regulated eIF2α kinase, is one of the four kinases identified to inhibit protein synthesis by means of eIF2α phosphorylation. HRI is predominantly expressed in reticulocytes and erythroid precursors[56,57], and it is known to regulate the synthesis of both α- and β-globins in RBCs and erythroid cells by phosphorylation of eIF2α[58]. Moreover, heme, the prosthetic group of hemoglobin, is known to inhibit eIF2α and therefore the transcription of globin genes through its binding to transcriptional factor Bach1. From our results, a decrease in *β-globin* gene transcripts levels during the course of viral exposure, accompanied with the observed phosphorylation of eIF2α, could suggest a possible heme regulation mechanism of eIF2 pathway in response to VHSV exposure in rainbow trout RBCs. The mechanism by which heme is altered in rainbow trout RBCs during VHSV exposure remains to be investigated.

An interesting mechanism found in rainbow trout RBCs in response to VHSV was the implication of protective antioxidant enzymes genes *gstp1*, *nkef* and *trx* in the defense of RBCs against the induction of ROS after VHSV exposure, since as the course of virus exposure increased ROS slightly augmented in parallel to transcript levels of these enzymes. It is known that ROS plays an important role in cell signalling and immunomodulation among others[85,86] as well as performing antimicrobial actions against pathogens[87]. However, oxidative stress due to imbalance in the production/elimination of ROS can have cytotoxic effects[88]. ROS scavengers are the major defense against oxidative stress produced in the cells[88]. These systems are known to contribute not only to repair the oxidative damage maintaining redox homeostasis, but also to the overall response of the cell to ROS by acting as oxidative sensors in signal transduction pathways[89]. However, although it has been said that ROS production contributes to eliminate pathogens, nowadays it is becoming evident that viruses, bacteria, and protozoans ROS induction can also promote pathogen burden[90]. In this regard, in relation to the implication of antioxidants activity against viral replication, it has been also described that antioxidants can suppress virus-induced oxidative stress and reduce RNA virus production[91]. GSTP1, NKEF and TRX are known antioxidant enzymes with implication and up-regulation in RNA-virus infections[92] and rhabdovirus infections[24,93]. However, whether these enzymes may contribute to halting or reducing viral replication remains to be studied. On the other hand, mammals' RBCs have an extensive array of antioxidant enzymes to counteract

oxidative stress and maintain redox homeostasis and RBCs survival[94]. However, to our knowledge this is the first report that implicates nucleated RBCs ROS scavengers in the antiviral immune response. Separately, these antioxidant enzymes are known NF-κβ antioxidant targets in response to inflammation stimulus (reviewed in Morgan and Liu, 2011[89]) and ROS can be sometimes produced in response to cytokines. Since NF-κβ appeared slightly activated in VHSV-exposed RBCs (Figure S7A and B), it is suggested that the cytokine response generated after VHSV exposure in rainbow trout RBCs would induce ROS production, and in turn this would modulate the NF-κβ response and NF-κβ target genes could attenuate ROS to promote RBCs survival. Apart from the observation of NF-κβ translocation to the nucleus in some of the RBCs, it is noteworthy that it is always accompanied by an increase in the protein levels of the p65 NF-κβ subunit in the cytoplasm. This phenomenon has been also observed in human foreskin fibroblasts during HCMV infection, where an increase in p65 mRNA levels correlated with the sustained increase in NF-kB activity during the course of infection[95]. Another fish rhabdovirus, the SVCV, has been reported to induce accumulation of ROS accompanied by the up-regulation of Nrf2 and its downstream genes (i.e. Heme Oxygenase-1 and thrioredoxin). The overexpression of Nrf2 has been also reported to significantly suppress either entry or replication of several viruses (reviewed in 96), and Shao *et al.*[96] also demonstrated that the activation of Nrf2 repressed the replication of SVCV. Therefore, future research could be directed to investigate the implication of the Nrf2 pathway in inhibiting VHSV replication in rainbow trout RBCs.

In summary, this study unveils previously unobserved but important mechanisms for fish nucleated RBCs in the contribution to the defense against a viral aggression not involving RBCs as targets. To our knowledge, this is the first report that implicates fish RBCs as antiviral mediators against viruses targeting other tissues or cells. The recognition of body circulating viruses and the subsequent generation of immune defenses by RBCs may largely contribute to fish survival, given the large volume of RBCs and its rapid and wide distribution to the whole body. We are further investigating if similar mechanisms operate *in vivo*, the molecules that trigger such immune responses or the cellular factors implicated in the interaction with the virus.

Data availability

F1000Research: Dataset 1. Excel file containing qPCR data. Each sheet contains the raw Ct values for the indicated figure numbers, organized by samples (rows) and genes (columns), 10.5256/f1000research.12985.d192872[99]

F1000Research: Dataset 2. Excel file containing the virus titration data. Each sheet contains the virus titer (PFU/mL) results of the indicated figure number, 10.5256/f1000research.12985.d182834[100]

F1000Research: Dataset 3. Flow cytometry data. Each folder contains the Flow Cytometry Standard (.fcs) format files. Source data files are organized by figure number, and then by antibody, sample number and condition, 10.5256/f1000research.12985.d192873[101]

F1000Research: Dataset 4. Excel file containing the computed peptide spectrum match (PSM) raw data, and the spectra recovered in the iTRAQ 4-plex analysis 10.5256/f1000research.12985.d182836[102]

F1000Research: Dataset 5. Excel file containing the iTRAQ 4-plex quantitative analysis raw data 10.5256/f1000research.12985.d182837[103]

F1000Research: Dataset 6. Excel file containing the densitometry raw data of eIF2α-P and α-Actin western blots. Related uncropped blots are included, 10.5256/f1000research.12985.d192879[104]

F1000Research: Dataset 7. Excel file containing DCFDA absorbance raw data, 10.5256/f1000research.12985.d182839[105]

Competing interests

No competing interests were disclosed.

Grant information

This work was supported by the European Research Council (ERC starting grant 2014 GA639249).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

Special acknowledgment is due to the co-author Dr. Amparo Estepa, passed away, who largely contributed to the initial ideas and enthusiasm behind this work. Thanks are due also to Beatriz Bonmati, Remedios Torres and Efren Lucas for their technical assistance. As well, thanks are due to Prof. Henny Gevers and Dr. Craig Plaisance for helping with English editing.

Supplementary material

Figure S1. Validation of the polyclonal antibody against *Onchorhynchus mykiss* BD1. Western blotting using the antibody developed in rabbit that recognizes the synthetic BD1 (41aa), in samples from *O. mykiss*: head kidney, muscle, untreated red blood cells (RBCs), VHSV-exposed RBCs, and the synthetic BD1.

Click here to access the data.

Figure S2. Validation of the polyclonal antibodies against *Onchorhynchus mykiss* IFNγ and IFN1. Left Panel: Prediction of three-dimensional structure of the molecules (by Phyre2). Right panel: Western blotting using the respective antibody. A: Validation of anti-IFNγ antibody. On the left 3D predicted structure of the recombinant protein rIFNγ used for the immunization. On the right the antibody

developed in mouse recognizes the rIFNγ. B: Validation of anti-IFN1 antibody, in red: DWIQHHFGHLSAEYLSQ (aa 25-42), the synthetic epitope sequence of IFN1 used for the immunization. On the right, the anti-epitope antibody recognizes the whole molecule expressed in a RT Gill cell line.

Click here to access the data.

Figure S3. Kinetics of VHSV RNA synthesis in RBCs exposed to the virus. RBCs were exposed to VHSV with a multiplicity of infection (MOI) of 1 at 14°C. Gene expression was quantified by RT-qPCR at time 0, 1, 3, and 72 hours postexposure (hpe). (**A**) Random hexamer-primed RT reaction (total RNA) (black bars) and oligo dT primed-RT reaction (mRNA) (dashed bars). Gene expression was normalized against endogenous *ef1α* gene expression and relativized to control cells (non-exposed, time 0, red line) (fold-change). Data is displayed as mean ± SD (n = 3).

Click here to access the data.

Figure S4. Time course of interferon-inducible antiviral genes *mx* and *pkr* in untreated rainbow trout red blood cells (RBCs). *mx1-3* (grey) and *pkr* (black) genes expression was quantified at time 0, 3, 6, 24, 72 hours post-ficoll. Gene expression was normalized against eukaryotic 18S rRNA and relativized to control cells (time 0, red line) (fold of increase). Data is displayed as mean ± SD (n = 2). Kruskal-Wallis Test with Dunn´s Multiple Comparison post-hoc test was performed among all conditions.

Click here to access the data.

Figure S5. Representative flow cytometry dotplots of immune protein responses of VHSV-exposed RBCs. RBCs were exposed to VHSV at MOI 1, at 14°C, and stained with anti-BD1 (**A**) and anti-IL8 (**B**), 72 hpe. Control (non-exposed) and VHSV-exposed RBCs dotplots are shown. Y axis represents side scattering (SSC-A) and X axis FITC fluorescence intensity (FITC-A).

Click here to access the data.

Figure S6. Pathway network of significantly over-represented GO-terms in VHSV-exposed rainbow trout RBCs protein iTRAQ profiling. Big nodes represent significantly differentially expressed (down-regulated) proteins that have similar function; edges represent pairwise interactions; small nodes represent the proteins associated to each function. Functional groups are labelled as follows: Blue = proteasome, pink = regulation of RNA stability, light green = cellular catabolic process, dark green= viral process, grey = proteins not associated to any function. A list of all over-represented terms is provided in Table S1.

Click here to access the data.

Figure S7. NF-kß p65 protein labelling in VHSV-exposed RBCs. (**A**) Protein expression levels calculated by the formula MRFI (Mean Relative Fluorescence Intensity) = fluorescence in VHSV-exposed RBCs / fluorescence in non-exposed RBCs, at MOI 1, 10 and 100, 72hpe, at 14°C, relative to control cells (non-exposed, red line). Data represent mean ± SD (n=3). Mann Whitney Test was performed for statistical analysis between the VHSV-exposed cells and control cells. (**B**) Representative immunofluorescences of control and VHSV-exposed RBCs stained with anti-NF-kß (FITC) and DAPI for nuclei (IF representative of 20 images).

Click here to access the data.

Table S1. List of significantly over-represented GO-terms in VHSV-exposed rainbow trout RBCs protein iTRAQ profiling.

Click here to access the data.

References

1. Magadan S, Sunyer OJ, Boudinot P: **Unique Features of Fish Immune Repertoires: Particularities of Adaptive Immunity Within the Largest Group of Vertebrates.** *Results Probl Cell Differ.* 2015; **57**: 235–64.
PubMed Abstract | Publisher Full Text | Free Full Text
2. Morera D, Roher N, Ribas L, *et al.*: **RNA-Seq reveals an integrated immune response in nucleated erythrocytes.** *PLoS One.* 2011; **6**(10): e26998.
PubMed Abstract | Publisher Full Text | Free Full Text
3. Morera D, Mackenzie SA: **Is there a direct role for erythrocytes in the immune response?** *Vet Res.* 2011; **42**(1): 89.
PubMed Abstract | Publisher Full Text | Free Full Text
4. Passantino L, Altamura M, Cianciotta A, *et al.*: **Maturation of fish erythrocytes coincides with changes in their morphology, enhanced ability to interact with *Candida albicans* and release of cytokine-like factors active upon autologous macrophages.** *Immunopharmacol Immunotoxicol.* 2004; **26**(4): 573–85.
PubMed Abstract | Publisher Full Text
5. Wessel Ø, Olsen CM, Rimstad E, *et al.*: **Piscine orthoreovirus (PRV) replicates in Atlantic salmon (*Salmo salar* L.) erythrocytes *ex vivo*.** *Vet Res.* 2015; **46**: 26.
PubMed Abstract | Publisher Full Text | Free Full Text
6. Dahle MK, Wessel Ø, Timmerhaus G, *et al.*: **Transcriptome analyses of Atlantic salmon (*Salmo salar* L.) erythrocytes infected with piscine orthoreovirus (PRV).** *Fish Shellfish Immunol.* 2015; **45**(2): 780–90.
PubMed Abstract | Publisher Full Text
7. Workenhe ST, Kibenge MJ, Wright GM, *et al.*: **Infectious salmon anaemia virus replication and induction of alpha interferon in Atlantic salmon erythrocytes.** *Virol J.* 2008; **5**: 36.
PubMed Abstract | Publisher Full Text | Free Full Text
8. Dhar AK, Manna SK, Thomas Allnutt FC: **Viral vaccines for farmed finfish.** *Virusdisease.* 2014; **25**(1): 1–17.
PubMed Abstract | Publisher Full Text | Free Full Text
9. Schütze H, Mundt E, Mettenleiter TC: **Complete genomic sequence of viral**

hemorrhagic septicemia virus, a fish rhabdovirus. *Virus Genes.* 1999; **19**(1): 59–65.
PubMed Abstract | Publisher Full Text

10. Bang Jensen B, Kristoffersen AB: **Risk factors for outbreaks of infectious pancreatic necrosis (IPN) and associated mortality in Norwegian salmonid farming.** *Dis Aquat Organ.* 2015; **114**(3): 177–87.
PubMed Abstract | Publisher Full Text

11. Kim R, Faisal M: **Emergence and resurgence of the viral hemorrhagic septicemia virus (*Novirhabdovirus*, *Rhabdoviridae*, *Mononegavirales*).** *J Adv Res.* 2011; **2**(1): 9–23.
Publisher Full Text

12. Bearzotti M, Delmas B, Lamoureux A, *et al.*: **Fish rhabdovirus cell entry is mediated by fibronectin.** *J Virol.* 1999; **73**(9): 7703–7709.
PubMed Abstract | Free Full Text

13. Daher KA, Selsted ME, Lehrer RI: **Direct inactivation of viruses by human granulocyte defensins.** *J Virol.* 1986; **60**(3): 1068–74.
PubMed Abstract | Free Full Text

14. Falco A, Mas V, Tafalla C, *et al.*: **Dual antiviral activity of human alpha-defensin-1 against viral haemorrhagic septicaemia rhabdovirus (VHSV): inactivation of virus particles and induction of a type I interferon-related response.** *Antiviral Res.* 2007; **76**(2): 111–23.
PubMed Abstract | Publisher Full Text

15. Chico V, Martinez-Lopez A, Ortega-Villaizan M, *et al.*: **Pepscan mapping of viral hemorrhagic septicemia virus glycoprotein G major lineal determinants implicated in triggering host cell antiviral responses mediated by type I interferon.** *J Virol.* 2010; **84**(14): 7140–50.
PubMed Abstract | Publisher Full Text | Free Full Text

16. Martinez-Lopez A, Garcia-Valtanen P, Ortega-Villaizan M, *et al.*: **VHSV G glycoprotein major determinants implicated in triggering the host type I IFN antiviral response as DNA vaccine molecular adjuvants.** *Vaccine.* 2014; **32**(45): 6012–9.
PubMed Abstract | Publisher Full Text

17. Ortega-Villaizan M, Chico V, Martinez-Lopez A, *et al.*: ***In vitro* analysis of the factors contributing to the antiviral state induced by a plasmid encoding the viral haemorrhagic septicaemia virus glycoprotein G in transfected trout cells.** *Vaccine.* 2011; **29**(4): 737–43.
PubMed Abstract | Publisher Full Text

18. Rojas V, Camus-Guerra H, Guzmán F, *et al.*: **Pro-inflammatory caspase-1 activation during the immune response in cells from rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) challenged with pathogen-associated molecular patterns.** *J Fish Dis.* 2015; **38**(11): 993–1003.
PubMed Abstract | Publisher Full Text

19. Schmitt P, Wacyk J, Morales-Lange B, *et al.*: **Immunomodulatory effect of cathelicidins in response to a β-glucan in intestinal epithelial cells from rainbow trout.** *Dev Comp Immunol.* 2015; **51**(1): 160–9.
PubMed Abstract | Publisher Full Text

20. Santana P, Palacios C, Narváez E, *et al.*: **Anti-peptide antibodies: A tool for detecting IL-8 in salmonids.** *Electron J Biotechnol.* 2012; **15**(5).
Publisher Full Text

21. Rojas V, Morales-Lange B, Guzmán F, *et al.*: **Immunological strategy for detecting the pro-inflammatory cytokine TNF-alpha in salmonids.** *Electron J Biotechnol.* 2012; **15**(5).
Publisher Full Text

22. Santana PA, Álvarez CA, Guzmán F, *et al.*: **Development of a sandwich ELISA for quantifying hepcidin in Rainbow trout.** *Fish Shellfish Immunol.* 2013; **35**(3): 748–55.
PubMed Abstract | Publisher Full Text

23. Bethke J, Rojas V, Berendsen J, *et al.*: **Development of a new antibody for detecting natural killer enhancing factor (NKEF)-like protein in infected salmonids.** *J Fish Dis.* 2012; **35**(5): 379–88.
PubMed Abstract | Publisher Full Text

24. Ordas MC, Cuesta A, Mercado L, *et al.*: **Viral hemorrhagic septicaemia virus (VHSV) up-regulates the cytotoxic activity and the perforin/granzyme pathway in the rainbow trout RTS11 cell line.** *Fish Shellfish Immunol.* 2011; **31**(2): 252–9.
PubMed Abstract | Publisher Full Text

25. Encinas P, Garcia-Valtanen P, Chinchilla B, *et al.*: **Identification of multipath genes differentially expressed in pathway-targeted microarrays in zebrafish infected and surviving spring viremia carp virus (SVCV) suggest preventive drug candidates.** *PLoS One.* 2013; **8**(9): e73553.
PubMed Abstract | Publisher Full Text | Free Full Text

26. García-Valtanen P, Martinez-Lopez A, Ortega-Villaizan M, *et al.*: **In addition to its antiviral and immunomodulatory properties, the zebrafish β-defensin 2 (zfBD2) is a potent viral DNA vaccine molecular adjuvant.** *Antiviral Res.* 2014; **101**: 136–47.
PubMed Abstract | Publisher Full Text

27. Abós B, Castro R, González Granja A, *et al.*: **Early activation of teleost B cells in response to rhabdovirus infection.** *J Virol.* 2015; **89**(3): 1768–80.
PubMed Abstract | Publisher Full Text | Free Full Text

28. Sanz F, Basurco B, Babin M, *et al.*: **Monoclonal antibodies against the structural proteins of viral haemorrhagic septicaemia virus isolates.** *J Fish Dis.* 1993; **16**(1): 53–63.
Publisher Full Text

29. Carracedo B: **Caracterización de cultivos celulares angiogénicos obtenidos de órganos linfoides de trucha arco iris, Oncorhynchus mykiss**. Ph.D. thesis: University of León. Spain. 2003.

30. Winton J, Batts W, deKinkelin P, *et al.*: **Current lineages of the *epithelioma papulosum cyprini* (EPC) cell line are contaminated with fathead minnow, *Pimephales promelas*, cells.** *J Fish Dis.* 2010; **33**(8): 701–4.
PubMed Abstract | Publisher Full Text

31. LeBerre M, De Kinkelin P, Metzger A: **Identification sérologique des rhabdovirus des salmonidés.** *Bull Off Int Epizoot.* 1977; **87**: 391–3.
Reference Source

32. Basurco B, Coll JM: **Spanish isolates and reference strains of viral *haemorrhagic septicaemia* virus show similar protein size patterns.** *Bull Eur Ass Fish Pathol.* 1989; **9**(4): 92–5.
Reference Source

33. Mas V, Pérez L, Encinar JA, *et al.*: **Salmonid viral haemorrhagic septicaemia virus: fusion-related enhancement of virus infectivity by peptides derived from viral glycoprotein G or a combinatorial library.** *J Gen Virol.* 2002; **83**(Pt 11): 2671–81.
PubMed Abstract | Publisher Full Text

34. García-Valtanen P, Ortega-Villaizán Mdel M, Martínez-López A, *et al.*: **Autophagy-inducing peptides from mammalian VSV and fish VHSV rhabdoviral G glycoproteins (G) as models for the development of new therapeutic molecules.** *Autophagy.* 2014; **10**(9): 1666–80.
PubMed Abstract | Publisher Full Text | Free Full Text

35. Chico V, Gomez N, Estepa A, *et al.*: **Rapid detection and quantitation of viral hemorrhagic septicemia virus in experimentally challenged rainbow trout by real-time RT-PCR.** *J Virol Methods.* 2006; **132**(1–2): 154–9.
PubMed Abstract | Publisher Full Text

36. Marroqui L, Estepa A, Perez L: **Assessment of the inhibitory effect of ribavirin on the rainbow trout rhabdovirus VHSV by real-time reverse-transcription PCR.** *Vet Microbiol.* 2007; **122**(1–2): 52–60.
PubMed Abstract | Publisher Full Text

37. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_{T}$ Method.** *Methods.* 2001; **25**(4): 402–8.
PubMed Abstract | Publisher Full Text

38. Walpurgis K, Kohler M, Thomas A, *et al.*: **Validated hemoglobin-depletion approach for red blood cell lysate proteome analysis by means of 2D PAGE and Orbitrap MS.** *Electrophoresis.* 2012; **33**(16): 2537–45.
PubMed Abstract | Publisher Full Text

39. Geer LY, Markey SP, Kowalak JA, *et al.*: **Open mass spectrometry search algorithm.** *J Proteome Res.* 2004; **3**(5): 958–64.
PubMed Abstract | Publisher Full Text

40. Craig R, Beavis RC: **TANDEM: matching proteins with tandem mass spectra.** *Bioinformatics.* 2004; **20**(9): 1466–7.
PubMed Abstract | Publisher Full Text

41. Tabb DL, Fernando CG, Chambers MC: **MyriMatch: highly accurate tandem mass spectral peptide identification by multivariate hypergeometric analysis.** *J Proteome Res.* 2007; **6**(2): 654–61.
PubMed Abstract | Publisher Full Text | Free Full Text

42. Ramos-Fernández A, Paradela A, Navajas R, *et al.*: **Generalized method for probability-based peptide and protein identification from tandem mass spectrometry data and sequence database searching.** *Mol Cell Proteomics.* 2008; **7**(9): 1748–54.
PubMed Abstract | Publisher Full Text | Free Full Text

43. Lopez-Serra P, Marcilla M, Villanueva A, *et al.*: **A *DERL3*-associated defect in the degradation of SLC2A1 mediates the Warburg effect.** *Nat Commun.* 2014; **5**: 3608.
PubMed Abstract | Publisher Full Text | Free Full Text

44. Bindea G, Mlecnik B, Hackl H, *et al.*: **ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks.** *Bioinformatics.* 2009; **25**(8): 1091–3.
PubMed Abstract | Publisher Full Text | Free Full Text

45. Bindea G, Galon J, Mlecnik B: **CluePedia Cytoscape plugin: pathway insights using integrated experimental and *in silico* data.** *Bioinformatics.* 2013; **29**(5): 661–3.
PubMed Abstract | Publisher Full Text | Free Full Text

46. Shannon P, Markiel A, Ozier O, *et al.*: **Cytoscape: a software environment for integrated models of biomolecular interaction networks.** *Genome Res.* 2003; **13**(11): 2498–504.
PubMed Abstract | Publisher Full Text | Free Full Text

47. Banerjee AK: **Transcription and replication of rhabdoviruses.** *Microbiol Rev.* 1987; **51**(1): 66–87.
PubMed Abstract | Free Full Text

48. Mas V, Rocha A, Perez L, *et al.*: **Reversible inhibition of spreading of *in vitro* infection and imbalance of viral protein accumulation at low pH in viral hemorrhagic septicemia rhabdovirus, a salmonid rhabdovirus.** *J Virol.* 2004; **78**(4): 1936–44.
PubMed Abstract | Publisher Full Text | Free Full Text

49. Schloemer RH, Wagner RR: **Cellular adsorption function of the sialoglycoprotein of vesicular stomatitis virus and its neuraminic acid.** *J Virol.* 1975; **15**(4): 882–93.
PubMed Abstract | Free Full Text

50. Catton WT: **Blood cell formation in certain teleost fishes.** *Blood.* 1951; **6**(1):

39–60.
PubMed Abstract

51. Fänge R, Nilsson S: **The fish spleen: structure and function.** *Experientia.* 1985; **41**(2): 152–8.
PubMed Abstract | Publisher Full Text

52. Ganassin RC, Bols NC: **A stromal cell line from rainbow trout spleen, RTS34ST, that supports the growth of rainbow trout macrophages and produces conditioned medium with mitogenic effects on leukocytes.** *In Vitro Cell Dev Biol Anim.* 1999; **35**(2): 80–6.
PubMed Abstract | Publisher Full Text

53. Liu L, Oliveira NM, Cheney KM, *et al.*: **A whole genome screen for HIV restriction factors.** *Retrovirology.* 2011; **8**: 94.
PubMed Abstract | Publisher Full Text | Free Full Text

54. Szklarczyk D, Franceschini A, Wyder S, *et al.*: **STRING v10: protein-protein interaction networks, integrated over the tree of life.** *Nucleic Acids Res.* 2015; **43**(Database issue): D447–52.
PubMed Abstract | Publisher Full Text | Free Full Text

55. Han AP, Yu C, Lu L, *et al.*: **Heme-regulated eIF2alpha kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency.** *EMBO J.* 2001; **20**(23): 6909–18.
PubMed Abstract | Publisher Full Text | Free Full Text

56. Grayzel AI, Hörchner P, London IM: **The stimulation of globin synthesis by heme.** *Proc Natl Acad Sci U S A.* 1966; **55**(3): 650–5.
PubMed Abstract | Publisher Full Text | Free Full Text

57. Legon S, Jackson RJ, Hunt T: **Control of protein synthesis in reticulocyte lysates by haemin.** *Nat New Biol.* 1973; **241**(109): 150–2.
PubMed Abstract | Publisher Full Text

58. Chen JJ: **Regulation of protein synthesis by the heme-regulated eIF2alpha kinase: relevance to anemias.** *Blood.* 2007; **109**(7): 2693–9.
PubMed Abstract | Publisher Full Text | Free Full Text

59. Tahara T, Sun J, Igarashi K, *et al.*: **Heme-dependent up-regulation of the alpha-globin gene expression by transcriptional repressor Bach1 in erythroid cells.** *Biochem Biophys Res Commun.* 2004; **324**(1): 77–85.
PubMed Abstract | Publisher Full Text

60. Camini FC, da Silva Caetano CC, Almeida LT, *et al.*: **Implications of oxidative stress on viral pathogenesis.** *Arch Virol.* 2017; **162**(4): 907–17.
PubMed Abstract | Publisher Full Text

61. Boukhvalova MS, Prince GA, Blanco JC: **Respiratory syncytial virus infects and abortively replicates in the lungs in spite of preexisting immunity.** *J Virol.* 2007; **81**(17): 9443–50.
PubMed Abstract | Publisher Full Text | Free Full Text

62. Gautier EF, Ducamp S, Leduc M, *et al.*: **Comprehensive Proteomic Analysis of Human Erythropoiesis.** *Cell Rep.* 2016; **16**(5): 1470–84.
PubMed Abstract | Publisher Full Text | Free Full Text

63. Cano I, Collet B, Pereira C, *et al.*: ***In vivo* virulence of viral haemorrhagic septicaemia virus (VHSV) in rainbow trout Oncorhynchus mykiss correlates inversely with *in vitro* Mx gene expression.** *Vet Microbiol.* 2016; **187**: 31–40.
PubMed Abstract | Publisher Full Text

64. Lorenzen E, Carstensen B, Olesen NJ: **Inter-laboratory comparison of cell lines for susceptibility to three viruses: VHSV, IHNV and IPNV.** *Dis Aquat Organ.* 1999; **37**(2): 81–8.
PubMed Abstract | Publisher Full Text

65. Estepa A, Frías D, Coll JM: ***In vitro* susceptibility of rainbow trout fin cells to viral haemorrhagic septicaemia virus.** *Dis Aquat Organ.* 1993; **15**: 35–9.
Publisher Full Text

66. Diago ML, Estepa A, López-Fierro P, *et al.*: **The *in vitro* infection of the hematopoietic stroma of trout kidney by hemorrhagic septicemia rhabdovirus.** *Viral Immunol.* 1993; **6**(3): 185–91.
PubMed Abstract | Publisher Full Text

67. Pham PH, Lumsden JS, Tafalla C, *et al.*: **Differential effects of viral hemorrhagic septicaemia virus (VHSV) genotypes IVa and IVb on gill epithelial and spleen macrophage cell lines from rainbow trout (*Oncorhynchus mykiss*).** *Fish Shellfish Immunol.* 2013; **34**(2): 632–40.
PubMed Abstract | Publisher Full Text

68. Wang W, Asim M, Yi L, *et al.*: **Abortive infection of snakehead fish vesiculovirus in ZF4 cells was associated with the RLRs pathway activation by viral replicative intermediates.** *Int J Mol Sci.* 2015; **16**(3): 6235–50.
PubMed Abstract | Publisher Full Text | Free Full Text

69. Pfefferkorn C, Kallfass C, Lienenklaus S, *et al.*: **Abortively Infected Astrocytes Appear To Represent the Main Source of Interferon Beta in the Virus-Infected Brain.** *J Virol.* 2016; **90**(4): 2031–8.
PubMed Abstract | Publisher Full Text | Free Full Text

70. Makris S, Bajorek M, Culley FJ, *et al.*: **Alveolar Macrophages Can Control Respiratory Syncytial Virus Infection in the Absence of Type I Interferons.** *J Innate Immun.* 2016; **8**(5): 452–63.
PubMed Abstract | Publisher Full Text | Free Full Text

71. Nombela I, Carrion A, Puente-Marin S, *et al.*: **Infectious pancreatic necrosis virus triggers antiviral immune response in rainbow trout red blood cells, despite not being infective [version 2; referees: 2 approved].** *F1000Res.* 2017; **6**: 1968.
PubMed Abstract | Publisher Full Text | Free Full Text

72. Tafalla C, Sanchez E, Lorenzen N, *et al.*: **Effects of viral hemorrhagic septicemia virus (VHSV) on the rainbow trout (*Oncorhynchus mykiss*) monocyte cell line RTS-11.** *Mol Immunol.* 2008; **45**(5): 1439–48.
PubMed Abstract | Publisher Full Text

73. Ivashkiv LB, Donlin LT: **Regulation of type I interferon responses.** *Nat Rev Immunol.* 2014; **14**(1): 36–49.
PubMed Abstract | Publisher Full Text | Free Full Text

74. Alvarez-Torres D, Garcia-Rosado E, Fernandez-Trujillo MA, *et al.*: **Antiviral specificity of the *Solea senegalensis* Mx protein constitutively expressed in CHSE-214 cells.** *Mar Biotechnol (NY).* 2013; **15**(2): 125–32.
PubMed Abstract | Publisher Full Text

75. Collet B, Boudinot P, Benmansour A, *et al.*: **An Mx1 promoter-reporter system to study interferon pathways in rainbow trout.** *Dev Comp Immunol.* 2004; **28**(7–8): 793–801.
PubMed Abstract | Publisher Full Text

76. Fierro-Castro C, Barrioluengo L, López-Fierro P, *et al.*: **Fish cell cultures as *in vitro* models of pro-inflammatory responses elicited by immunostimulants.** *Fish Shellfish Immunol.* 2012; **33**(2): 389–400.
PubMed Abstract | Publisher Full Text

77. Fierro-Castro C, Barrioluengo L, López-Fierro P, *et al.*: **Fish cell cultures as *in vitro* models of inflammatory responses elicited by immunostimulants. Expression of regulatory genes of the innate immune response.** *Fish Shellfish Immunol.* 2013; **35**(3): 979–87.
PubMed Abstract | Publisher Full Text

78. Anthony SM, Howard ME, Hailemichael Y, *et al.*: **Soluble interleukin-15 complexes are generated *in vivo* by type I interferon dependent and independent pathways.** *PLoS One.* 2015; **10**(3): e0120274.
PubMed Abstract | Publisher Full Text | Free Full Text

79. Boudinot P, Massin P, Blanco M, *et al.*: ***vig-1*, a new fish gene induced by the rhabdovirus glycoprotein, has a virus-induced homologue in humans and shares conserved motifs with the MoaA family.** *J Virol.* 1999; **73**(3): 1846–52.
PubMed Abstract | Free Full Text

80. Konig R, Zhou Y, Elleder D, *et al.*: **Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication.** *Cell.* 2008; **135**(1): 49–60.
PubMed Abstract | Publisher Full Text | Free Full Text

81. Paz S, Lu ML, Takata H, *et al.*: **SRSF1 RNA Recognition Motifs Are Strong Inhibitors of HIV-1 Replication.** *J Virol.* 2015; **89**(12): 6275–86.
PubMed Abstract | Publisher Full Text | Free Full Text

82. Hadian K, Vincendeau M, Mausbacher N, *et al.*: **Identification of a heterogeneous nuclear ribonucleoprotein-recognition region in the HIV Rev protein.** *J Biol Chem.* 2009; **284**(48): 33384–91.
PubMed Abstract | Publisher Full Text | Free Full Text

83. Schneider RJ, Mohr I: **Translation initiation and viral tricks.** *Trends Biochem Sci.* 2003; **28**(3): 130–6.
PubMed Abstract | Publisher Full Text

84. Connor JH, Lyles DS: **Inhibition of host and viral translation during vesicular stomatitis virus infection. eIF2 is responsible for the inhibition of viral but not host translation.** *J Biol Chem.* 2005; **280**(14): 13512–9.
PubMed Abstract | Publisher Full Text

85. Lander HM: **An essential role for free radicals and derived species in signal transduction.** *FASEB J.* 1997; **11**(2): 118–24.
PubMed Abstract | Publisher Full Text

86. Olagnier D, Peri S, Steel C, *et al.*: **Cellular oxidative stress response controls the antiviral and apoptotic programs in dengue virus-infected dendritic cells.** *PLoS Pathog.* 2014; **10**(12): e1004566.
PubMed Abstract | Publisher Full Text | Free Full Text

87. Fang FC: **Antimicrobial actions of reactive oxygen species.** *mBio.* 2011; **2**(5): pii: e00141-11.
PubMed Abstract | Publisher Full Text | Free Full Text

88. Birben E, Sahiner UM, Sackesen C, *et al.*: **Oxidative stress and antioxidant defense.** *World Allergy Organ J.* 2012; **5**(1): 9–19.
PubMed Abstract | Publisher Full Text | Free Full Text

89. Morgan MJ, Liu ZG: **Crosstalk of reactive oxygen species and NF-κB signaling.** *Cell Res.* 2011; **21**(1): 103–15.
PubMed Abstract | Publisher Full Text | Free Full Text

90. Paiva CN, Bozza MT: **Are reactive oxygen species always detrimental to pathogens?** *Antioxid Redox Signal.* 2014; **20**(6): 1000–37.
PubMed Abstract | Publisher Full Text | Free Full Text

91. Gullberg RC, Jordan Steel J, Moon SL, *et al.*: **Oxidative stress influences positive strand RNA virus genome synthesis and capping.** *Virology.* 2015; **475**: 219–29.
PubMed Abstract | Publisher Full Text | Free Full Text

92. Kan F, Ye T, Yan T, *et al.*: **Proteomic and transcriptomic studies of HBV-associated liver fibrosis of an AAV-HBV-infected mouse model.** *BMC Genomics.* 2017; **18**(1): 641.
PubMed Abstract | Publisher Full Text | Free Full Text

93. De Zoysa M, Whang I, Nikapitiya C, *et al.*: **Transcriptional analysis of disk abalone (*Haliotis discus discus*) antioxidant enzymes against marine bacteria and virus challenge.** *Fish Shellfish Immunol.* 2011; **3**(1): 155–60.
PubMed Abstract | Publisher Full Text

94. Hattangadi SM, Lodish HF: **Regulation of erythrocyte lifespan: do reactive oxygen species set the clock?** *J Clin Invest.* 2007; **117**(8): 2075–7.
PubMed Abstract | Publisher Full Text | Free Full Text

95. Yurochko AD, Kowalik TF, Huong SM, *et al.*: **Human cytomegalovirus upregulates NF-kappa B activity by transactivating the NF-kappa B p105/p50**

and p65 promoters. *J Virol.* 1995; **69**(9): 5391–400.
PubMed Abstract | Free Full Text

96. Shao J, Huang J, Guo Y, *et al.*: **Up-regulation of nuclear factor E2-related factor 2 (Nrf2) represses the replication of SVCV.** *Fish Shellfish Immunol.* 2016; **58**: 474–82.
PubMed Abstract | Publisher Full Text

97. Raida MK, Buchmann K: **Temperature-dependent expression of immune-relevant genes in rainbow trout following *Yersinia ruckeri* vaccination.** *Dis Aquat Organ.* 2007; **77**(1): 41–52.
PubMed Abstract | Publisher Full Text

98. Ortega-Villaizan M, Chico V, Martinez-Lopez A, *et al.*: ***In vitro* analysis of the factors contributing to the antiviral state induced by a plasmid encoding the viral haemorrhagic septicaemia virus glycoprotein G in transfected trout cells.** *Vaccine.* 2011; **29**(4): 737–43.
PubMed Abstract | Publisher Full Text

99. Nombela I, Puente-Marin S, Chico V, *et al.*: **Dataset 1 in: Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus.** *F1000Research.* 2017.
Data Source

100. Nombela I, Puente-Marin S, Chico V, *et al.*: **Dataset 2 in: Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus.** *F1000Research.* 2017.
Data Source

101. Nombela I, Puente-Marin S, Chico V, *et al.*: **Dataset 3 in: Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus.** *F1000Research.* 2017.
Data Source

102. Nombela I, Puente-Marin S, Chico V, *et al.*: **Dataset 4 in: Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus.** *F1000Research.* 2017.
Data Source

103. Nombela I, Puente-Marin S, Chico V, *et al.*: **Dataset 5 in: Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus.** *F1000Research.* 2017.
Data Source

104. Nombela I, Puente-Marin S, Chico V, *et al.*: **Dataset 6 in: Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus.** *F1000Research.* 2017.
Data Source

105. Nombela I, Puente-Marin S, Chico V, *et al.*: **Dataset 7 in: Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus.** *F1000Research.* 2017.
Data Source

Open Peer Review

Current Referee Status: ✔ ✔ ?

Version 2

Referee Report 19 February 2018

doi:10.5256/f1000research.15111.r30735

✔ **Johannes M. Dijkstra**
Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Japan

The authors performed some additional experiments and rewrote some parts of the manuscript. This improved the article. I am still not overly enthusiastic about the study, because the provided evidence for the several storylines is quite thin. However, I realize that for the type of presented data it is hard to get more convincing results, and believe that also this type of data deserves to be indexed. Therefore, I recommend indexing of the article, although I simultaneously advise the readers to read all reviewer's reports so that they have an understanding of the critical issues of the study.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 16 February 2018

doi:10.5256/f1000research.15111.r30734

✔ **Aleksei Krasnov**
Nofima AS, Ås, Norway

I am satisfied with the revision.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Referee Report 12 December 2017

doi:10.5256/f1000research.14081.r27746

Uwe Fischer
Friedrich Loeffler Institute, Greifswald-Insel Riems, Germany

Summarized statement
This is a comprehensive piece of work with a number of interesting experiments.
However, I suggest to not accepting the paper in its present form.

To my opinion the authors have not convincingly shown an initial viral replication in RBCs, although this represents their primary experimental basis. All consecutive data are based on this initial assumption and this makes the whole story challengeable.

In contrast to the assumption of viral replication detection of viral RNA by RT-PCR could have been simply based on inoculated virus that could have been absorbed to the surface of RBCs or internalized by them. In order to prove viral replication de novo synthetized viral RNA or protein expression must be determined.
In this system viral RNA replication can be determined on the mRNA level. For the RT step e.g. oligo(dT) primers could be used that only bind to the polyadenylated tail of mRNAs. The resulting cDNA must then be thoroughly treated with RNAse. This experiment is essential to make the experiments reliable.

Alternatively, I suggest modifying the whole story and stressing the obvious responses of RBCs to the virus (avoiding the claim of virus replication). Thus the title could be changed into something like e.g. “Response of rainbow trout RBCs to VHSV”.

Specific comments
Introduction:
The authors claim: "To compensate for those immune deficiencies, fish have unique phagocytic B lymphocytes".
This is not fully true since also B lymphocytes of some mammalians can execute phagocytosis.

They further claim: "To compensate for those immune deficiencies, … and stronger innate immune responses, as shown in survivors of viral infection".
This is highly speculative and can’t be concluded from a single paper.

Material and methods:
The anti-β-defensin (BD1) anti -IFN1 and anti -IFNγ antibodies have not been described or characterized elsewhere. Their suitability can be questioned.
The anti-Mx3 antiserum has been produced against a predicted Mx peptide. However, I did not find information on the characterization of this antiserum.
The anti-IL1β and the NKEF antibodies have only been tested against the immunizing peptide and there is no further information if the antibodies react with the respective native proteins.
The rabbit polyclonal antibody against human NF-κβ p65 has not been shown to be suitable in any fish species. Abcam only declares that it reacts with mouse, rat, chicken, human and Indian muntjac (Heterocephalus glaber). Those species are phylogenetically not closely related to teleosts.

The authors describe: "Separately, NVHSV RT-qPCR was also used to quantify the viral RNA inside VHSV-exposed RBCs …."
This is not a proof that virus was inside erythrocytes. The virus could have been simply attached/absorbed to RBCs.
To show viral replication RT-PCR with mRNA is required.

"RBCs were fixed with 4% paraformaldehyde (PFA)…"
Unfixed RBCs should have been used in addition to fixed RBCs in order to distinguish between intracellular and membrane bound virus.

Results:
The authors conclude: "…to monitor the replication of VHSV in trout RBCs. … Clearly, the expressions of NVHSV gene were significantly upregulated at 3 hours postexposure.".
Again, I can't support this statement since replication can't be shown by RT-PCR for an RNA virus. Here simply increasing attachment to RBCs might have occurred which explains lower Cq values (higher amounts of viral RNA, respectively). This needs to be verified on the mRNA level.

The authors write: "On the other hand, after VHSV enters the cell, the first gene that starts to transcribe is the NVHSV gene,…".
This observation has been made in cell cultures that are permissive for VHSV. However, it has not convincingly been shown in this paper that N gene transcription took place in RBCs (see my comments above).

"NVHSV RT-qPCR also confirmed the presence of viral RNA in VHSV-infected RBCs (Figure 2B)."
Similarly to the above mentioned, this just says that VHSV was associated with RBCs, but not if the virus was inside RBCs. Attached virus could have resulted in positive RT-PCR results as well.

To further illustrate my concerns regarding viral replication in RBCs I have a few comments regarding Figure 1:
The authors write that "The initial VHSV inoculum titer declined ~3-logs after 3 days".
In Figure 1H, however, I see a 4 log (from 10-to-the-6 to 10-to-the-2) reduction after 3 days (72 hours).

As for Figure 1H a negative control is missing where the same inoculum should have been added to the corresponding amount of cell-free medium or, even better, to inactivated (irradiated) erythrocytes. At 14°C, viral titers would probably also drop in the absence of cells (RBCs).

In Figure 1H the viral titers with RBCs drop within 72 hours by log4. However, in Figure 1D I can see an increase (although minor) in viral titers between 72 hours and 6 days in untreated cells. Is this statistically approved?

In Figure 1D the authors claim that "VHSV internalization in trout RBCs is NH4Cl-sensitive." I can't follow this conclusion. If internalization was NH4Cl-sensitive why it did not fully block virus internalization? The difference between untreated and treated RBCs is about only 2 fold. Is this statistically approved?
Why the decrease in internalization wasn't checked 3 hours after exposure when the N gene expression was claimed to be highest in RBCs, and why was it recorded 3 days post exposure when viral titers have been dropped by 4 logs anyway?

The NH4Cl blocking experiment should have been done with RTG-2 cells as a positive control?

Ethical statement
The anti-IL1β antibody and the NKEF antibodies have been produced by ascitic tumor induction. This method is not in line with European standards of animal welfare.
Since there are already two reports on this manuscript by two other referees, which I agree on, I will not repeat their objections and suggestions.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 Jan 2018

Maria del Mar Ortega-Villaizan Romo, Universidad Miguel Hernandez, Spain

Dear Dr. Uwe Fischer,
Thank you for your revision and comments on the manuscript. We have included your corrections in the new version of the manuscript hoping that now the manuscript will be suitable for publication. Please find below the response to your comments:

———

This is a comprehensive piece of work with a number of interesting experiments.
However, I suggest to not accepting the paper in its present form.

To my opinion the authors have not convincingly shown an initial viral replication in RBCs, although this represents their primary experimental basis. All consecutive data are based on this initial assumption and this makes the whole story challengeable.

In contrast to the assumption of viral replication detection of viral RNA by RT-PCR could have been simply based on inoculated virus that could have been absorbed to the surface of RBCs or internalized by them. In order to prove viral replication de novo synthetized viral RNA or protein expression must be determined.
In this system viral RNA replication can be determined on the mRNA level. For the RT step e.g. oligo(dT) primers could be used that only bind to the polyadenylated tail of mRNAs. The resulting cDNA must then be thoroughly treated with RNAse. This experiment is essential to make the

experiments reliable.

Response: De novo synthetized viral RNA has been determined by Oligo(dT) RT-qPCR. We have included this result as supplementary Figure (Figure S1). As it can be observed, the kinetics of VHSV mRNA expression closely matched total virus RNA synthesis.

Alternatively, I suggest modifying the whole story and stressing the obvious responses of RBCs to the virus (avoiding the claim of virus replication). Thus the title could be changed into something like e.g. “Response of rainbow trout RBCs to VHSV”.

Response: We appreciate the suggestion, however, in view of other reviewers’ comments, we would like to maintain the title as it is.

Specific comments
Introduction:
The authors claim: "To compensate for those immune deficiencies, fish have unique phagocytic B lymphocytes".
This is not fully true since also B lymphocytes of some mammalians can execute phagocytosis.

Response: We have removed this sentence.

They further claim: "To compensate for those immune deficiencies, … and stronger innate immune responses, as shown in survivors of viral infection".
This is highly speculative and can’t be concluded from a single paper.

Response: We have removed this sentence.

Material and methods:
The anti-β-defensin (BD1) anti -IFN1 and anti -IFNγ antibodies have not been described or characterized elsewhere. Their suitability can be questioned.

Response: The antiserum against β-defensin has been validated by our laboratory. Figure S2 depicts western blotting validation for anti-BD1 antibody. anti -IFN1 and anti -IFNγ antibodies have been validated by Dr. Luis Mercado laboratory. Figure S3 depicts western blotting validation for anti -IFN1 and anti –IFNγ.

The anti-Mx3 antiserum has been produced against a predicted Mx peptide. However, I did not find information on the characterization of this antiserum.

Response: anti-Mx3 antiserum production was described in Chico et al. 2010, Journal of Virology. In Martinez-Lopez et al. 2014 and Ortega-Villaizan et al. 2011 they showed the Mx protein expression in stimulated RTG-2 cells, using anti-Mx3 antibody.

The anti-IL1β and the NKEF antibodies have only been tested against the immunizing peptide and there is no further information if the antibodies react with the respective native proteins.

Response: For anti-IL1β, in Schmitt et al. 2015 and Rojas et al. 2015, we can find the expression of IL1β in RTS11 and RTgutGC cells, respectively, by immunofluorescence and western blot. For anti-NKEF, Bethke et al. 2012, show western blotting of NKEF protein in serum of

Atlantic salmon. Also Tafalla et al. 2011 show the expression of NKEF in RTS11 as evaluated by western blotting and by flow cytometry.

The rabbit polyclonal antibody against human NF-κβ p65 has not been shown to be suitable in any fish species. Abcam only declares that it reacts with mouse, rat, chicken, human and Indian muntjac (Heterocephalus glaber). Those species are phylogenetically not closely related to teleosts.

Response: We indicated references where this antibody has been used for teleost species:
Encinas P, Garcia-Valtanen P, Chinchilla B, et al.: Identification of multipath genes differentially expressed in pathway-targeted microarrays in zebrafish infected and surviving spring viremia carp virus (SVCV) suggest preventive drug candidates. PLoS One. 2013;8(9):e73553. 24069208 10.1371/journal.pone.0073553 3772095
García-Valtanen P, Martinez-Lopez A, Ortega-Villaizan M, et al.: In addition to its antiviral and immunomodulatory properties, the zebrafish β-defensin 2 (zfBD2) is a potent viral DNA vaccine molecular adjuvant. Antiviral Res. 2014;101:136–47. 24286781 10.1016/j.antiviral.2013.11.009
Abós B, Castro R, González Granja A, et al.: Early activation of teleost B cells in response to rhabdovirus infection. J Virol. 2015;89(3):1768–80. 25410870 10.1128/JVI.03080-14 4300759

The authors describe: "Separately, NVHSV RT-qPCR was also used to quantify the viral RNA inside VHSV-exposed RBCs …."
This is not a proof that virus was inside erythrocytes. The virus could have been simply attached/absorbed to RBCs.
To show viral replication RT-PCR with mRNA is required.

Response: As previously indicated, we have included that result as supplementary Figure (Figure S1). As it can be observed, the kinetics of VHSV mRNA expression closely matched total virus RNA synthesis.

"RBCs were fixed with 4% paraformaldehyde (PFA)…"
Unfixed RBCs should have been used in addition to fixed RBCs in order to distinguish between intracellular and membrane bound virus.

Response: Yes, it is right. However, we have tried to do immune staining of unfixed RBCs and it is very difficult since RBCs are lysed during the antibody incubation.

Results:
The authors conclude: "…to monitor the replication of VHSV in trout RBCs. … Clearly, the expressions of NVHSV gene were significantly upregulated at 3 hours postexposure.".
Again, I can't support this statement since replication can't be shown by RT-PCR for an RNA virus. Here simply increasing attachment to RBCs might have occurred which explains lower Cq values (higher amounts of viral RNA, respectively). This needs to be verified on the mRNA level.

Response: As previously indicated, we have included that result as supplementary Figure (Figure S1). As it can be observed, the kinetics of VHSV mRNA expression closely matched total virus RNA synthesis.

The authors write: "On the other hand, after VHSV enters the cell, the first gene that starts to transcribe is the NVHSV gene,…".

This observation has been made in cell cultures that are permissive for VHSV. However, it has not convincingly been shown in this paper that N gene transcription took place in RBCs (see my comments above).
“NVHSV RT-qPCR also confirmed the presence of viral RNA in VHSV-infected RBCs (Figure 2B).” Similarly to the above mentioned, this just says that VHSV was associated with RBCs, but not if the virus was inside RBCs. Attached virus could have resulted in positive RT-PCR results as well.

Response: The reviewer is correct, RT-qPCR data do not tell if the virus is inside or attached to the cell. But we would like to highlight that Figure 1E shows that in RBCs exposed to UV-inactivated VHSV, Nvhsv levels did not increase in comparison with live VHSV exposed RBCs. This experiment demonstrates that live VHSV replicated early post-exposure, in comparison with UV-inactivated VHSV. In order to emphasize this result we have re-written its explanation.

To further illustrate my concerns regarding viral replication in RBCs I have a few comments regarding Figure 1:
The authors write that “The initial VHSV inoculum titer declined ~3-logs after 3 days".
In Figure 1H, however, I see a 4 log (from 10-to-the-6 to 10-to-the-2) reduction after 3 days (72 hours).

Response: It was a mistyping error, we have corrected it.

As for Figure 1H a negative control is missing where the same inoculum should have been added to the corresponding amount of cell-free medium or, even better, to inactivated (irradiated) erythrocytes. At 14°C, viral titers would probably also drop in the absence of cells (RBCs).

Response: VHSV titer in cell-free medium 3 d at 14ºC does not drop more than one log.

In Figure 1H the viral titers with RBCs drop within 72 hours by log4. However, in Figure 1D I can see an increase (although minor) in viral titers between 72 hours and 6 days in untreated cells. Is this statistically approved?

Response: Yes, we observed a minor increase at 6 days post exposure (from 200 to 300 PFU/ml), and not statistically significant.

In Figure 1D the authors claim that “VHSV internalization in trout RBCs is NH4Cl-sensitive.” I can’t follow this conclusion. If internalization was NH4Cl-sensitive why it did not fully block virus internalization? The difference between untreated and treated RBCs is about only 2 fold. Is this statistically approved?
Why the decrease in internalization wasn’t checked 3 hours after exposure when the N gene expression was claimed to be highest in RBCs, and why was it recorded 3 days post exposure when viral titers have been dropped by 4 logs anyway?

Response: You are right, the sentence is quite confusing, we have re-written it. We would like to clarify that NH4Cl blocks fusion of viruses to cell membrane, not internalization. On the other hand, the concentration used for NH4Cl (7 mM) was selected since it is non-cytotoxic for RBCs and other cells, such as EPC and RTG-2, but effective for reducing VHSV infectivity by 40% in EPC cells (Mas et al. 2002, Journal of General Virology), similar to the reduction that we observe in RBCs.

The NH4Cl blocking experiment should have been done with RTG-2 cells as a positive control?

Response: We have previously carried out this assay in RTG-2 cells and observed that, at 10 mM NH4Cl, VHSV infection is reduced by 90% in RTG-2 cells, quantified by Nvhsv RT-qPCR. However, a reduction of 50% was observed by Mas et al. 2002 at the same NH4Cl concentration, in EPC cells, although in this case it was quantified by FFU/ml.

Ethical statement
The anti-IL1β antibody and the NKEF antibodies have been produced by ascitic tumor induction. This method is not in line with European standards of animal welfare.

Response: Only anti-NKEF, produced at the laboratory of Dr. Luis Mercado (Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile), was developed in ascities fluids, because this antibody was obtained in 2011, when this technique was allowed in Chile. Nowadays, anti-NKEF is no longer produced by ascitic tumor induction, following the Chilean standards for animal welfare.

Competing Interests: No competing interests were disclosed.

Referee Report 30 November 2017

doi:10.5256/f1000research.14081.r27745

? **Johannes M. Dijkstra**
Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Japan

Nombela et al. describe in what they claim to be defense mechanisms in trout red blood cells in response to halted replication of VHSV virus.

Major comments:

The study suffers from the effort to simultaneously answer a few unknowns. Can VHSV infect trout erythrocytes? Does infection by, or exposure to, VHSV virus modify the expression of genes with an anti-virus function? Do those altered gene expression levels have a measurable immune effect (which in this article is measured by the effect on cell line TSS)?

In my opinion, the authors show understanding of the complexity of those questions, but do not take sufficiently control of them. I am not satisfied with any of the story lines. My main concerns are that it is unclear for many of the presented data (i) whether the enhanced expression of immune molecules are due to RBC infection or due to other stimulations of the RBCs by the virus preparation, (ii) whether the changes in immune molecule expression are due to stimulation by virus preparation or due to time of culturing, and (iii) whether the expression levels of immune molecules reach meaningful levels or are just variation within what could be considered as “non-functional background levels”.

At the very positive side, the authors addressed an important question, and delivered an honest and elaborate piece of work. Therefore, I will not reject the paper, but I do request the addition of experimental data that in my opinion are necessary for better interpretation of the currently presented data in relation to actual VHS disease.

The authors should infect rainbow trout cells which they deem (sufficiently representative of) the natural

host cells of VHSV, and use the UV-inactivated supernatant for stimulation of trout RBCs and compare the effect on RBC immune molecule expression with the effect after RBC incubation with VHSV. Alternatively, they can use inactivated serum of VHSV-infected trout. My guess is that the released cytokines have a much stronger effect on those erythrocytes than the viruses to which the erythrocytes are hardly receptive.

The authors should also use those supernatants of natural host cells, or sera from infected trout, for stimulation of TSS cells, and compare the effects quantitatively with those after stimulation with the supernatant of VHSV-exposed erythrocytes.

The above requested set of experiments (or modifications thereof, depending on the preferences of the authors) should help to quantitatively estimate the direct effect of VHSV on erythrocytes, and the effect of VHSV-stimulated erythrocytes on other cells, in comparison with other routes of immune stimulation during VHSV infection.

Detailed comments:

Why did the authors use an MOI of 1? Even if such MOI is achieved, only half of the cells are expected to be infected. In this case the actual MOI for red blood cells probably was far below 1, because the MOI was calculated based on infection of the receptive EPC cells.

How were the viruses prepared? It seems that they were generated on EPC cells, but the details are important. Namely, other than the viruses, the infected cells also release cytokines which may have a cross-species effect.

The only presented data that I find convincing for that red blood cells were infected were the experiments shown in Fig. 1E and Fig. 1F, namely after pretreatment with neuraminidase (Fig. 1E) and infection with an MOI=100 (Fig. 1G; even in that case only 1/6 cells shows infection). However, none of the experiments on immune molecule expression was done under those conditions.

In the experiments, expression levels are compared with those of “control cells”. In some cases those control cells are not specified, while in other cases they are said to be the T=0 cells. However, this does not take into account that also the time of culture can have a significant effect on gene and protein expression levels. Most of the expression level effects reported in this article are quite small (e.g. from very low to only two-fold higher), and a possible “culture-time effect” should have been excluded.

The introduction should give detailed descriptions of what is known or unknown (i) about natural target cells and receptors used by VHSV for infection, and (ii) about fish erythrocytes and to what extent they have a normal metabolism. The introduction should also give an indication of the abundance of erythrocytes, which is relevant because many small amounts of cytokine could make a big amount, and also because any “intelligent” virus will do its best to avoid interaction with this abundant and for the virus non-productive cell type.

In the title, shouldn’t it be “in response to halted replication of VHS virus.”?

In the abstract and in the text: “after 6 hours postexposure” is double.

In the abstract, in the sentence “Co-culture assays of RBCs with TSS”, it should be made clear that those RBCs were stimulated with UV-inactivated VHSV.

In the introduction, a number of speculations are presented as facts:

- Fish poikilothermic nature results in a delayed antigen affinity maturation, memory and lymphocyte proliferation.
- Fish have unique phagocytic B lymphocytes. (later than the reference, also mammalian B cells with phagocytic ability have been found)
- Fish have stronger innate immune responses.
- To compensate for those immune deficiencies, fish have unique phagocytic B lymphocytes and stronger innate immune responses.

I don't understand the "Thus" in the sentence "Thus, fish RBCs generate a wide variety of immune-related gene transcripts when viruses highly replicate inside them".

In Fig. 1A, how was the PCR value for N gene determined at T=0? Was that before or after addition of the viruses, and could the difference between T=0 and the other time points be explained by amplification from RNA in virions?

Fig. 1B seems to argue against the assumption that the RNAs amplified in Fig. 1A were derived from an infection (see also my previous point). In addition, although the relative comparison between the RTG2 and RBC results as presented in Fig. 1B should be OK, it is unclear to me from the materials and methods section how the absolute quantitative statement "However, a ratio of 2 was observed in RBCs, compared to the ratio of 8 found in RTG-2 cells, at 1 and 3 hpe (Figure 1B)" can be made.

I am not convinced that Fig. 1D is evidence for replication between days 3 and 6, since the titer goes down >5000-fold from day 0 to day 3, and then stays very low. The authors should make clearer whether they feel that the small increase in virus titer between days 3 and 6 is only suggestive of virus replication, or that such replication is supported by proper statistics.
As for the NH4Cl effect observed in Fig. 1D. Can a chemical effect of NH4CL on the integrity of virions stuck to the outside of RBCs be excluded from explaining the results? Furthermore, I would like the authors to elaborate, possibly in the introduction section, on endocytosis in regard to erythrocytes and VHSV infection. Could it be that only immature erythrocytes are expected to display efficient endocytosis, and might the Fig. 1F result be explained by differences in erythrocyte subpopulations?

I don't understand the sentence "As a result, the VHSV RNA inside RBCs was increased about ten times at 3 hpe", because the increase seems to be from around 0.6 to 3.4, which is closer to a six-fold increase.

For discussion of the Fig. 1F result, the authors should explain the intracellular organization of RBCs (which are unusual cells), and where VHSV is expected. The sentence "along the cytoplasm and nucleus" can't be understood, and gives the impression that the authors do not know where to expect (normal) cytoplasm in RBCs. Although they observe "along the nucleus", which I think is the correct observation, the authors discuss the possibility of N protein being present in the nucleus. It is not wrong to present that as a possibility to partially explain their observations, but the authors should declare clearer that their observations do not necessitate that N protein is present in the nucleus. To superficial readers it now looks as if they claim detection of N protein in the RBC nucleus.

In Fig. 3 legend, the B and C order should be altered.

(writing error) were co-culture with > were co-cultured with

In the Transwell system, the authors tried to get rid of RBC-attached virions with a single non-stringent wash. I doubt that such was sufficient for complete removal, and in the following 24 hours of co-incubation some virions or viral products may have diffused to the TSS cells.

As for Fig. 4. Is it OK to assume linear regression based on only two time points? Wouldn't it be more proper to indicate all individual observations with dots? What is a 0-fold increase? In Figs. 4A and 4C, all three lines need explanation.

I can't trust the claims based on Fig. 8. In Fig. 8A, why were protein amounts loaded in the Control and VHSV lanes so different? I don't believe that densiometry analysis technique for Western blot bands is sufficiently sensitive, especially not if comparing different ranges of band densities, to reliably claim an about 15% difference as done in Fig. 8B. In addition, for densiometry analysis, the Fig. 8A Actin blot is a horrible result because only half of the lane was properly exposed to the Western treatment. If the Fig. 8A result truly is a "representative" result, as claimed by the authors, the Fig. 8 based conclusions can't be taken seriously.

Trout erythrocytes are known to express MHC class I (Dijkstra *et al.* (2003)[1]; Sarder *et al*. (2003)[2]). Because MHC class I is a molecule upregulated during virus infection, it would be interesting to see the effect on its expression in RBCs by exposure to VHSV. Likewise, and especially because Nombela et al. discuss the proteasome, it would be interesting to see the regulation of the genes for the immuno-proteasome specific subunits.

References

1. Dijkstra J, Köllner B, Aoyagi K, Sawamoto Y, Kuroda A, Ototake M, Nakanishi T, Fischer U: The rainbow trout classical MHC class I molecule Onmy-UBA*501 is expressed in similar cell types as mammalian classical MHC class I molecules. *Fish & Shellfish Immunology*. 2003; **14** (1): 1-23 Publisher Full Text

2. Sarder MR, Fischer U, Dijkstra JM, Kiryu I, Yoshiura Y, Azuma T, Köllner B, Ototake M: The MHC class I linkage group is a major determinant in the in vivo rejection of allogeneic erythrocytes in rainbow trout (Oncorhynchus mykiss).*Immunogenetics*. 2003; **55** (5): 315-24 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 Jan 2018

Maria del Mar Ortega-Villaizan Romo, Universidad Miguel Hernandez, Spain

Dear Dr. Johannes M. Dijkstra,
We appreciate very much your detailed revision and constructive comments and suggestions on the manuscript. We have included your corrections in the new version of the manuscript hoping that now the manuscript will be suitable for publication.
Please find below the response to your comments:

_____

Major comments:

The study suffers from the effort to simultaneously answer a few unknowns. Can VHSV infect trout erythrocytes? Does infection by, or exposure to, VHSV virus modify the expression of genes with an anti-virus function? Do those altered gene expression levels have a measurable immune effect (which in this article is measured by the effect on cell line TSS)?

In my opinion, the authors show understanding of the complexity of those questions, but do not take sufficiently control of them. I am not satisfied with any of the story lines. My main concerns are that it is unclear for many of the presented data (i) whether the enhanced expression of immune molecules are due to RBC infection or due to other stimulations of the RBCs by the virus preparation, (ii) whether the changes in immune molecule expression are due to stimulation by virus preparation or due to time of culturing, and (iii) whether the expression levels of immune molecules reach meaningful levels or are just variation within what could be considered as “non-functional background levels”.

At the very positive side, the authors addressed an important question, and delivered an honest and elaborate piece of work. Therefore, I will not reject the paper, but I do request the addition of experimental data that in my opinion are necessary for better interpretation of the currently presented data in relation to actual VHS disease.

The authors should infect rainbow trout cells which they deem (sufficiently representative of) the natural host cells of VHSV, and use the UV-inactivated supernatant for stimulation of trout RBCs and compare the effect on RBC immune molecule expression with the effect after RBC incubation with VHSV. Alternatively, they can use inactivated serum of VHSV-infected trout. My guess is that the released cytokines have a much stronger effect on those erythrocytes than the viruses to which the erythrocytes are hardly receptive.
The authors should also use those supernatants of natural host cells, or sera from infected trout, for stimulation of TSS cells, and compare the effects quantitatively with those after stimulation with the supernatant of VHSV-exposed erythrocytes.
The above requested set of experiments (or modifications thereof, depending on the preferences of the authors) should help to quantitatively estimate the direct effect of VHSV on erythrocytes, and

the effect of VHSV-stimulated erythrocytes on other cells, in comparison with other routes of immune stimulation during VHSV infection.

Response: This is an interesting suggestion. To answer this question we have carried out an experiment where RTG-2 cells were treated with UV-inactivated VHSV during 24h, after that UV-VHSV was removed and cells were culture 24h in RPMI fresh medium. This conditioned medium was used to stimulate rainbow trout RBCs, during 24h. RTG-2 is a known cell line susceptible to VHSV infection and with a high interferon response to VHSV, therefore we considered it as a proper cell line for this experiment. Please see the results in Figure 4D.
In relation to the last sentence, we did not proceed with that assay since the results obtained with RTG-2 conditioned media on RBCs were conclusive.

Detailed comments:

Why did the authors use an MOI of 1? Even if such MOI is achieved, only half of the cells are expected to be infected. In this case the actual MOI for red blood cells probably was far below 1, because the MOI was calculated based on infection of the receptive EPC cells.

Response: We have also infected RBCs with lower and higher MOIs, as can be observed in Figure 1C, in order to monitor course of infection. However, for immune response experiments we decided to use MOI 1 since over this value more defecting interfering virus will be present. Moreover, MOI1 is already a high titer in experiments of immune response to the infection.

How were the viruses prepared? It seems that they were generated on EPC cells, but the details are important. Namely, other than the viruses, the infected cells also release cytokines which may have a cross-species effect.

Response: VHSV was prepared in EPC cells as indicated in Methods. As suggested, we have expanded the details about it. As Dr. Dijkstra comments, we are aware that the clarified supernatant contains cytokines that may have a cross-species effect. However, the volume of supernatant/inoculum needed to infect may be less than 0.01 ul, since we usually have virus stock titter of 10^7 ffu/ml. Therefore, cytokines from EPC cells are diluted 1:100.

The only presented data that I find convincing for that red blood cells were infected were the experiments shown in Fig. 1E and Fig. 1F, namely after pretreatment with neuraminidase (Fig. 1E) and infection with an MOI=100 (Fig. 1G; even in that case only 1/6 cells shows infection). However, none of the experiments on immune molecule expression was done under those conditions.

Response: Yes, MOI values over 100 are needed to detect VHSV inside RBCs by immunofluorescence. However, MOI1 is already a high titer in experiments of immune response, since one or few viral particles per cell can induce detectable immune response.

In the experiments, expression levels are compared with those of “control cells”. In some cases those control cells are not specified, while in other cases they are said to be the T=0 cells. However, this does not take into account that also the time of culture can have a significant effect on gene and protein expression levels. Most of the expression level effects reported in this article are quite small (e.g. from very low to only two-fold higher), and a possible “culture-time effect” should have been excluded.

Response: For all the experiments, except for time course assays, control cells are uninfected cells culture in RPMI 2%FBS (viral infection medium), in the same plate as infected cells, and incubated the same time. To better explain it, we have indicated it in each figure legend. In the case of time course experiment (Figure 2), control cells refer to time=0h. For this assay, in order to evaluate the culture-time effect in the expression of *mx* and *pkr* genes, we have analysed gene expression in non-stimulated RBCs along the time course and observed that it does not change during the three days of culture ex vivo. We have added a Supplementary figure with these results (Figure S4), and commented it in the manuscript.

The introduction should give detailed descriptions of what is known or unknown (i) about natural target cells and receptors used by VHSV for infection, and (ii) about fish erythrocytes and to what extent they have a normal metabolism. The introduction should also give an indication of the abundance of erythrocytes, which is relevant because many small amounts of cytokine could make a big amount, and also because any "intelligent" virus will do its best to avoid interaction with this abundant and for the virus non-productive cell type.

Response: As advised, we have include information about VHSV targets and cell receptors in the introduction. About RBCs metabolism, we have talked about oxidative stress in the discussion. About the last item ("abundance of erythrocytes, which is relevant because many small amounts of cytokine could make a big amount"), we have talked about it the introduction and discussion.

In the title, shouldn't it be "in response to halted replication of VHS virus."?

Response: Yes, we have corrected it.

In the abstract and in the text: "after 6 hours postexposure" is double.

Response: We have corrected it.

In the abstract, in the sentence "Co-culture assays of RBCs with TSS", it should be made clear that those RBCs were stimulated with UV-inactivated VHSV.

Response: Yes, we have added it.

In the introduction, a number of speculations are presented as facts:

- Fish poikilothermic nature results in a delayed antigen affinity maturation, memory and lymphocyte proliferation.

Response: We have deleted it to avoid misunderstanding.

- Fish have unique phagocytic B lymphocytes. (later than the reference, also mammalian B cells with phagocytic ability have been found)

Response: We have deleted it to avoid misunderstanding.

- Fish have stronger innate immune responses.

Response: We have deleted it to avoid misunderstanding.

- To compensate for those immune deficiencies, fish have unique phagocytic B lymphocytes and stronger innate immune responses.

Response: We have deleted it to avoid misunderstanding.

I don't understand the "Thus" in the sentence "Thus, fish RBCs generate a wide variety of immune-related gene transcripts when viruses highly replicate inside them".

Response: We have deleted it.

In Fig. 1A, how was the PCR value for N gene determined at T=0? Was that before or after addition of the viruses, and could the difference between T=0 and the other time points be explained by amplification from RNA in virions?

Response: Control cells are cells non-exposed to the virus, at t=0. We have better explained it in figure legend.

Fig. 1B seems to argue against the assumption that the RNAs amplified in Fig. 1A were derived from an infection (see also my previous point). In addition, although the relative comparison between the RTG2 and RBC results as presented in Fig. 1B should be OK, it is unclear to me from the materials and methods section how the absolute quantitative statement “However, a ratio of 2 was observed in RBCs, compared to the ratio of 8 found in RTG-2 cells, at 1 and 3 hpe (Figure 1B)” can be made.

Response: It is not clear to me why Fig. 1B argues that the RNAs amplified in Fig. 1A were derived from an infection. Please take into account that Fig. 1A is in log scale and the Nvhsv RNA level increment in RBCs at 3 hpe was low.
About N:G genes ratio, which indicates RNA virus transcription, it was calculated as $2^{-\Delta\Delta Ct}$ N_{VHSV} : $2^{-\Delta\Delta Ct}$ G_{VHSV}. We have indicated it in the Figure 1B legend.

I am not convinced that Fig. 1D is evidence for replication between days 3 and 6, since the titer goes down >5000-fold from day 0 to day 3, and then stays very low. The authors should make clearer whether they feel that the small increase in virus titer between days 3 and 6 is only suggestive of virus replication, or that such replication is supported by proper statistics.

Response: This result is not statistically significant. VHSV titers are maintained in RBCs but do not increase. We have explained it in the manuscript.

As for the NH4Cl effect observed in Fig. 1D. Can a chemical effect of NH4CL on the integrity of virions stuck to the outside of RBCs be excluded from explaining the results? Furthermore, I would like the authors to elaborate, possibly in the introduction section, on endocytosis in regard to erythrocytes and VHSV infection. Could it be that only immature erythrocytes are expected to display efficient endocytosis, and might the Fig. 1F result be explained by differences in erythrocyte subpopulations?

Response: 7mM NH4Cl slightly increases cell pH and therefore inhibits endosome acidification. Thus, virus is kept into the endosome and not released into the cytoplasm. On the other hand, high alkaline pH (about 9) is needed to remove virus binded to cytoplasmic membrane. With 7mM NH4Cl, pH is only slightly increased to 7.5.
In relation to RBCs endocytosis and virus endocytosis, as advised, we have talked about it in the introduction. However, in relation to the differences of endocytosis among RBCs subpopulations, it would be very interesting to evaluate but we do not have any evidence or reference about it.

I don’t understand the sentence “As a result, the VHSV RNA inside RBCs was increased about ten times at 3 hpe”, because the increase seems to be from around 0.6 to 3.4, which is closer to a six-fold increase.

Response: This comment refers to Figure 1E. Yes, we have now specified that 10 times increment at 3 hpe is in relation to VHSV-UV treated cells.

For discussion of the Fig. 1F result, the authors should explain the intracellular organization of RBCs (which are unusual cells), and where VHSV is expected. The sentence “along the cytoplasm and nucleus” can’t be understood, and gives the impression that the authors do not know where to expect (normal) cytoplasm in RBCs. Although they observe “along the nucleus”, which I think is the correct observation, the authors discuss the possibility of N protein being present in the nucleus. It is not wrong to present that as a possibility to partially explain their observations, but the authors should declare clearer that their observations do not necessitate that N protein is present in the nucleus. To superficial readers it now looks as if they claim detection of N protein in the RBC nucleus.

Response: Yes, thank you for your observation. After your observation, and in order to avoid misunderstandings, we prefer to eliminate the discussion about the localization of the N protein in the nucleus, and show it as an observation of intracellular localization.

In Fig. 3 legend, the B and C order should be altered.

Response: We have corrected it in figure 3 legend.

(writing error) were co-culture with > were co-cultured with

Response: We have corrected it.

In the Transwell system, the authors tried to get rid of RBC-attached virions with a single non-stringent wash. I doubt that such was sufficient for complete removal, and in the following 24 hours of co-incubation some virions or viral products may have diffused to the TSS cells.

Response: Yes, reviewer is right. However, more stringent washing conditions resulted to be cytotoxic for RBCs.

As for Fig. 4. Is it OK to assume linear regression based on only two time points? Wouldn’t it be more proper to indicate all individual observations with dots? What is a 0-fold increase? In Figs. 4A and 4C, all three lines need explanation.

Response: We have included individual observations and separated each line. In relation to your question about 0 fold-increase, for that point, TSS vig1 gene expression is 0.15 fold.

I can’t trust the claims based on Fig. 8. In Fig. 8A, why were protein amounts loaded in the Control and VHSV lanes so different? I don’t believe that densiometry analysis technique for Western blot bands is sufficiently sensitive, especially not if comparing different ranges of band densities, to reliably claim an about 15% difference as done in Fig. 8B. In addition, for densiometry analysis, the Fig. 8A Actin blot is a horrible result because only half of the lane was properly exposed to the Western treatment. If the Fig. 8A result truly is a “representative” result, as claimed by the authors, the Fig. 8 based conclusions can’t be taken seriously.

Response: We have replaced it by a better Western Blot.

Trout erythrocytes are known to express MHC class I (Dijkstra *et al.* (2003)[1]; Sarder *et al*. (2003)[2]). Because MHC class I is a molecule upregulated during virus infection, it would be interesting to see the effect on its expression in RBCs by exposure to VHSV. Likewise, and especially because Nombela et al. discuss the proteasome, it would be interesting to see the regulation of the genes for the immuno-proteasome specific subunits.

Response: Yes, it is an interesting observation. In fact we are working on it and the results wil be published in a different manuscript.

Competing Interests: No competing interests were disclosed.

Referee Report 27 November 2017

doi:10.5256/f1000research.14081.r27680

Aleksei Krasnov
Nofima AS, Ås, Norway

The paper reports studies with red blood cells (RBC) of rainbow trout infected with Viral Haemorrhagic Septicaemia virus (VHSV). RBC most likely do not support propagation of VHSV and immune responses to the pathogen were small by magnitude. Still, experiments and analyses were well designed and implemented, applied diverse methods and therefore publication will be useful and interesting for experts in the area.

Major comment
Suppression of innate antiviral immunity in infected RBC is included in the abstract as one of the key findings. However, of several genes analysed with qPCR only ifn1 showed down-regulation and only at one time-point. Other genes exhibited at best a slight tendency and differences from control were small. Reference to high variation is not convincing and does not overcome the lack of significance. Delete sentence “It is noteworthy to highlight the elevated inter-individual variability found in trout RBCs immune response, for most of the proteins and genes assayed, which could prevent to obtain statistical significance in most of the cases although regulations were apparent” (pages 9-10), this statement is trivial. M&M do not tell if each RBC culture was from an individual animal. If not, then variation was technical by character suggesting problems with methods. I also suggest to delete or at least shorten discussion of viral suppression of IFN-dependent responses in fish (page 16, 1st paragraph). I would emphasize strong induction of ROS scavengers as most impressive result of this study.

Specific comments

- Figure 2. Indicate method in the legend – qPCR? Change label of Y-axis: fold instead of fold of increase.

- Figure 4. The number of replicates (n = 6) is too small for regression and correlation analyses. I strongly suggest to plot empirical data, trend lines alone are not convincing. Judging from the figures, ifn1 levels were in the range from 0 to 15. Units should be explained. Furthermore, it is

unclear how such differences was achieved taking into account minor responses of ifn1 to IHNV in trout RBC.

- Figure 10. Explain grey and black bars in the legend.
- Page 16. Delete paragraph “It is noticeable that the iTRAQ-based protein…” – no need to explain that proteomic analyses fail to detect low abundance proteins including cytokines.
- Page 17, 1st paragraph. NFkB can be mentioned but extensive discussion is not warranted since study did not produce any experimental data for this gene or protein.
- “It is evident that the antiviral response of RBCs is low compared to other cells of the immune system” – this statement is wrong. Virus infected fish RBC develop immune responses of huge magnitude. “Inter-individual variability” – if RBC cultures represented individuals, this must be explicitly stated in M&M (see comment above). Given small number of replicates, discussion of high and low responders is not supported with data.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
No source data required

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 Jan 2018

Maria del Mar Ortega-Villaizan Romo, Universidad Miguel Hernandez, Spain

Dear Dr. Aleksei Krasnov,
We appreciate very much your thorough revision and positive and constructive comments on the manuscript. We have included your corrections and suggestions in the new version of the manuscript hoping that now the manuscript will be suitable for publication.

Please find below the response to your comments:

_____

The paper reports studies with red blood cells (RBC) of rainbow trout infected with Viral Haemorrhagic Septicaemia virus (VHSV). RBC most likely do not support propagation of VHSV and immune responses to the pathogen were small by magnitude. Still, experiments and analyses were well designed and implemented, applied diverse methods and therefore publication will be useful and interesting for experts in the area.

Major comment
Suppression of innate antiviral immunity in infected RBC is included in the abstract as one of the key findings. However, of several genes analysed with qPCR only ifn1 showed down-regulation and only at one time-point. Other genes exhibited at best a slight tendency and differences from control were small. Reference to high variation is not convincing and does not overcome the lack of significance. Delete sentence “It is noteworthy to highlight the elevated inter-individual variability found in trout RBCs immune response, for most of the proteins and genes assayed, which could prevent to obtain statistical significance in most of the cases although regulations were apparent” (pages 9-10), this statement is trivial.

Response: We have deleted this sentence in order to avoid misunderstanding.

M&M do not tell if each RBC culture was from an individual animal. If not, then variation was technical by character suggesting problems with methods.

Response: We have indicated in Methods, Animals section, the following: The number of individuals used is indicated by an “n” in each experiment.

I also suggest to delete or at least shorten discussion of viral suppression of IFN-dependent responses in fish (page 16, 1st paragraph).

Response: As advised, we have shortened the discussion of viral suppression of IFN-dependent responses in fish (page 16, 1st paragraph).

I would emphasize strong induction of ROS scavengers as most impressive result of this study.

Response: As advised, we have emphasize the discussion related to ROS and antioxidant response in RBCs.

Specific comments

- Figure 2. Indicate method in the legend – qPCR? Change label of Y-axis: fold instead of fold of increase.

Response: We have indicated it as advised, in Figure 2 and in other figures as well.

Figure 4. The number of replicates (n = 6) is too small for regression and correlation analyses. I strongly suggest to plot empirical data, trend lines alone are not convincing. Judging from the figures, ifn1 levels were in the range from 0 to 15. Units should be explained. Furthermore, it is

unclear how such differences was achieved taking into account minor responses of ifn1 to IHNV in trout RBC.

Response: As indicated we have included the individual values. The ifn1 response is normally downregulated in RBCs after VHSV-exposure. However, in a few individuals (outliers) we could find ifn1 upregulation, which correlated with the upregulation in TPS-2.

- Figure 10. Explain grey and black bars in the legend.

Response: We have corrected it and explained it.

- Page 16. Delete paragraph "It is noticeable that the iTRAQ-based protein…" – no need to explain that proteomic analyses fail to detect low abundance proteins including cytokines.

Response: We have deleted this sentence in order to avoid misunderstanding.

Page 17, 1st paragraph. NFkB can be mentioned but extensive discussion is not warranted since study did not produce any experimental data for this gene or protein.

Response: As advised, we have shortened the discussion related to NFkB.

- "It is evident that the antiviral response of RBCs is low compared to other cells of the immune system" – this statement is wrong. Virus infected fish RBC develop immune responses of huge magnitude. "Inter-individual variability" – if RBC cultures represented individuals, this must be explicitly stated in M&M (see comment above). Given small number of replicates, discussion of high and low responders is not supported with data.

Response: As indicated before, we have indicated in Methods, Animals section, the following: The number of individuals used is indicated by an "n" in each experiment. Separately, in order to avoid misunderstanding we have eliminated the sentence "It is evident that the antiviral response of RBCs is low compared to other cells of the immune system", "Inter-individual variability" and high and low responders discussion.

Competing Interests: No competing interests were disclosed.

PUBLICACIÓN 2

TÍTULO: Infectious pancreatic necrosis virus triggers antiviral immune response in rainbow trout red blood cells, despite not being infective

AUTORES: Ivan Nombela, Aurora Carrion, Sara Puente-Marin, Veronica Chico, Luis Mercado, Luis Perez, Julio Coll y Maria del Mar Ortega-Villaizan

REVISTA: *F1000Research*

DOI: 10.12688/f1000research.12994.2

RESEARCH ARTICLE

REVISED Infectious pancreatic necrosis virus triggers antiviral immune response in rainbow trout red blood cells, despite not being infective [version 2; referees: 2 approved]

Previously titled: "Piscine birnavirus triggers antiviral immune response in trout red blood cells, despite not being infective"

Ivan Nombela [1], Aurora Carrion[1], Sara Puente-Marin[1], Verónica Chico[1], Luis Mercado[2], Luis Perez [1], Julio Coll[3], Maria del Mar Ortega-Villaizan [1]

[1]Instituto de Biología Molecular y Celular, Miguel Hernández University, Elche, Spain
[2]Institute of Biology, Catholic Pontifical University of Valparaiso, Valparaiso, Chile
[3]Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain

v2 **First published:** 07 Nov 2017, **6**:1968 (doi: 10.12688/f1000research.12994.1)
Latest published: 13 Dec 2017, **6**:1968 (doi: 10.12688/f1000research.12994.2)

Abstract

Background: Some fish viruses, such as piscine orthoreovirus and infectious salmon anemia virus, target red blood cells (RBCs), replicate inside them and induce an immune response. However, the roles of RBCs in the context of infectious pancreatic necrosis virus (IPNV) infection have not been studied yet.
Methods: Ex vivo rainbow trout RBCs were obtained from peripheral blood, Ficoll purified and exposed to IPNV in order to analyze infectivity and immune response using RT-qPCR, immune fluorescence imaging, flow cytometry and western-blotting techniques.
Results: IPNV could not infect RBCs; however, IPNV increased the expression of the INF1-related genes *ifn-1*, *pkr* and *mx* genes. Moreover, conditioned media from IPNV-exposed RBCs conferred protection against IPNV infection in CHSE-214 fish cell line.
Conclusions: Despite not being infected, rainbow trout RBCs could respond to IPNV with increased expression of antiviral genes. Fish RBCs could be considered as mediators of the antiviral response and therefore targets of new strategies against fish viral infections. Further research is ongoing to completely understand the molecular mechanism that triggers this antiviral response in rainbow trout RBCs.

Open Peer Review

Referee Status: ✓ ✓

	Invited Referees 1	2
REVISED version 2 published 13 Dec 2017	✓ report	
version 1 published 07 Nov 2017	? report	✓ report

1 **Espen Rimstad**, Norwegian University of Life Sciences, Norway
Maria Dahle, Norwegian Veterinary Institute, Norway

2 **Niels C. Bols**, University of Waterloo, Canada

Discuss this article

Comments (0)

Corresponding author: Maria del Mar Ortega-Villaizan (mortega-villaizan@umh.es)

Author roles: Nombela I: Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Carrion A**: Formal Analysis, Investigation; **Puente-Marin S**: Investigation; **Chico V**: Formal Analysis, Investigation, Writing – Review & Editing; **Mercado L**: Resources; **Perez L**: Writing – Review & Editing; **Coll J**: Writing – Review & Editing; **Ortega-Villaizan MdM**: Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

How to cite this article: Nombela I, Carrion A, Puente-Marin S *et al.* **Infectious pancreatic necrosis virus triggers antiviral immune response in rainbow trout red blood cells, despite not being infective [version 2; referees: 2 approved]** *F1000Research* 2017, **6**:1968 (doi: 10.12688/f1000research.12994.2)

Grant information: This work was supported by the European Research Council (ERC starting grant 2014 GA639249).
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

First published: 07 Nov 2017, **6**:1968 (doi: 10.12688/f1000research.12994.1)

REVISED **Amendments from Version 1**

In this new version of the manuscript we have included the corrections of Dr. Rimstad and Dr. Bols along the manuscript, in order to improve manuscript understanding. The line of the manuscript has not changed, but the manuscript comprehension has been improved. We have corrected several mistyping and linguistic errors, as well as clarifying some points in the manuscript and eliminating some confusing citations. Therefore, some references have been eliminated and one reference previously unpublished is now updated. Besides, as Dr. Bols suggested, in Figure 3A, FFU/ml has been removed from the y axis and only mentioned in the legend of the figure.

See referee reports

Introduction

Fish viral infections cause significant losses in aquaculture. Infectious pancreatic necrosis (IPN) is a highly contagious viral disease with a high impact on salmonid aquaculture industry. Infectious pancreatic necrosis virus (IPNV) is the causative agent of IPN and was the first fish virus isolated in cell culture[1]. IPNV outbreaks are usually related to high mortality rates in salmonid aquaculture, especially in young individuals[2,3], highlighting the urgent necessity for the development of efficient strategies in vaccination. IPNV belongs to the *Aquabirnavirus* genus within the *Birnaviridae* family. Viruses of this family are non-enveloped particles with a double stranded RNA genome. This genome consists of two segments: the A segment contains the information to encode a protein that is post-translationally cleaved into VP2, VP3 and VP4 viral proteins; the B segment encodes the viral RNA polymerase VP1[4]. VP2 and VP3 are the major structural and immunogenic proteins, as they represent 64% of the total proteins of the virion[5].

In contrast to mammals, fish, reptiles and avian red blood cells (RBCs) are nucleated. Typically, the role associated with RBCs has been the transport of O_2 to different tissues and gas exchange. However, a whole set of biological processes related to the immune response has been recently reported for nucleated RBCs from different species: recognition of pathogen associated molecular patterns[6,7] through expression of pattern recognition receptors, such as toll-like receptors (TLRs)[8]; production of cytokine-like factors[7,9–11]; phagocytosis[12]; and formation of complement immune complexes[13]. Fish RBCs are known to be the target of some viruses, such as infectious salmon anemia virus (ISAV)[11] and piscine orthoreovirus (PRV)[14,15]. Furthermore, both viruses can induce immune responses in infected RBCs, characterized by the expression of genes related to IFN-1 (type I interferon) pathway. Besides, recently it has been shown that viral hemorrhagic septicemia virus (VHSV) halted replication in rainbow trout RBCs could induce cytokine production[16].

In view of the aforementioned evidence, this study was aimed to evaluate the immune response of rainbow trout RBCs against IPNV, one of the most ubiquitous viral fish pathogens. To achieve this objective, we first analyzed the infectivity of IPNV in rainbow trout RBCs. Then, RBCs immune response was evaluated after *ex vivo* exposure to IPNV, by means of antiviral gene and protein expression analysis. Finally, we evaluated the ability of RBCs to confer protection against IPNV in CHSE-214 cells, which are susceptible to IPNV infection. To summarize, here we report the regulation of the immune response of rainbow trout RBCs by IPNV, a non-infective virus in this cell type. This immune response was characterized by the expression of genes related to the IFN-1 pathway, Mx production and induction of an antiviral state to IPNV in CHSE-214 cells.

Methods

Animals

Rainbow trout (*Oncorhynchus mykiss*) individuals of approximately 10 g were obtained from a commercial fish farm (PISZOLLA S.L., CIMBALLA FISH FARM, Zaragoza, Spain). Fish were maintained at the University Miguel Hernandez (UMH) facilities with a re-circulating dechlorinated-water system, at a stocking density of 1 fish/3L, at 14°C, and fed daily with a commercial diet (SKRETTING, Burgos, Spain). Fish were acclimatized to laboratory conditions over 2 weeks before experimentation. The number of fish used is indicated for each experiment/figure.

RBCs purification

Rainbow trout were sacrificed by overexposure to tricaine methanesulfonate (Sigma-Aldrich, Madrid, Spain) at 0.2 g/L. Peripheral blood was sampled from the caudal vein using insulin syringes (NIPRO Bridgewater, NJ). Approximately 100 µL of blood was diluted in RPMI-1640 medium (Dutch modification) (Gibco, Thermo Fischer Scientific Inc., Carlsbad, CA) supplemented with 10% FBS (Cultek, Madrid, Spain), 1 mM pyruvate (Gibco), 2 mM L-glutamine (Gibco), 50 µg/mL gentamicin (Gibco), 2 µg/mL fungizone (Gibco) and 100 U/mL penicillin/streptomycin (Sigma-Aldrich). Then, RBCs were purified by two consecutive density gradient centrifugations with Histopaque 1077 (7206g, Ficoll 1.007; Sigma-Aldrich). Finally, RBCs were washed twice with RPMI 2% FBS. Purity of RBCs of 99.9% was estimated by optical microscopy evaluation. Then, purified RBCs were cultured in the above indicated medium at a density of 10^7 cells/mL, in cell culture flasks, at 14°C, overnight.

Viral infection assays

Ex vivo rainbow trout RBCs along with CHSE-214 cell line (Chinook Salmon Embryo, ATCC CRL-1681) were infected using IPNV Sp strain[17]. IPNV was grown as previously described[18]. *Ex vivo* RBCs exposure to IPNV was performed by incubating RBCs with diluted IPNV at the indicated MOI (multiplicity of infection) in RPMI 2% FBS. After three hours of incubation at 14°C, RBCs were centrifuged at 1600 rpm for 5 minutes and then washed with medium to completely eliminate the non-adsorbed excess of virus. Finally, RBCs were placed in 24 well plates (Corning Costar, Sigma-Aldrich, Madrid, Spain) with 500 µl of RPMI 2% FBS. The whole process was done at 14°C. Infection of the CHSE-214 cell line was done by incubating IPNV diluted in RPMI 2% FBS at the desired MOI for 1 hour at 14°C. After that, medium was removed and RPMI 2% FBS was added to each well. Infected CHSE-214 cells were maintained at 14°C[18].

In time course experiments, the initial supernatant with IPNV was not removed. When each of the time points was reached, RBCs were washed with cell culture medium and CHSE-214 cells with PBS supplemented with calcium.

Viral titration assay

The virus titer in IPNV-exposed RBCs supernatants was quantified by $TCID_{50}$ and by RT-qPCR. Briefly, different dilutions of the supernatants (from 10^{-1} to 10^{-4}) were added to CHSE-214 cell monolayers, and incubated at 14°C for 90 minutes. Then, the virus was removed and infected CHSE-214 cell monolayers covered with a solution of RPMI 2% FBS. Cell plates were incubated at 14°C for 7 days. For RT-qPCR titration, 30 µL of IPNV with known titer (10^9 $TCID_{50}$/mL) and 30 µL of IPNV-exposed RBCs supernatants were used to extract RNA and synthetize cDNA, as explained hereafter. Ten-fold serial dilutions from 10^8 to 10^2 $TCID_{50}$/mL were done to obtain IPNV cDNA and create a standard line.

RNA isolation and DNAse treatment

The E.Z.N.A.® Total RNA Kit (Omega Bio-Tek Inc., Norcross, GA) was used for total RNA extraction, in accordance with manufacturer's instructions. DNAse treatment was done in order to eliminate residual genomic DNA using TURBO™ DNase (Ambion, Thermo Fischer Scientific Inc.), following the manufacturer's instructions. RNA was quantified with a NanoDrop® 377 Spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Gene expression by RT-qPCR

cDNA was synthesized from RNA using M-MLV reverse transcriptase (Invitrogen, Thermo Fischer Scientific Inc.), as previously described[19]. Final concentration of cDNA was 6 ng/µL. RT-qPCR reactions were performed in a total volume of 20 µl using 12 ng of cDNA, 10 µl of TaqMan universal PCR master mix (Thermo Fischer Scientific), 900 nM final concentration of each primer (300 nM for IPNV segment A) and 300 nM of probe (150 nM for IPNV segment A). RT-qPCR was performed using the ABI PRISM 7300 System (Thermo Fischer Scientific). Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Gene expression was analyzed by the $2^{-\Delta\Delta}$Ct method[20]. The eukaryotic 18S rRNA gene (Cat#4310893E, Thermo Fischer Scientific) was used as endogenous control. Primers and probes are listed in Table 1.

Antibodies

Several antibodies were used to stain cells for cytokines and to measure polypeptides in RBCs extracts by western blotting. They are briefly described below and their Research Resource Identifiers (RRIDs) given. For intracellular staining, mouse polyclonal antibodies against rainbow trout IL1β (RRID: AB_2716269)[21,22], IL8 (RRID: AB_2716272)[23] and TNF-α (RRID: AB_2716270)[24] were produced at the laboratory of Dr. Luis Mercado. Rabbit polyclonal antibody against rainbow trout Mx3 (RRID: ABA_2716267)[25,26] was produced at the laboratory of Dr. Amparo Estepa. Anti-IPNV-VP3 monoclonal antibody 2F12 (RRID: AB_2716296) was used for IPNV labelling[27]. Anti-rabbit IgG (H+L) CF™ 488 antibody produced in goat and anti-mouse IgG (H+L) CF™ 488 antibody produced in goat were used as secondary antibodies for proteins and anti-mouse IgG (H+L) CF™ 647 produced in goat to detect 2F12 antibody.

For western blotting, rabbit polyclonal antibody against human eIF2α-P (Cat# E2152, RRID:AB_259283) and rabbit polyclonal antibody against human α-Actin (Cat#2066, RRID: AB_476693) were purchased from Sigma-Aldrich.

Western blot

Control and IPNV-exposed RBCs pellets (≈10^7 cells) were used for protein extraction. Cell pellets were washed 3 times with PBS and then resuspended in 30 µl of PBS with a cocktail of protease inhibitors (Sigma-Aldrich). Then, cells were frozen/thawed 3 times and lysed using an eppendorf micropistile (Eppendorf, Hamburg, Germany). Samples were loaded in Tris–Glycine sodium dodecyl sulfate 12% polyacrylamide gels under reducing conditions. Electrophoresis was performed at 200 V for 60 min. For blotting, the proteins in the gel were transferred for 80 min at 100 V in transfer buffer (2.5 mM Tris, 9 mM glycine, 20% methanol) to nitrocellulose membranes (BioRad, Madrid, Spain). Then, membranes were blocked with 8% dry milk and 1% Tween-20 in PBS and incubated with eIF2α-P or α-Actin antibodies, at the recommended dilutions in PBS containing 0.5% dry milk and 0.5% Tween-20 at 4°C and overnight. Incubation with secondary antibody GAR-Po (Sigma-Aldrich) was done in 0.5% milk 0.5% Tween-20 in PBS for 45 min. Membranes were washed 3 times with PBS containing 1% dry milk 0.5% Tween-20 for 15 min after every antibody incubation. Finally, the membrane was washed 3 times with PBS before analysis of the peroxidase activity. Peroxidase activity was detected using ECL chemiluminescence reagents (Amersham Biosciences, Buckinghamshire, UK) and revealed by exposure to X-ray. Protein bands from western blotting were analysed by densitometry using the *Scion Image 4.0.2* Software (RRID: SCR_008673) (www.scionorg.com).

Intracellular immunofluorescence stain and flow cytometry

RBCs were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) and 0.08% glutaraldehyde (Sigma-Aldrich) diluted in RPMI medium for 20 minutes. Then, RBCs were incubated with permeabilization buffer containing 0.05% saponin (Sigma-Aldrich) in RPMI, for 15 minutes. Primary antibodies were used at 1/50 dilution for IL-1β, IL-8 and TNF-α, 1/300 for Mx and 1/500 for 2F12 in permeabilization buffer and incubated for 60 minutes at room temperature. Secondary antibodies were incubated for 30 minutes at 1/200 dilution. RBCs were washed with permeabilization buffer after antibody incubations. Finally, RBCs were kept in PFA 1% in PBS. For nuclear staining, RBCs were stained with 1 µg/mL of 4′-6-408 Diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 5 minutes. Flow cytometry (FC) analysis was done in a BD FACSCanto™ II (BD Biosciences) flow cytometer. Immunofluorescence (IF) images were performed in the INCell Analyzer 6000 Cell imaging system (GE Healthcare, Little Chalfont, UK).

Antiviral activity of conditioned medium

Conditioned medium (CM) was obtained from control and IPNV-exposed RBCs at MOI 0.5, during 3 days. The CMs were clarified at 1600 rpm for 5 min. IPNV titer in the

Table 1. List of primers and probes.

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')	Probe (5' – 3')	Reference or accession number
IPNV SA	TCTCCCGGGCAGTTCAAGT	CGGTTTCACGATGGGTTGTT	CCAGAACCAGGTGACGAGTATGAGGACTACAT	18
tlr3	ACTCGGTGGTGCTGGTCTTC	GAGGAGGCAATTTGGACGAA	CAAGTTGTCCCGCTGTCTGCTCCTG	NM_001124578.1
irf7	CCCAGGGTTCAGCTCCACTA	GGTCTGGCAACCCGTCAGT	TCGAGCCAAACACCAGCCCCT	AJ829673
ifn1	ACCAGATGGGAGGAGATATCACA	GTCCTCAAACTCAGCATCATCTATGT	AATGCCCCAGTCCTTTTCCCAAATC	AM489418.1
mx1–3	TGAAGCCCAGGATGAAATGG	TGGCAGGTCGATGAGTGTGA	ACCTCATCAGCCTAGAGATTGGCTCCCC	28
pkr	GACACCGCGTACCGATGTG	GGACGAACTGCTGCCTGAAT	CACCACCTCTGAGAGCGACACCACTTC	NM_001145891.1
il8	AGAGACACTGAGATCATTGCCAC	CCCTCTTCATTTGTTGTTGGC		29
ifnγ	CAAACTGAAAGTCCACTATAAGATCTCCA	TCCTGAATTTTCCCCTTGACATATTT		30
tnfα	AGCATGGAAGACCGTCAACGAT	ACCCTCTAAATGGATGGCTGCTT		31

supernatants of IPNV-exposed RBCs resulted in 10 $TCID_{50}$/mL or less, therefore viral presence in the supernatants was obviated. To test the antiviral activity of the CM, confluent CHSE-214 cells (7.8×10^4 cells/well), seeded in 96 well plates, were pre-treated with 100 µL of each supernatant at the indicated dilutions for 24 hours. After that, CHSE-214 cells were infected, as described previously, with IPNV at MOI 0.05, for 24 hours. Finally, intracellular staining of IPNV foci was carried out.

Intracellular staining of IPNV foci

CHSE-214 cells were fixed with PFA diluted at 4% in PBS followed by a second fixation with cold methanol. Each fixation step lasted 15 minutes. Cells were washed with PBS after each fixation step. Blocking buffer containing 5% goat serum (Sigma-Aldrich) and 0.3% Triton X-100 (Sigma-Aldrich) was added to each well with the cells for 1 hour. Then, anti-VP3 2F12 antibody was diluted 1/500 in antibody dilution buffer (1% BSA (Sigma-Aldrich), 0.3% Triton X-100) and was incubated for 1 hour. FITC-labelled goat anti-rabbit was used as secondary antibody at 1/300 dilution. Cells were washed three times after each antibody incubation with PBS. IF images were taken INCell Analyzer 6000 imaging system. *IN Cell Developer Toolbox 1.9.2* (RRID: SCR_015790; GE Healthcare, Little Chalfont, UK) was used to count number of IPNV foci (positive areas after image segmentation were selected when >21000 fluorescence units and >2500 μm^2 criteria was reached).

MTT assays

Cell viability was tested using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay[32]. Briefly, 25 µl of MTT at a final concentration of 1.9 mg/mL were added to previously treated CHSE-214 cells monolayers, seeded in 96 well plates. Cells were incubated for 3 hours at 21°C with the reagent. Then, the medium was removed from the wells. Formazan crystals were dissolved in 100 µl of 100% DMSO, incubated for 30 minutes. Absorbance was read at 570 nm in the EON™ microplate spectrophotometer (Biotek, Winooski, VT). Percentage of viable cells was calculated as follows: absorbance treated cells/absorbance non-treated cells) x100.

Software and statistics

All the figures and graphics show the mean and standard deviation of the data. P-values associated with each graphic are represented by the legends: *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001, ****, p-value < 0.0001. *Graphpad Prism 6* (RRID: SCR_002798, www.graphpad.com) (Graphpad Software Inc., San Diego, A) was used for preparing graphs and preforming statistical calculations. FC data were analyzed using *Flowing Software 2.5.1* (RRID: SCR_015781)(www.flowingsoftware.com) to obtain Mean Fluorescence Intensity (MFI) and Mean Relative Fluorescence Intensity (MRFI) (relative to control cells) values.

Ethics approval

Methodology was carried out in accordance with the Spanish Royal Decree RD 53/2013 and EU Directive 2010/63/EU for animals used in research experimentation. All experimental protocols involving animal handling were also reviewed and approved by the Animal Welfare Body and the Research Ethics Committee at the Miguel Hernandez University (approval number 2014.205.E.OEP; 2016.221.E.OEP) and performed by qualified research personnel.

Results

IPNV did not infect rainbow trout RBCs

To evaluate the infectivity of IPNV in rainbow trout RBCs, RBCs were exposed to IPNV at MOI 0.5 and the viral RNA was evaluated by RT-qPCR in the cell pellet at different times post-exposure. IPNV infectivity was also evaluated in parallel in the CHSE-214 cell line, used as a positive control of infection. IPNV segment A (IPNV-A) RNA levels inside RBCs and CHSE-214 cell line were similar at 1 and 3 hours post-exposure (hpe) (Figure 1A). After 6 hpe, IPNV-A RNA level was 3 logarithms lower in RBCs in comparison with CHSE-214 cells. On the other hand, the titer of IPNV in the supernatants from IPNV-exposed RBCs at a MOI of 0.5 and 5, was evaluated by $TCID_{50}$, at 3 days post-exposure (dpe), and showed a recovered titer of 5 and 4 logarithms lower, respectively (Figure 1B). Furthermore, the supernatants titrated by RT-qPCR, were below the lowest limit of detection 10^2 $TCID_{50}$ (Table 2). Moreover, FC analysis of control and IPNV-exposed RBCs for IPNV VP3 protein did not show significant differences (Figure 1C and D). Therefore, IPNV did not infect rainbow trout RBCs.

IPNV exposure increased the expression of interferon-related antiviral genes and proteins in rainbow trout RBCs

To determine if IPNV would induce an antiviral response in RBCs, RT-qPCR analysis of IFN-related antiviral genes was performed for IPNV-exposed RBCs. The results showed that *mx1–3* and *pkr* genes were significantly expressed at 72 hpe. On the other hand, *ifn1* gene presented a tendency to increase its expression after 6 hpe, having a peak at 24 hpe. Also, *tlr3* gene expression tended to be upregulated at 24 hpe, whereas *irf7* expression was upregulated at 72 hpe (Figure 2A). Three and six dpe with IPNV, RBCs were stained intracellularly with an anti-Mx antibody and analyzed by FC and immunofluorescence imaging (IF). The results showed a significant increment in the expression of Mx protein at 6 dpe by both FC an IF (Figure 2B and D). FC histograms showed, at 6 dpe, that RBCs depicted distinct peaks of Mx expression, showing that the expression of Mx in RBCs was heterogeneous in the total RBCs population (Figure 2C).

Conditioned medium from IPNV-exposed RBCs protected CHSE-214 cells against IPNV infection

To analyze if IPNV-exposed RBCs could secrete factors that were capable to protect other fish cells against IPNV infection, conditioned medium (CM) from control and IPNV-exposed RBCs (with IPNV titer <10 $TCID_{50}$/mL) were added to CHSE-214 cells prior to infection. Figure 3A shows a significant decrease in the number of IPNV infective focus forming units

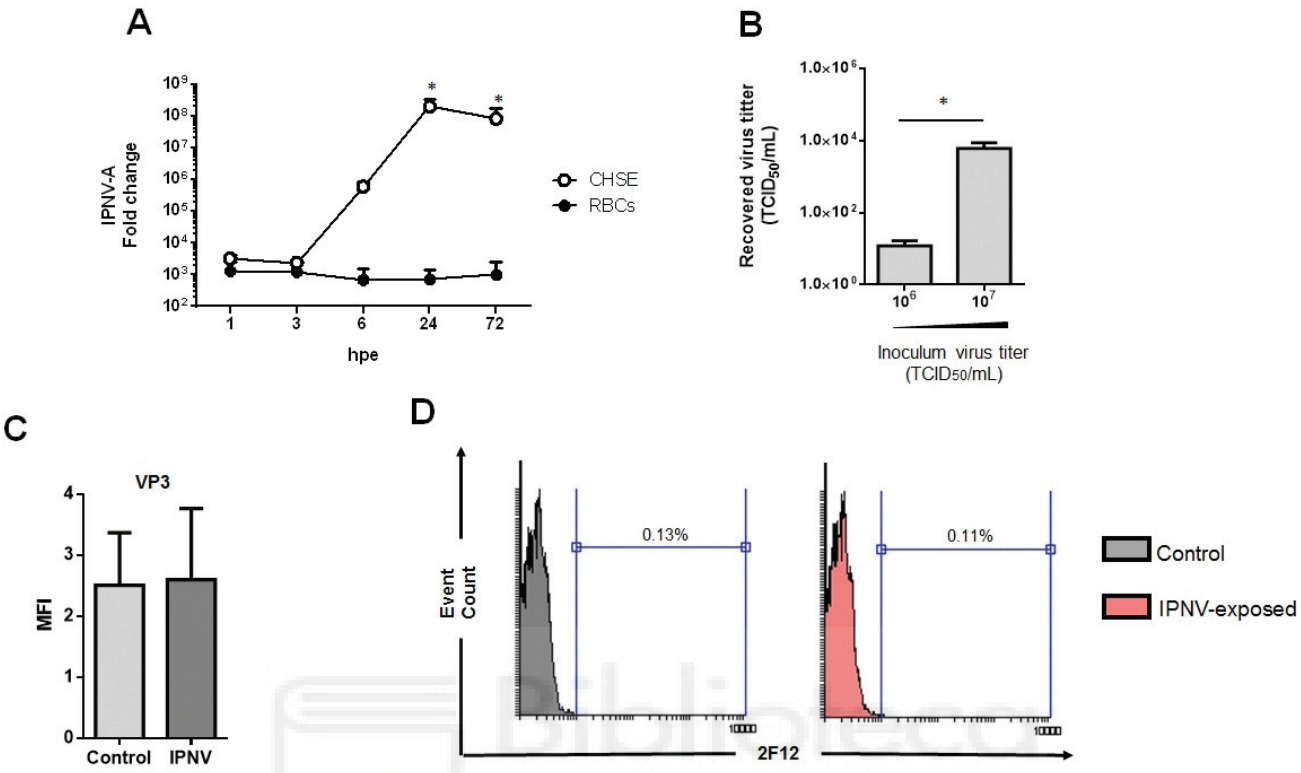


Figure 1. Infectivity of IPNV in RBCs. (**A**) Time-course experiment of the expression of IPNV segment A (IPNV-A) in RBCs (●) (n = 6) and CHSE-214 cells (○) (n = 2) at MOI 0.5. Data is represented as mean±SD. Kruskal-Wallis Test with Dunn´s Multiple Comparison post-hoc test was performed among all time-points post-exposure in comparison with control time point (0 hpi) (*, p-value < 0.05). (**B**) Recovered virus titer in supernatants from IPNV-exposed RBCs with an inoculum titer of 10^6 (MOI 0.5) and 10^7 (MOI 5) $TCID_{50}$/mL obtained after 72 hpe (n = 5). Data is represented as mean±SD. Mann-Whitney test was performed among both conditions (*, p-value < 0.05). (**C**) MFI (mean fluorescence intensity) of viral protein VP3 in control and IPNV-exposed RBCs at MOI 0.5 and 3 dpe (n = 6) Mann-Whitney test was performed among both conditions. (**D**) Representative flow cytometry histograms of IPNV VP3 protein detection in control and IPNV-exposed RBCs at MOI 0.5 and 3 dpi.

Table 2. Rt-qPCR virus titration. Ct value ± standard deviation from standard line points (10^8 to 10^2 dilutions) and supernatants from IPNV-exposed RBCs at MOI 0.5, at 3 and 6 dpe. (n=7 individuals).

Sample	Ct value ± SD
10^8 $TCID_{50}$	25,885 ± 0,052
10^7 $TCID_{50}$	29,856 ± 0,117
10^6 $TCID_{50}$	33,165 ± 0,168
10^5 $TCID_{50}$	36,057 ± 0,11
10^4 $TCID_{50}$	39,126 ± 0.873
10^3 $TCID_{50}$	Undetected
10^2 $TCID_{50}$	Undetected
RBCs #1 3 dpe	Undetected
RBCs #1 6 dpe	Undetected
RBCs #2 3 dpe	Undetected
RBCs #2 6 dpe	Undetected
RBCs #3 3 dpe	Undetected
RBCs #3 6 dpe	Undetected
RBCs #4 3 dpe	Undetected
RBCs #4 6 dpe	Undetected
RBCs #5 3 dpe	Undetected
RBCs #5 6 dpe	Undetected
RBCs #6 3 dpe	Undetected
RBCs #6 6 dpe	Undetected
RBCs #7 3 dpe	Undetected
RBCs #7 6 dpe	Undetected
NTC	Undetected

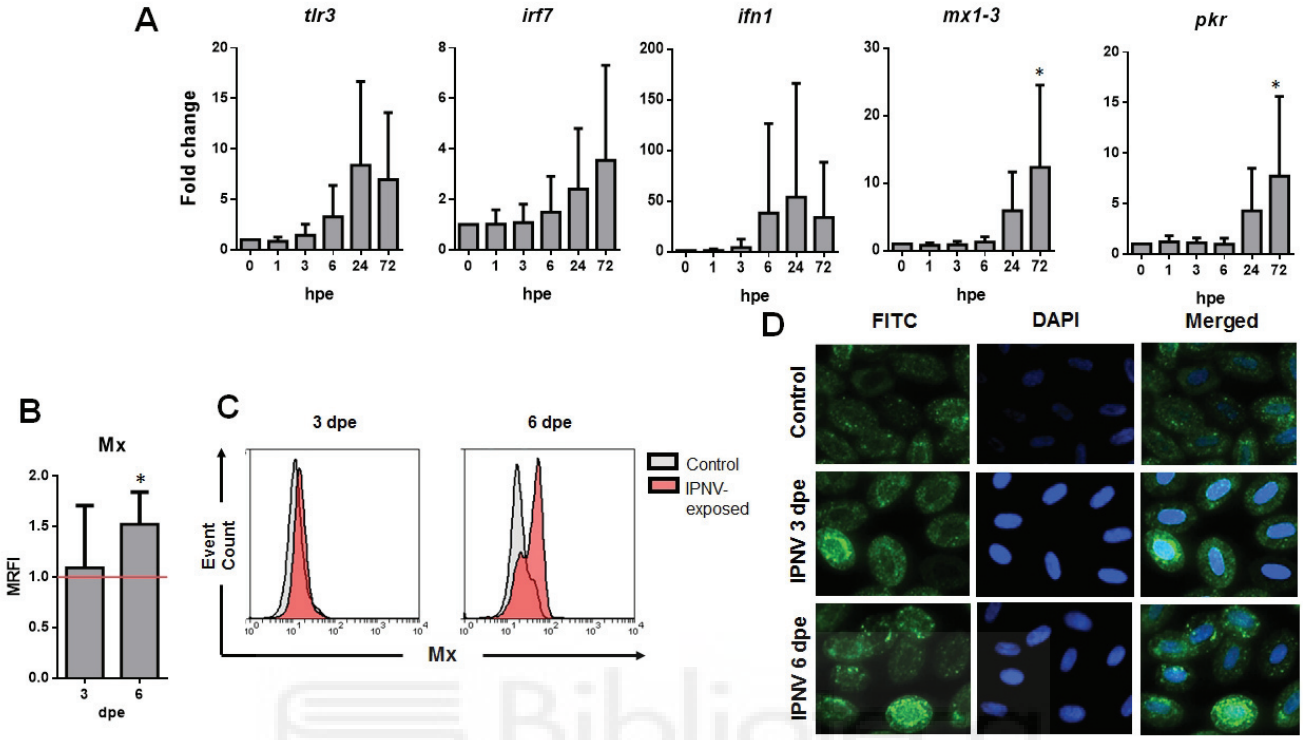


Figure 2. RBCs IFN-related antiviral response against IPNV. (**A**) Gene expression of *tlr3*, *irf7*, *inf1*, *mx1–3* and *pkr* in IPNV-exposed RBCs at the indicated times post-infection and MOI 0.5, measured by RT-qPCR. Data represent mean±SD (n = 6). Kruskal-Wallis Test with Dunn´s Multiple Comparison post-hoc test was performed among all time-points post-exposure in comparison with control time point (0 hpi) (*, p-value < 0.05). (**B**) Mx protein MRFI (mean relative fluorescent intensity, relative to control cells) in IPNV-exposed RBCs at MOI 0.5 (n = 5). (**C**) Flow cytometry histograms of Mx protein expression from control (grey) and IPNV-exposed (red) RBCs at MOI 0.5 and the indicated days post-exposure (dpe). (**D**) Representative immunofluorescence images of Mx protein expression in control and IPNV-exposed RBCs at MOI 0.5 (FITC – Mx protein expression, DAPI - Nuclei) (IF representative of 40 images).

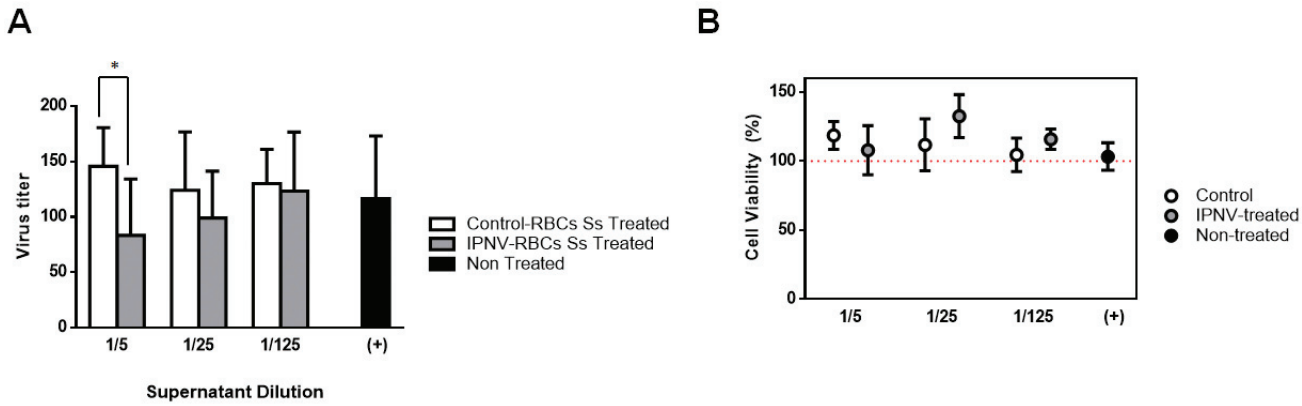


Figure 3. Antiviral activity of the conditioned media from IPNV-exposed RBCs. (**A**) Viral titers (FFU/mL) in CHSE-214 cells infected with IPNV at MOI 0.05 previously non-treated (black) or treated with either supernatants from control RBCs (white) or IPNV-exposed RBCs (grey), during 24 hours, at the indicated dilutions (n = 4, performing triplicates from each individual). Two-way ANOVA, with Sidak´s multiple comparison test, was performed among the different dilutions and conditions to test statistical differences. (**B**) Percentage of viable CHSE-214 cells pre-treated with conditioned medium from control and IPNV-exposed RBCs, during 24 hours, and relative to non-treated CHSE-214 cells. Percentage of viable cells was calculated as follows: absorbance treated cells/absorbance non-treated cells) x100.

(FFU/mL) when pre-treating with 1/5 diluted CM from IPNV-exposed RBCs. CHSE-214 cells viability, by means of an MTT colorimetric assay, was not affected by the exposure to CM (Figure 3B).

IPNV exposure decreased the expression of cytokines in rainbow trout RBCs

To evaluate whether *ex vivo* rainbow trout RBCs could produce cytokines in response to IPNV exposure, RBCs were exposed to IPNV and IL-1 β, IL-8 and TNF-α protein levels were evaluated by means of FC and IF in control and IPNV-exposed cell cultures. The results showed a decrease in the protein expression of IL-1β, IL-8 and TNFα in IPNV-exposed RBCs (Figure 4A).

IPNV exposure did not induce phosphorylation of the α-subunit of the eukaryotic translational initiation factor 2 (eIF2α) in rainbow trout RBCs

The phosphorylation of the translation initiation factor eIF2α is a key mechanism of global inhibition of translational initiation[33] and it has been described to happen after IPNV infection in the permissive cell line CHSE-214 cells[34]. In this sense, since IPNV-exposed RBCs depicted a small downregulation of the evaluated cytokines protein levels, we further investigated whether IPNV exposure could reduce protein translation in RBCs by triggering the phosphorylation of eIF2α. However, the results revealed no changes in the phosphorylation of eIF2α (Figure 4B).

Dataset 1. Excel file containing qPCR data

http://dx.doi.org/10.5256/f1000research.12994.d182842

Each sheet contains the raw Ct values for the indicated figure numbers, organized by samples (rows) and genes (columns).

Dataset 2. Excel file containing the virus titration data

http://dx.doi.org/10.5256/f1000research.12994.d182843

Contains the virus titer ($TCID_{50}$/mL) results of the indicated figure number.

Dataset 3. Flow cytometry data

http://dx.doi.org/10.5256/f1000research.12994.d182844

Each folder contains the Flow Cytometry Standard (.fcs) format files. Source data files are organized by figure number, and then by sample number, condition and antibody.

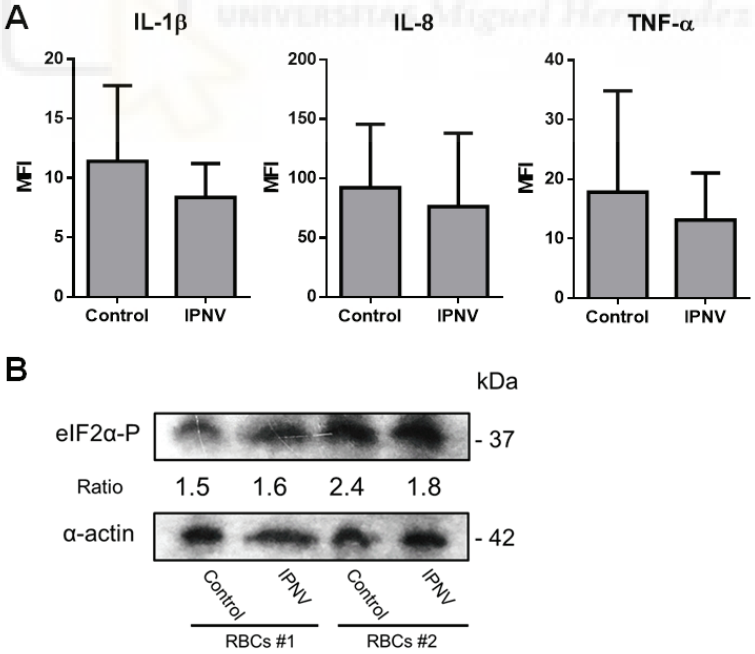


Figure 4. IPNV-exposure decreased cytokine levels in rainbow trout RBCs. (**A**) Intracellular MFI (mean fluorescent intensity) values of IL-1β, IL-8 and TNFα from control and IPNV-exposed RBCs at MOI 0.5 and 3 dpe measured by FC (flow cytometry)(n = 6). Mann-Whitney test was performed among both conditions. (**B**) Phosphorylation of translation initiation factor eIF2α in IPNV-exposed RBCs. Representative western blot of eIF2α-P in control and IPNV-exposed RBCs from two individuals at MOI 0.5, 3 dpe. Densitometry ratios were done relativizing to α-actin. Mann-Whitney test was performed among both conditions.

Dataset 4. Excel file containing the Focus Forming Units (FFU) counting for Figure 3A

http://dx.doi.org/10.5256/f1000research.12994.d182845

Dataset 5. Excel file containing MTT absorbance raw data

http://dx.doi.org/10.5256/f1000research.12994.d182846

Dataset 6. Excel file containing the densitometry raw data of eIF2α-P and α-Actin western blots

http://dx.doi.org/10.5256/f1000research.12994.d182847

Related uncropped blots are included.

Discussion

Previously, we have demonstrated that rainbow trout RBCs can respond to VHSV, a ssRNA virus not targeting RBCs, halting its replication, downregulating type I interferon-related genes, showing global protein downregulation in the cell and phosphorylation of the translation initiation factor eIF2α[16].

It is known that IPNV primarily targets pancreatic and liver cells[35]. It has been also reported that IPNV was detectable in kidney hematopoietic tissue, corpuscles of Stannius, in Islets of Langherhans, in the lamina propria of the pyloric caeca, the gill arch connective tissue, the ventricle of the heart and dermis of the skin[35]. Our results showed that IPNV did not replicate in RBCs, although small amounts of IPNV were persistently found inside RBCs after 3 dpe (≈ 10^3 $TCID_{50}$/mL). Similarly, IPNV has been shown to enter mammalian cells, without significant levels of replication, being this entry suggested to be receptor mediated[36]. From our results, the persistence of IPNV in RBCs after 72 hpe could point out the entry of the virus inside RBCs. However, we could not further verify the presence of the IPNV inside RBCs (Figure 1).

Nevertheless, despite the lack of replication of IPNV in RBCs, IPNV could induce an antiviral gene expression mediated by the IFN pathway, as it has been observed in RBCs productive infections with ISAV[11] and PRV[14]. As shown by our results, *ifn1* and IFN-1 related genes (*irf7*, *pkr* and *mx*) expression levels were increased time-dependently in response to IPNV-exposure. High inter-individual variability was observed, similarly to that found in the RBCs from salmons challenged with PRV[37]. In addition, although we could not verify the entry and uncoating of IPNV inside RBCs, we could observe an increment in the expression of the *tlr3* gene in parallel to the expression of the other IFN-related genes in IPNV-exposed RBCs. This could indicate the activation of the TLR3/IFN pathway by the presence of intracellular viral dsRNA.

IFN-1 leads to the expression of interferon stimulated genes (ISGs)[38]. Among ISGs, the antiviral protein Mx has a well characterized antiviral role. Confirming those expectations, our results showed the significant upregulation of the Mx protein 6 dpe, after having a peak of its gene expression at 3 dpe. Previously, a positive correlation between the expression of Mx protein and the inhibition of IPNV in CHSE-214 cells has been established[39]. Therefore, Mx protein production in IPNV-exposed RBCs could be involved in the low IPNV titers observed. The high basal levels of Mx protein detected inside RBCs (Figure 2D), much elevated than those for CHSE-214 cells (Figure S1), could be implicated in the early disappearance of IPNV inside RBCs. A similar hypothesis has been made in the abortive infection of VHSV in the RTS-11 cell line[40] and in rainbow trout RBCs[16], where upregulation or high constitutive expression of *mx* genes was speculated to be related to the inhibition of the virus.

Moreover, our results showed that CM from RBCs exposed to IPNV could partially protect CHSE-214 cells from IPNV infection. Similar to other cell types, this antiviral activity has been also observed in CM of RTS11 and RTG-2 cells exposed to Poly (I:C) (polyinosinic:polycytidylic acid) and/or infected with chum salmon reovirus[41]. The fact that RBCs can secrete factors that confer protection against IPNV infection in other cell lines could indicate that RBCs, despite not being permissive to IPNV infection, may exhibit an antiviral response. Besides, we evaluated the production of cytokines in IPNV-exposed RBCs. Previously, the expression of IL-1β in salmon gill and head kidney tissues[42], IL-8 in rainbow trout head kidney tissue[43] and TNFα in zebrafish embryonic cells[44] have been implicated in the immune response against IPNV; therefore, we chose these cytokines to evaluate the immune response of rainbow trout RBCs to IPNV exposure. However, our results showed a reduction trend of these proteins in IPNV-exposed RBCs.

A shutdown in protein synthesis by phosphorylation of eIF2α has been reported in CHSE-214 cells infected with IPNV[34]. So far, in rainbow trout RBCs exposed to IPNV, although a trend to cytokine protein reduction was observed, no phosphorylation of eIF2α was detected and Mx protein expression was increased. IFN-1 has been reported to inhibit the production of IL-1β[45], therefore, the cytokine reduction trend observed could have been a result of the related IFN-1 pathway upregulation. In contrast, in rainbow trout RBCs, VHSV rhabdovirus induced phosphorylation of eIF2α and a cell shut-off characterized by the downregulation of the proteome[16].

Further studies are needed to completely understand the molecular mechanism through which IPNV triggers this immune response in rainbow trout RBCs. However, the lack of commercial antibodies against fish proteins involved in cell signaling networks limits the study of this area. The implication of RBCs during *in vivo* IPNV infection and the response against different strains of IPNV remains to be evaluated.

Finally, one of the potential applications of these results is that fish RBCs could be considered mediators of the antiviral response and therefore targets of novel DNA vaccines and of new strategies against fish viral infections.

Data availability

Dataset 1. Excel file containing qPCR data. Each sheet contains the raw Ct values for the indicated figure numbers, organized by samples (rows) and genes (columns). doi, 10.5256/f1000research.12994.d182842[46]

Dataset 2. Excel file containing the virus titration data. Contains the virus titer ($TCID_{50}$/mL) results of the indicated figure number. doi, 10.5256/f1000research.12994.d182843[47]

Dataset 3. Flow cytometry data. Each folder contains the Flow Cytometry Standard (.fcs) format files. Source data files are organized by figure number, and then by sample number, condition and antibody. doi, 10.5256/f1000research.12994.d182844[48]

Dataset 4. Excel file containing the Focus Forming Units (FFU) counting for Figure 3A. doi, 10.5256/f1000research.12994.d182845[49]

Dataset 5. Excel file containing MTT absorbance raw data. doi, 10.5256/f1000research.12994.d182846[50]

Dataset 6. Excel file containing the densitometry raw data of eIF2α-P and α-Actin western blots. Related uncropped blots are included. doi, 10.5256/f1000research.12994.d182847[51]

Competing interests

No competing interests were disclosed.

Grant information

This work was supported by the European Research Council (ERC starting grant 2014 GA639249).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

Special thanks to Remedios Torres and Efren Lucas for their technical assistance.

Supplementary material

Figure S1. qPCR Amplification plot of mx1-3 gene expression in RBCs and CHSE-214 cells.

Click here to access the data.

References

1. Wolf K, Snieszko SF, Dunbar CE, *et al.*: **Virus nature of infectious pancreatic necrosis in trout.** *Proc Soc Exp Biol Med.* 1960; **104**: 105–8.
PubMed Abstract | Publisher Full Text
2. Zhu L, Wang X, Wang K, *et al.*: **Outbreak of infectious pancreatic necrosis virus (IPNV) in farmed rainbow trout in China.** *Acta Trop.* 2017; **170**: 63–9.
PubMed Abstract | Publisher Full Text
3. Snieszko SF: **Your fishes' health: Diseases of fishes.** *Trop Fish Hobbyist.* 1981; **30**(3).
Reference Source
4. Dobos P, Hill BJ, Hallett R, *et al.*: **Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes.** *J Virol.* 1979; **32**(2): 593–605.
PubMed Abstract | Free Full Text
5. Dobos P: **The molecular biology of infectious pancreatic necrosis virus (IPNV).** *Ann Rev Fish Dis.* 1995; **5**: 25–54.
Publisher Full Text
6. Baum J, Ward RH, Conway DJ: **Natural selection on the erythrocyte surface.** *Mol Biol Evol.* 2002; **19**(3): 223–9.
PubMed Abstract | Publisher Full Text
7. Morera D, Roher N, Ribas L, *et al.*: **RNA-Seq reveals an integrated immune response in nucleated erythrocytes.** *PLoS One.* 2011; **6**(10): e26998.
PubMed Abstract | Publisher Full Text | Free Full Text
8. Rodriguez MF, Wiens GD, Purcell MK, *et al.*: **Characterization of Toll-like receptor 3 gene in rainbow trout (*Oncorhynchus mykiss*).** *Immunogenetics.* 2005; **57**(7): 510–9.
PubMed Abstract | Publisher Full Text
9. Passantino L, Massaro MA, Jirillo F, *et al.*: **Antigenically activated avian erythrocytes release cytokine-like factors: a conserved phylogenetic function discovered in fish.** *Immunopharmacol Immunotoxicol.* 2007; **29**(1): 141–52.
PubMed Abstract | Publisher Full Text
10. Passantino L, Altamura M, Cianciotta A, *et al.*: **Maturation of fish erythrocytes coincides with changes in their morphology, enhanced ability to interact with *Candida albicans* and release of cytokine-like factors active upon autologous macrophages.** *Immunopharmacol Immunotoxicol.* 2004; **26**(4): 573–85.
PubMed Abstract | Publisher Full Text
11. Workenhe ST, Kibenge MJ, Wright GM, *et al.*: **Infectious salmon anaemia virus replication and induction of alpha interferon in Atlantic salmon erythrocytes.** *Virol J.* 2008; **5**: 36.
PubMed Abstract | Publisher Full Text | Free Full Text
12. Passantino L, Altamura M, Cianciotta A, *et al.*: **Fish immunology. I. Binding and engulfment of *Candida albicans* by erythrocytes of rainbow trout (*Salmo gairdneri* Richardson).** *Immunopharmacol Immunotoxicol.* 2002; **24**(4): 665–78.
PubMed Abstract | Publisher Full Text
13. Schraml B, Baker MA, Reilly BD: **A complement receptor for opsonized immune complexes on erythrocytes from *Oncorhynchus mykiss* but not *Ictalarus punctatus*.** *Mol Immunol.* 2006; **43**(10): 1595–603.
PubMed Abstract | Publisher Full Text
14. Finstad OW, Dahle MK, Lindholm TH, *et al.*: **Piscine orthoreovirus (PRV) infects Atlantic salmon erythrocytes.** *Vet Res.* 2014; **45**: 35.
PubMed Abstract | Publisher Full Text | Free Full Text
15. Wessel Ø, Olsen CM, Rimstad E, *et al.*: **Piscine orthoreovirus (PRV) replicates in Atlantic salmon (*Salmo salar* L.) erythrocytes *ex vivo*.** *Vet Res.* 2015; **46**: 26.
PubMed Abstract | Publisher Full Text | Free Full Text
16. Nombela I, Puente-Marin S, Chico V, *et al.*: **Identification of diverse defense mechanisms in trout red blood cells in response to VHSV halted viral replication [version 1; referees: 3 approved with reservations].** *F1000Res.*

2017; **6**: 1958.
Publisher Full Text

17. Hill BJ, Way K: **Serological classification of infectious pancreatic necrosis (IPN) virus and other aquatic birnaviruses.** *Annu Rev Fish Dis.* 1995; **5**: 55–77.
Publisher Full Text

18. Marroquí L, Estepa A, Perez L: **Inhibitory effect of mycophenolic acid on the replication of infectious pancreatic necrosis virus and viral hemorrhagic septicemia virus.** *Antiviral Res.* 2008; **80**(3): 332–8.
PubMed Abstract | Publisher Full Text

19. Chico V, Gomez N, Estepa A, *et al.*: **Rapid detection and quantitation of viral hemorrhagic septicemia virus in experimentally challenged rainbow trout by real-time RT-PCR.** *J Virol Methods.* 2006; **132**(1–2): 154–9.
PubMed Abstract | Publisher Full Text

20. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.** *Methods.* 2001; **25**(4): 402–8.
PubMed Abstract | Publisher Full Text

21. Rojas V, Camus-Guerra H, Guzmán F, *et al.*: **Pro-inflammatory caspase-1 activation during the immune response in cells from rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) challenged with pathogen-associated molecular patterns.** *J Fish Dis.* 2015; **38**(11): 993–1003.
PubMed Abstract | Publisher Full Text

22. Schmitt P, Wacyk J, Morales-Lange B, *et al.*: **Immunomodulatory effect of cathelicidins in response to a β-glucan in intestinal epithelial cells from rainbow trout.** *Dev Comp Immunol.* 2015; **51**(1): 160–9.
PubMed Abstract | Publisher Full Text

23. Santana P, Palacios C, Narváez E, *et al.*: **Anti-peptide antibodies: A tool for detecting IL-8 in salmonids.** *Electron J Biotechnol.* 2012; **15**(5).
Publisher Full Text

24. Rojas V, Morales-Lange B, Guzmán F, *et al.*: **Immunological strategy for detecting the pro-inflammatory cytokine TNF-alpha in salmonids.** *Electron J Biotechnol.* 2012; **15**(5).
Publisher Full Text

25. Chico V, Martinez-Lopez A, Ortega-Villaizan M, *et al.*: **Pepscan mapping of viral hemorrhagic septicemia virus glycoprotein G major lineal determinants implicated in triggering host cell antiviral responses mediated by type I interferon.** *J Virol.* 2010; **84**(14): 7140–50.
PubMed Abstract | Publisher Full Text | Free Full Text

26. Martinez-Lopez A, Garcia-Valtanen P, Ortega-Villaizan M, *et al.*: **VHSV G glycoprotein major determinants implicated in triggering the host type I IFN antiviral response as DNA vaccine molecular adjuvants.** *Vaccine.* 2014; **32**(45): 6012–9.
PubMed Abstract | Publisher Full Text

27. Domínguez J, Hedrick RP, Sánchez-Vizcaino JM: **Use of monoclonal-antibodies for detection of infectious pancreatic necrosis virus by the enzyme-linked-immunosorbent-assay (ELISA).** *Dis Aquat Organ.* 1990; **8**: 157–63.
Publisher Full Text

28. Ortega-Villaizan M, Chico V, Martinez-Lopez A, *et al.*: ***In vitro* analysis of the factors contributing to the antiviral state induced by a plasmid encoding the viral haemorrhagic septicaemia virus glycoprotein G in transfected trout cells.** *Vaccine.* 2011; **29**(4): 737–43.
PubMed Abstract | Publisher Full Text

29. Wang T, Bird S, Koussounadis A, *et al.*: **Identification of a novel IL-1 cytokine family member in teleost fish.** *J Immunol.* 2009; **183**(2): 962–74.
PubMed Abstract | Publisher Full Text

30. Wang T, Diaz-Rosales P, Costa MM, *et al.*: **Functional characterization of a nonmammalian IL-21: rainbow trout *Oncorhynchus mykiss* IL-21 upregulates the expression of the Th cell signature cytokines IFN-gamma, IL-10, and IL-22.** *J Immunol.* 2011; **186**(2): 708–21.
PubMed Abstract | Publisher Full Text

31. Purcell MK, Nichols KM, Winton JR, *et al.*: **Comprehensive gene expression profiling following DNA vaccination of rainbow trout against infectious hematopoietic necrosis virus.** *Mol Immunol.* 2006; **43**(13): 2089–106.
PubMed Abstract | Publisher Full Text

32. Roehm NW, Rodgers GH, Hatfield SM, *et al.*: **An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT.** *J Immunol Methods.* 1991; **142**(2): 257–65.
PubMed Abstract | Publisher Full Text

33. Levin D, London IM: **Regulation of protein synthesis: activation by double-stranded RNA of a protein kinase that phosphorylates eukaryotic initiation factor 2.** *Proc Natl Acad Sci U S A.* 1978; **75**(3): 1121–5.
PubMed Abstract | Publisher Full Text | Free Full Text

34. Gamil AA, Mutoloki S, Evensen Ø: **A piscine birnavirus induces inhibition of protein synthesis in CHSE-214 cells primarily through the induction of eIF2α phosphorylation.** *Viruses.* 2015; **7**(4): 1987–2005.
PubMed Abstract | Publisher Full Text | Free Full Text

35. Ellis AE, Cavaco A, Petrie A, *et al.*: **Histology, immunocytochemistry and qRT-PCR analysis of Atlantic salmon, *Salmo salar* L., post-smolts following infection with infectious pancreatic necrosis virus (IPNV).** *J Fish Dis.* 2010; **33**(10): 803–18.
PubMed Abstract | Publisher Full Text

36. Ørpetveit I, Küntziger T, Sindre H, *et al.*: **Infectious pancreatic necrosis virus (IPNV) from salmonid fish enters, but does not replicate in, mammalian cells.** *Virol J.* 2012; **9**: 228.
PubMed Abstract | Publisher Full Text | Free Full Text

37. Dahle MK, Wessel Ø, Timmerhaus G, *et al.*: **Transcriptome analyses of Atlantic salmon (*Salmo salar* L.) erythrocytes infected with piscine orthoreovirus (PRV).** *Fish Shellfish Immunol.* 2015; **45**(2): 780–90.
PubMed Abstract | Publisher Full Text

38. Schoggins JW, Rice CM: **Interferon-stimulated genes and their antiviral effector functions.** *Curr Opin Virol.* 2011; **1**(6): 519–25.
PubMed Abstract | Publisher Full Text | Free Full Text

39. Larsen R, Røkenes TP, Robertsen B: **Inhibition of infectious pancreatic necrosis virus replication by atlantic salmon Mx1 protein.** *J Virol.* 2004; **78**(15): 7938–44.
PubMed Abstract | Publisher Full Text | Free Full Text

40. Pham PH, Lumsden JS, Tafalla C, *et al.*: **Differential effects of viral hemorrhagic septicaemia virus (VHSV) genotypes IVa and IVb on gill epithelial and spleen macrophage cell lines from rainbow trout (*Oncorhynchus mykiss*).** *Fish Shellfish Immunol.* 2013; **34**(2): 632–40.
PubMed Abstract | Publisher Full Text

41. DeWitte-Orr SJ, Leong JA, Bols NC: **Induction of antiviral genes, Mx and vig-1, by dsRNA and Chum salmon reovirus in rainbow trout monocyte/macrophage and fibroblast cell lines.** *Fish Shellfish Immunol.* 2007; **23**(3): 670–82.
PubMed Abstract | Publisher Full Text

42. Ingerslev HC, Rønneseth A, Pettersen EF, *et al.*: **Differential expression of immune genes in Atlantic salmon (*Salmo salar* L.) challenged intraperitoneally or by cohabitation with IPNV.** *Scand J Immunol.* 2009; **69**(2): 90–8.
PubMed Abstract | Publisher Full Text

43. Reyes-Cerpa S, Reyes-López F, Toro-Ascuy D, *et al.*: **Induction of anti-inflammatory cytokine expression by IPNV in persistent infection.** *Fish Shellfish Immunol.* 2014; **41**(2): 172–82.
PubMed Abstract | Publisher Full Text

44. Wang WL, Liu W, Gong HY, *et al.*: **Activation of cytokine expression occurs through the TNFα/NF-κB-mediated pathway in birnavirus-infected cells.** *Fish Shellfish Immunol.* 2011; **31**(1): 10–21.
PubMed Abstract | Publisher Full Text

45. Guarda G, Braun M, Staehli F, *et al.*: **Type I interferon inhibits interleukin-1 production and inflammasome activation.** *Immunity.* 2011; **34**(2): 213–23.
PubMed Abstract | Publisher Full Text

46. Nombela I, Carrion A, Puente-Marin S, *et al.*: **Dataset 1 in: Piscine birnavirus triggers antiviral immune response in trout red blood cells, despite not being infective.** *F1000Research.* 2017.
Data Source

47. Nombela I, Carrion A, Puente-Marin S, *et al.*: **Dataset 2 in: Piscine birnavirus triggers antiviral immune response in trout red blood cells, despite not being infective.** *F1000Research.* 2017.
Data Source

48. Nombela I, Carrion A, Puente-Marin S, *et al.*: **Dataset 3 in: Piscine birnavirus triggers antiviral immune response in trout red blood cells, despite not being infective.** *F1000Research.* 2017.
Data Source

49. Nombela I, Carrion A, Puente-Marin S, *et al.*: **Dataset 4 in: Piscine birnavirus triggers antiviral immune response in trout red blood cells, despite not being infective.** *F1000Research.* 2017.
Data Source

50. Nombela I, Carrion A, Puente-Marin S, *et al.*: **Dataset 5 in: Piscine birnavirus triggers antiviral immune response in trout red blood cells, despite not being infective.** *F1000Research.* 2017.
Data Source

51. Nombela I, Carrion A, Puente-Marin S, *et al.*: **Dataset 6 in: Piscine birnavirus triggers antiviral immune response in trout red blood cells, despite not being infective.** *F1000Research.* 2017.
Data Source

Open Peer Review

Current Referee Status: ✔ ✔

Version 2

Referee Report 21 December 2017

doi:10.5256/f1000research.14494.r29012

✔ **Espen Rimstad** [1], **Maria Dahle** [2]
[1] Department of Food Safety and Infection Biology, Norwegian University of Life Sciences, Oslo, Norway
[2] Norwegian Veterinary Institute, Oslo, Norway

The authors have responded well to each individual point raised. We would recommend that fish populations used in experimental infections including immunological studies are routinely screened for pathogens before entering experimental infections, and not just assessed by measuring mortality. The lack of mortality may not warrant freedom of infection. The presence of low virulent variants of virus may be missed, but they could still have impact on parameters used for measuring immune responses.

Competing Interests: No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Referee Report 23 November 2017

doi:10.5256/f1000research.14090.r27697

✔ **Niels C. Bols**
Department of Biology, University of Waterloo, Waterloo, ON, Canada

The manuscript by Maria de Mar Ortega-Villaizan Romo provides evidence that trout red blood cells can express antiviral mechanisms. The discovery is very intriguing, as red blood cells appear to have addition functions beyond carry gases, and this might open up new ways to enhance fish health. Therefore, I recommend indexing. As documented below, I do have many minor suggestions for improving the clarity and aesthetics of the manuscript.

ABSTRACT
Background
Remove “highly”
I suggest “However, the roles of RBCs in birnavirus….”
Methods:

Remove “induced”
Results:
Remove “-exposed RBCs”
Conclusions: I suggest recasting a few sentences as shown below.
“Despite not being infected, trout RBCs respond to IPNV with increased expression of antiviral genes.”
In the last sentence, I suggest “that triggers this antiviral response in trout RBCs”

INTRODUCTION
1st paragraph 2nd line remove “Among all of the viral diseases,”
It sounds awkward and the point being made is made again in the 4th paragraph.
2nd paragraph
Remove “This implies that nucleated RBCs can support viral replication.” Over 40 years ago, enucleated cells were shown to support replication of some viruses[1]. Therefore, having a nucleus in RBCs might have nothing to do with their capacity to support the replication of some viruses. For example, human RBCs might also have roles in viral infections. Therefore, the first 2 sentences of this paragraph could be eliminated and the next sentence, after removing “for example”, would become the topic sentence.

3rd paragraph
Remove the sentence that begins “Recently, it has been shown that viral hemorrhagic septicemia virus (VHSV) can induce ….”
I don’t see how it fits the subject of the paragraph or point out in the sentence that RBCs as well as stromal cells were studied.
4th paragraph
recast the last 2 sentences
“However, we report that even though not infecting RBCs, IPNV induces in them the expression of antiviral genes in the INF-1 pathway.” I am not sure that I would emphasize so strongly “protection against IPNV infection in CHSE-214 cells”. This appears to be based on the 1/5 bar in Figure 3A. I would like to have seen if protection also arose from 1 to 3 supernatant dilution or 1 to 10 supernatant dilution. If either one or both of these dilutions did protect, it would strongly indicate that protection can be repeatability demonstrated.

METHODS
Animals
Space between 1 and fish.
Space after Spain)

RBC purification
Was no anticoagulant used in the collection of the blood? Please clarify. Would thrombocytes be present among the RBCs?

In the 3rd last sentence I wonder if something should follow the 2%. RPMI with 2 % FBS? This also comes in the next section. Maybe define it here as RPMI with 2 % FBS (RPMI/FBS) and use in RPMI/FBS in subsequent section.

Viral infection assays
No need to repeat *Oncorhynchus mykiss* here as the genus species name are mentioned earlier.
In the 2nd last sentence, I believe that the RPMI was “removed” rather than “retired”.
Were RBC infected at 14 °C like CHSE-214 were?

Viral titration assay
In the first sentence, “supernatants were quantified by TCID50 and by RT-qPCR.”

RNA isolation and DNAse treatment
no suggestions

Gene expression by RT-qPCR
“synthesized”

Antibodies
I suggest the following topic sentence for this section.
“Several antibodies were used to stain cells for cytokines and to measure polypeptides in RBC extracts by western blotting and they are briefly described below and their Research Resource Identifiers (RRIDs) given.”

Western blot
no suggestions

Intracellular immunofluorescence staining and flow cytometry
no suggestions

Antiviral activity of conditioned medium
Would not the conditioned medium (CM) have virus that had not bound to RBC and thus not pelleted at 1600 rpm and so was in the CM? Please briefly discuss.

Is ‘staining’ rather than “stain” meant in the following sentence and the next heading?
“Finally, intracellular stain of IPNV foci was carried out.”

Intracellular stain of IPNV foci
Add to the end of the 3rd sentence “for 1 hour with the cells”.

MTT assays
At the end of this section, a statement of how the absorbance readings are converted to percent viability should be given. I know that it is mentioned in the legend for Figure 3.

Software and statistics
I suggest the following modification to the 2nd sentence.
“was used for preparing graphs and preforming statistical calculations”.

Ethics approval
no suggestions

RESULTS
IPNV did not infect trout RBCs
I suggest the following recasting of the last sentence and addition of a new last sentence.
“Moreover, flow cytometry analysis of control and IPNV-exposed RBCs for IPNV VP3 protein did not show significant differences (Figure 1C and D). Therefore, IPNV did not infect trout RBCs.”

IPNV exposure increased the expression of interferon-related antiviral immune genes and proteins in RBCs
The first sentence should be shortened as follows
"to determine if IPNV would induce an antiviral response in RBCs."
Are there antiviral genes that are not immune genes?
In other words, can the description be simplified to just 'antivrial genes"?

DISCUSSION
2nd paragraph
The last two sentences could refer to the data of the ms for support.

4th paragraph
I wonder if the authors could briefly discuss the apparent constitutive expression of Mx in RBCs as seen in Panel D of Figure 2. Also could Mx be released from RBCs? In cattle Mx1 is found in exosomes (see Racicot K et al., 2012, Am J Reprod Immunol) and exosomes can be released by RBCs (see Danesh A et al., 2014, Blood).

FIGURES AND LEGENDS

Figure 1
In panel A write out hpe on the X axis.

Figure 2
Is the staining in the control of interest or just background? I mentioned this earlier under the Discussion.
Could any of the cells in panel D be thrombocytes?

Figure 3
Could FFU be written out here in the legend so figure stands alone? The legend mentions doing a two-way ANOVA but no mention of a post test is given, although the 1/5 supernatant dilution is identified with an asterisk?

Figure 4 Title legend has a spelling mistake and is a bit misleading because panel B is not covered by the current title. I suggest the following title.
"Reduction in RBC cytokine levels by IPNV and a possible mechanism"
The legend should give more information so the figure can better stand by itself.
Write out MFI (Mean Fluorescence Intensity) and FC (Flow Cytometry).
At two places in the legend, mention is made of the Mann-Whitney test but the outcome is not clearly stated.

References
1. Follett EA, Pringle CR, Wunner WH, Skehel JJ: Virus replication in enucleate cells: vesicular stomatitis virus and influenza virus.*J Virol*. 1974; **13** (2): 394-9 PubMed Abstract

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: Fish cell line development, fish virology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 01 Dec 2017

Maria del Mar Ortega-Villaizan Romo, Universidad Miguel Hernandez, Spain

Dear Dr. Niels Bols,
Thank you very much for your positive review and suggestions for the manuscript. We have included your corrections and suggestions in the new version of the manuscript.

ABSTRACT
Background
Remove “highly”
Author´s response: It has been corrected.

I suggest “However, the roles of RBCs in birnavirus….”
Author´s response: It has been corrected.

Methods:
Remove “induced”
Author´s response: It has been corrected.

Results:
Remove “-exposed RBCs”
Author´s response: It has been corrected.

Conclusions: I suggest recasting a few sentences as shown below.
“Despite not being infected, trout RBCs respond to IPNV with increased expression of antiviral genes.”
In the last sentence, I suggest “that triggers this antiviral response in trout RBCs”
Author´s response: It has been corrected.

INTRODUCTION

1st paragraph 2nd line remove "Among all of the viral diseases,"
It sounds awkward and the point being made is made again in the 4th paragraph.
Author´s response: It has been corrected.

2nd paragraph
Remove "This implies that nucleated RBCs can support viral replication." Over 40 years ago, enucleated cells were shown to support replication of some viruses[1]. Therefore, having a nucleus in RBCs might have nothing to do with their capacity to support the replication of some viruses. For example, human RBCs might also have roles in viral infections. Therefore, the first 2 sentences of this paragraph could be eliminated and the next sentence, after removing "for example", would become the topic sentence.
Author´s response: We have removed these sentences.

3rd paragraph
Remove the sentence that begins "Recently, it has been shown that viral hemorrhagic septicemia virus (VHSV) can induce …."
I don't see how it fits the subject of the paragraph or point out in the sentence that RBCs as well as stromal cells were studied.
Author´s response: We have changed this sentence to: "Besides, recently it has been shown that viral hemorrhagic septicemia virus (VHSV) halted replication in rainbow trout RBCs could induce cytokine production" as another reference of immune response by RBCs against virus.

4th paragraph
recast the last 2 sentences
"However, we report that even though not infecting RBCs, IPNV induces in them the expression of antiviral genes in the INF-1 pathway." I am not sure that I would emphasize so strongly "protection against IPNV infection in CHSE-214 cells". This appears to be based on the 1/5 bar in Figure 3A. I would like to have seen if protection also arose from 1 to 3 supernatant dilution or 1 to 10 supernatant dilution. If either one or both of these dilutions did protect, it would strongly indicate that protection can be repeatability demonstrated.
Author´s response: The protection conferred by the CM from IPNV-exposed RBCs diminished as the dilution increased from 1/5 to 1/25 and 1/125. However, we have not tried 1/3 or 1/10 dilutions. As Dr. Bols pointed out, it would have been interesting to check with lower dilution ranges. In relation to repeatability, this assay was done using the supernatants from 4 ex vivo infections, in triplicate each of them. Therefore, the repeatability could have been demonstrated by the assay itself?
We have rewritten the sentence to do not emphasize so strongly "protection" as follows: "the induction of an antiviral state to IPNV in CHSE-214 cells".

METHODS
Animals
Space between 1 and fish.
Space after Spain)
Author´s response: It has been corrected.

RBC purification
Was no anticoagulant used in the collection of the blood? Please clarify. Would thrombocytes be present among the RBCs?

Author´s response: We did not use any anti-coagulant. The blood was immediately diluted in RPMI 10%FBS and slightly resuspended. In this way, no coagulation occurs. On the other hand, thrombocytes were eliminated with the double Ficoll purification.

In the 3rd last sentence I wonder if something should follow the 2%. RPMI with 2 % FBS? This also comes in the next section. Maybe define it here as RPMI with 2 % FBS (RPMI/FBS) and use in RPMI/FBS in subsequent section.
Author´s response: It has been corrected.

Viral infection assays
No need to repeat *Oncorhynchus mykiss* here as the genus species name are mentioned earlier.
In the 2nd last sentence, I believe that the RPMI was "removed" rather than "retired".
Were RBC infected at 14 °C like CHSE-214 were?
Author´s response: It has been corrected and 14 °C added to RBCs infection protocol.

Viral titration assay
In the first sentence, "supernatants were quantified by TCID50 and by RT-qPCR."
Author´s response: It has been corrected.

RNA isolation and DNAse treatment
no suggestions

Gene expression by RT-qPCR
"synthesized"
Author´s response: It has been corrected.

Antibodies
I suggest the following topic sentence for this section.
"Several antibodies were used to stain cells for cytokines and to measure polypeptides in RBC extracts by western blotting and they are briefly described below and their Research Resource Identifiers (RRIDs) given."
Author´s response: We have included this sentence.

Western blot
no suggestions

Intracellular immunofluorescence staining and flow cytometry
no suggestions

Antiviral activity of conditioned medium
Would not the conditioned medium (CM) have virus that had not bound to RBC and thus not pelleted at 1600 rpm and so was in the CM? Please briefly discuss.
Author´s response: The supernatants of RBCs exposed to IPNV were titrated and resulted in approximately 10 TCID50/mL or less. We have added the following sentence to clarify this item: "IPNV titer in the supernatants of IPNV-exposed RBCs resulted in 10 TCID50/mL or less, therefore viral presence in the supernatants was obviated".

Is 'staining' rather than "stain" meant in the following sentence and the next heading?
"Finally, intracellular stain of IPNV foci was carried out."

Author´s response: It has been corrected.

Intracellular stain of IPNV foci
Add to the end of the 3rd sentence “for 1 hour with the cells”.
Author´s response: It has been corrected.

MTT assays
At the end of this section, a statement of how the absorbance readings are converted to percent viability should be given. I know that it is mentioned in the legend for Figure 3.
Author´s response: The formula has been added.

Software and statistics
I suggest the following modification to the 2nd sentence.
“was used for preparing graphs and preforming statistical calculations”.
Author´s response: It has been corrected.

Ethics approval
no suggestions

RESULTS
IPNV did not infect trout RBCs
I suggest the following recasting of the last sentence and addition of a new last sentence.
“Moreover, flow cytometry analysis of control and IPNV-exposed RBCs for IPNV VP3 protein did not show significant differences (Figure 1C and D). Therefore, IPNV did not infect trout RBCs.”
Author´s response: We have recasted the sentence as advised.

IPNV exposure increased the expression of interferon-related antiviral immune genes and proteins in RBCs
The first sentence should be shortened as follows
“to determine if IPNV would induce an antiviral response in RBCs.”
Are there antiviral genes that are not immune genes?
In other words, can the description be simplified to just ‘antivrial genes”?
Author´s response: We have shortened the sentence and the description simplified to antiviral genes

DISCUSSION
2nd paragraph
The last two sentences could refer to the data of the ms for support.
Author´s response: We have referred this affirmation to Figure 1.

4th paragraph
I wonder if the authors could briefly discuss the apparent constitutive expression of Mx in RBCs as seen in Panel D of Figure 2. Also could Mx be released from RBCs? In cattle Mx1 is found in exosomes (see Racicot K et al., 2012, Am J Reprod Immunol) and exosomes can be released by RBCs (see Danesh A et al., 2014, Blood).
Author´s response: Yes, Figure S1 also highlights the elevated constitutive expression of Mx in RBCs, compared to other CHSE-214.
Mx in RBCs exosomes is very interesting appreciation. However, Racicot et al (2012) concluded that “The results presented here show MX1 could play a role in the formation of vesicles secreted

into the uterus and suggest involvement in basic cellular processes independent of its role during viral infections". Therefore, although it is an interesting topic of investigation we would rather not to mention the possible link between Mx and RBCs exosomes in this manuscript since it is unknown whether it could be related to Mx antiviral function.

FIGURES AND LEGENDS

Figure 1
In panel A write out hpe on the X axis.
Author´s response: It has been written

Figure 2
Is the staining in the control of interest or just background? I mentioned this earlier under the Discussion. Could any of the cells in panel D be thrombocytes?
Author´s response: Yes, we have discussed about it in the discussion section. In relation to panel D, none of those cells are thrombocytes.

Figure 3
Could FFU be written out here in the legend so figure stands alone? The legend mentions doing a two-way ANOVA but no mention of a post test is given, although the 1/5 supernatant dilution is identified with an asterisk?
Author´s response: Yes, FFU/mL has been deleted from panel A, x axis, as it is indicated in the figure legend. On the other hand, Sidak´s multiple comparison test was performed and has been added to the legend.

Figure 4 Title legend has a spelling mistake and is a bit misleading because panel B is not covered by the current title. I suggest the following title.
"Reduction in RBC cytokine levels by IPNV and a possible mechanism"
The legend should give more information so the figure can better stand by itself.
Write out MFI (Mean Fluorescence Intensity) and FC (Flow Cytometry).
At two places in the legend, mention is made of the Mann-Whitney test but the outcome is not clearly stated.
Author´s response: Title of Figure 4 has been changed as follows: IPNV-exposure decreased cytokine levels in rainbow trout RBCs, and MFI and FC explained. In relation to the statistical analysis, Mann-Whitney test was performed for Fig4A and 4b, however, statistically significant differences were not observed.

Competing Interests: No competing interests were disclosed.

Referee Report 20 November 2017

doi:10.5256/f1000research.14090.r27699

? **Espen Rimstad** [1], **Maria Dahle** [2]
[1] Department of Food Safety and Infection Biology, Norwegian University of Life Sciences, Oslo, Norway
[2] Norwegian Veterinary Institute, Oslo, Norway

This is an interesting paper showing that purified rainbow trout RBC exposed to IPNV do not get infected by the virus, but nevertheless raise an innate antiviral response. The latter is shown by the induction of IFN and a few IFN related genes and that conditioned media from IPNV exposed RBC inhibits IPNV infection of the susceptibe cell line CHSE-214. Many methods are used to approach the hypotheses and the results appear provide new information on the very intriguing role of fish red blood cells in interaction with viruses, in particular by showing that the cells can induce an antiviral immune response without being infected.
The main comments are related to how the authors have interpreted the results far beyond what they have shown, by drawing links to adaptive immune mechanisms and vaccination approaches, while they could have discussed antiviral protection mechanisms in further detail and with more scientific basis. There are also some missing information regarding the experimental details, and far too extensive use of an unpublished paper in the discussion of the data.

Comments:

1. The title is generalizing beyond the scope of the paper, and should specify the virus and the host studied (IPNV and onchorhynchus mykiss).
2. Abstract and Discussion: The statement that antiviral responses in RBCs could be targets for DNA vaccines is not linked to the data presented here. A vaccination effect requires antigen presentation and adaptive immune activation for long term protection, which is not touched upon at all in this paper. If this point should be made, the authors should in the discussion provide some explanation and scientific basis for how they think antiviral responses in RBCs to an uninfecting virus make them promising DNA vaccination targets, and how directing DNA vaccines to RBC should be performed.
3. Introduction/Discussion: An unpublished paper: Nombela I, Puente-Marin S et al. is referred to in introduction and several times in the discussion. The content of this paper cannot be evaluated. The authors should primarily use published work in their discussion.
4. Introduction. There are many language mistakes in the introduction (and elsewhere). Please have the manuscript corrected linguistically. Trout is not a specific annotation.
5. Please specify that the work is performed for rainbow trout. Diseases are not agents. Diseases cause losses not loses. Viral titer not titters. *Aquabirnavirus* genus not *Aquabirnaviridae* genera, it is wrong suffix and it is not plural. etc.
6. The role of IPNV - VP3 is not completely unknown, there are several papers describing its function.
7. *"The VP2 epitopes are the target for antibodies against different serotypes."* Not *against* but distinguishing between. It should be noted that the serotyping system is based upon the use of rabbit antisera, it is not based on the immune response from fish.
8. No observations of viral factories has been observed for ISAV.
9. *"The fact that these cells are nucleated implies that fish RBCs can respond to different stimulus and control cellular processes".* Not really, having a nucleus doesn't really prove that the cell can react various stimuli, that have to shown.
10. Materials and Methods: Number of fish used not given. Unclear if the fish used were tested for previous viral infections.
11. 18S is given as the reference gene target in the text, but in the table EF1a primers are given. Results is not clear on what was used.
12. Results: Unclear if the "n" in experiments is number of fish or parallel ex vivo infections.
13. *"IPNV segment A (IPNV-A) RNA levels inside RBCs and CHSE-214 cell line were similar at 1 and 3 hours post-exposure (hpe) (Figure 1A). After 3 hpe, IPNV-A RNA level was 4 logarithms lower in RBCs in comparison with CHSE-214 cells."* Do you mean from 6 hpe and not after 3 hpe? It is not 4 logs difference according to Fig 1 it is 3 log at 6 hpe and 5 log at 26 hpe. It is nevenr 4 logs?
14. What is the detection limit for VP3 in flow cytometri?

15. Discussion:
    1. The small trend towards a difference in cytokine levels Fig 4A should not be discussed as “downregulation”, and cannot be interpreted as a cell shut-off of the proteome by the infection. Most likely the slight trend towards down-regulation is just an effect of the relative upregulation of the IFN-I pathway (ISGs)
    2. Authors say in the discussion regarding the protective effect of conditioned media: “*To elucidate which factors could be implicated in this protection, we evaluated the production of cytokines in IPNV-exposed RBCs*” It is well known that IFN-I, and not to the same degree cytokines, confers a direct antiviral response on surrounding cells. The upregulation of IFN-I is already shown at the transcriptional level, and the upregulation of ISGs directly indicates IFN activity. The main role of cytokines is to attract and activate immune cells and mobilize an immune response in tissue in vivo. The authors should clarify this to show that they understand the difference between local antiviral protection and crosstalk with the immune system.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response 01 Dec 2017

Maria del Mar Ortega-Villaizan Romo, Universidad Miguel Hernandez, Spain

Dear Dr. Espen Rimstad and Dr. Maria Dahle,
We appreciate very much your revision and constructive comments on the manuscript. We have included your corrections in the new version of the manuscript hoping that now the manuscript will be suitable for publication.
Please find below the response to your comments:

1. The title is generalizing beyond the scope of the paper, and should specify the virus and the host studied (IPNV and onchorhynchus mykiss).
Author's response: The title have been changed as advised.

2. Abstract and Discussion: The statement that antiviral responses in RBCs could be targets for DNA vaccines is not linked to the data presented here. A vaccination effect requires antigen presentation and adaptive immune activation for long term protection, which is not touched upon at all in this paper. If this point should be made, the authors should in the discussion provide some explanation and scientific basis for how they think antiviral responses in RBCs to an uninfecting virus make them promising DNA vaccination targets, and how directing DNA vaccines to RBC should be performed.
Author's response: In order to avoid misunderstanding we have deleted DNA vaccines from abstract

3. Introduction/Discussion: An unpublished paper: Nombela I, Puente-Marin S et al. is referred to in introduction and several times in the discussion. The content of this paper cannot be evaluated. The authors should primarily use published work in their discussion.
Author's response: The paper reference can be included now and has been included.

4. Introduction. There are many language mistakes in the introduction (and elsewhere). Please have the manuscript corrected linguistically. Trout is not a specific annotation.
Author's response: the manuscript has been linguistically corrected and trout has been properly annotated as rainbow trout.

5. Please specify that the work is performed for rainbow trout. Diseases are not agents. Diseases cause losses not loses. Viral titer not titters. Aquabirnavirus genus not Aquabirnaviridae genera, it is wrong suffix and it is not plural. etc.
Author's response: we have specified that the work has been done for rainbow trout. Titer has been written instead of titters. Aquabirnavirus genus has been written instead of Aquabirnaviridae genera.

6. The role of IPNV - VP3 is not completely unknown, there are several papers describing its function.
Author's response: This sentence has been deleted since it is not the target of our study.

7. "The VP2 epitopes are the target for antibodies against different serotypes." Not against but distinguishing between. It should be noted that the serotyping system is based upon the use of rabbit antisera, it is not based on the immune response from fish.
Author's response: This sentence has been deleted since it is not the target of our study.

8. No observations of viral factories has been observed for ISAV.
Author's response: We have deleted that sentence.

9. "The fact that these cells are nucleated implies that fish RBCs can respond to different stimulus and control cellular processes". Not really, having a nucleus doesn't really prove that the cell can react various stimuli, that have to shown.
Author's response: We have deleted that sentence.

10. Materials and Methods: Number of fish used not given. Unclear if the fish used were tested

for previous viral infections.
Author's response: The number of fish is indicated in each experiment/figure. On the other hand, the fish has not been tested for previous infection. However, the fish farm appears to be free of IPNV and VHSV, since they do not have associated mortalities.

11. 18S is given as the reference gene target in the text, but in the table EF1a primers are given. Results is not clear on what was used.
Author's response: Yes, this has been an error. EF1a has been removed from the primers table.

12. Results: Unclear if the "n" in experiments is number of fish or parallel ex vivo infections.
Author's response: Yes, "n" refers to number of fish/ ex vivo infections

13. "IPNV segment A (IPNV-A) RNA levels inside RBCs and CHSE-214 cell line were similar at 1 and 3 hours post-exposure (hpe) (Figure 1A). After 3 hpe, IPNV-A RNA level was 4 logarithms lower in RBCs in comparison with CHSE-214 cells." Do you mean from 6 hpe and not after 3 hpe? It is not 4 logs difference according to Fig 1 it is 3 log at 6 hpe and 5 log at 26 hpe. It is nevenr 4 logs?
Author's response: Yes, please excuse the error. We have corrected it as "After 6 hpe, IPNV-A RNA level was 3 logarithms lower"

14. What is the detection limit for VP3 in flow cytometri?
Author's response: We do not know the limit of detection for VP3 by flow cytometry. However, by ELISA, it has been described that the limit of the detection for 2F12 + 3B12 antibody mix (anti-VP3) is 104 TCID50/ml (Domínguez J, Hedrick RP, Sánchez-Vizcaino JM: Use of monoclonal-antibodies for detection of infectious pancreatic necrosis virus by the enzyme-linked-immunosorbent-assay (ELISA). Dis Aquat Organ. 1990; 8: 157–63. Publisher Full Text).

15. Discussion:

1. The small trend towards a difference in cytokine levels Fig 4A should not be discussed as "downregulation", and cannot be interpreted as a cell shut-off of the proteome by the infection. Most likely the slight trend towards down-regulation is just an effect of the relative upregulation of the IFN-I pathway (ISGs)
Author's response: We have included this appreciation in the discussion.

2. Authors say in the discussion regarding the protective effect of conditioned media: "To elucidate which factors could be implicated in this protection, we evaluated the production of cytokines in IPNV-exposed RBCs" It is well known that IFN-I, and not to the same degree cytokines, confers a direct antiviral response on surrounding cells. The upregulation of IFN-I is already shown at the transcriptional level, and the upregulation of ISGs directly indicates IFN activity. The main role of cytokines is to attract and activate immune cells and mobilize an immune response in tissue in vivo. The authors should clarify this to show that they understand the difference between local antiviral protection and crosstalk with the immune system.
Author's response: In order to avoid misunderstandings, and due to lack of information about il8, il1b and tnfa receptor pathways in CHSE-214, we have deleted in the manuscript the relation between il8, il1b and tnfa with the protection of CHSE against IPNV.

Competing Interests: No competing interests were disclosed.

PUBLICACIÓN 3

TÍTULO: 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

AUTORES: Ivan Nombela, Ricardo Requena-Platek, Byron Morales-Lange, Veronica Chico, Sara Puente-Marin, Sergio Ciordia, Maria Carmen Mena, Julio Coll, Luis Perez, Luis Mercado and Maria del Mar Ortega-Villaizan

REVISTA: *Cells*

DOI: 10.3390/cells8050386

cells

MDPI

Article

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

Ivan Nombela [1], Ricardo Requena-Platek [1], Byron Morales-Lange [2], Veronica Chico [1], Sara Puente-Marin [1], Sergio Ciordia [3], Maria Carmen Mena [3], Julio Coll [4], Luis Perez [1], Luis Mercado [2] and Maria del Mar Ortega-Villaizan [1,*]

1 Instituto de Investigación, Desarrollo e Innovación en Biotecnología Sanitaria de Elche (IDiBE) and Instituto de Biología Molecular y Celular (IBMC), Universidad Miguel Hernández (UMH), 03202 Elche, Spain; nombela@umh.es (I.N.); ricardorequena.p@gmail.com (R.R.-P.); vchico@umh.es (V.C.); spuente@umh.es (S.P.-M.); luis.perez@umh.es (L.P.)

2 Instituto de Biología, Pontificia Universidad Católica de Valparaiso, 2373223 Valparaiso, Chile; byron.morales@pucv.cl (B.M.-L.); luis.mercado@pucv.cl (L.M.)

3 Unidad de Proteómica, Centro Nacional de Biotecnología (CNB- CSIC), 28049 Madrid, Spain; sciordia@cnb.csic.es (S.C.); mcmena@cnb.csic.es (M.C.M.)

4 Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), 28040 Madrid, Spain; juliocoll@inia.es

* Correspondence: mortega-villaizan@umh.es

Received: 11 March 2019; Accepted: 24 April 2019; Published: 27 April 2019

Abstract: Nucleated teleost red blood cells (RBCs) are known to express molecules from the major histocompatibility complex and peptide-generating processes such as autophagy and proteasomes, but the role of RBCs in antigen presentation of viruses have not been studied yet. In this study, RBCs exposed ex vivo to viral hemorrhagic septicemia virus (VHSV) were evaluated by means of transcriptomic and proteomic approaches. Genes and proteins related to antigen presentation molecules, proteasome degradation, and autophagy were up-regulated. VHSV induced accumulation of ubiquitinated proteins in ex vivo VHSV-exposed RBCs and showed at the same time a decrease of proteasome activity. Furthermore, induction of autophagy was detected by evaluating LC3 protein levels. Sequestosome-1/p62 underwent degradation early after VHSV exposure, and it may be a link between ubiquitination and autophagy activation. Inhibition of autophagosome degradation with niclosamide resulted in intracellular detection of N protein of VHSV (NVHSV) and p62 accumulation. In addition, antigen presentation cell markers, such as major histocompatibility complex (MHC) class I & II, CD83, and CD86, increased at the transcriptional and translational level in rainbow trout RBCs exposed to VHSV. In summary, we show that nucleated rainbow trout RBCs can degrade VHSV while displaying an antigen-presenting cell (APC)-like profile.

Keywords: rainbow trout; erythrocytes; red blood cells; VHSV; transcriptome; proteome; antigen presentation; autophagy; ubiquitination

1. Introduction

Nucleated red blood cells (RBCs) can develop immune responses to viruses that directly target these cells, such as infectious salmonid anemia virus (ISAV) [1] and piscine orthoreovirus (PRV) [2–6], which mainly results in the up-regulation of the interferon (IFN)-α gene and interferon-stimulated genes. Recently, we reported that rainbow trout RBCs can mount an antiviral response against viral

hemorrhagic septicemia virus (VHSV) [7]. Also, we have reported that RBCs can be stimulated by infectious pancreatic necrosis virus (IPNV), where up-regulation of IFN type 1-related genes leads to expression of antiviral myxovirus resistance protein Mx [8]. However, rainbow trout RBCs are nonpermissive to VHSV and IPNV infections, and the cellular mechanisms that make the infection nonpermissive are being studied [9].

Autophagy is an evolutionarily conserved mechanism in which intracellular material is enveloped in double-membrane vesicles and targeted for fusion with lysosomes for degradation. Numerous pathogens have been known to cause autophagy, including viruses [10]. The role of autophagy in the context of viral infections is still controversial and can have either antiviral or proviral functions depending on the virus and host cell [11]. Autophagy can contribute to the innate immune response by delivering viral pathogen-associated molecular pattern (PAMPs) to endosomal Toll-like receptors (TLRs) [12,13] through vesicle trafficking. Related to VHSV, it was found that rhabdoviral infections, including VHSV, can be inhibited when autophagy is activated [14]. Moreover, the viral glycoprotein G is sufficient to induce autophagy [14] and a Pepscan technique has successfully identified the peptides involved in autophagy activation [15]. In teleosts, VHSV infection in turbot RBCs led to expression of NK-lysin, an antimicrobial peptide, associated with LC3 protein in autophagosomes [16].

Recently, groups have reported on selective autophagy mechanisms, suggesting that autophagy is far from being a nonselective degradative process [17]. Autophagy uses adaptors known as SLRs (sequestosome 1/p62-like receptors) that can selectively target pathogens for degradation in autophagosomes [18]. p62 contains domains that interact with both ubiquitinated proteins and autophagy-specific light chain 3 (LC3) modifier [19] in the inner face of the autophagosome; in this way, p62 is involved in delivering ubiquitinated proteins marked for proteasome degradation to autophagosomes. Ubiquitination is a process mediated by the E3 ligases, in which a series of three different enzymes are involved in the activation, conjugation and ligation of ubiquitin to the proteins targeted for degradation [20]. Ubiquitinated proteins are primarily degraded by the proteasome. The ubiquitin-proteasome system (UPS) plays an important role in cell homeostasis by ensuring the quality of newly synthetized proteins and the regulation of levels of proteins performing critical functions in the cell. Functional 20S proteasomes have been identified in human [21] and rainbow trout [7] RBCs. As with autophagy, the UPS plays a double role in the context of viral infections: it can be manipulated by viruses to bypass host defenses mechanisms or participate in the elimination of viral components [22]. The UPS has been named as the principal source of antigenic peptides for the major histocompatibility complex (MHC) of the immune system [23]. Autophagy is also known to be involved in antigen degradation and delivery to MHC class I and II molecules, which could trigger the adaptive immune response [24–26].

Antigen presentation is a key process to activate T cells. This process is mediated by antigen-presenting cells (APCs) such as dendritic cells (DCs). DCs act as an important link between the innate and adaptive immune responses and are involved in patrolling tissues, pathogen engulfment, degradation, movement to lymphoid tissues, and T cell stimulation. However, the presence of APCs, and specifically DCs, was largely unknown in fish until recently, when a subset of APCs resembling those of mammals was identified in zebrafish [27] and rainbow trout [28]. APCs are characterized through cell markers such as CD86 and CD83, which serve as costimulatory molecules, and MHC molecules. Among them, MHC molecules are some of the most important proteins involved in the antigen presentation process, as they display pathogen-derived fragments on the cell surface to allow recognition by T cells. Expression of MHC molecules indicates that a cell can play an APC role. MHC class I (MHCI) protein expression has been detected in rainbow trout RBCs [29,30] and MHC class II (MHCII) transcriptional expression has been recently reported in nucleated rainbow trout [31,32] and chicken [33] RBCs. However, the role of RBCs in viral antigen presentation is unknown. APCs are classified as professional or atypical [34]. Professional APCs constitutively express MHC molecules, possess machinery to process antigens, and can localize to tissues and T cell zones, whereas atypical

APCs up-regulate MHC expression under certain conditions. Little evidence exists regarding atypical APCs priming T cells in an antigen-specific manner [34].

The aim of this study was to elucidate whether APCs cell markers regulation occurred in nucleated teleost RBCs after VHSV exposure, while also analyzing potential autophagy and UPS implications. These processes have been reported to generate peptides used by MHC molecules for antigen presentation [35]. Recently, we found that RBCs are nonpermissive to VHSV infection [7], but the cause of this abortive infection is being studied [9]. Our results show an increase in ubiquitination and autophagy activation in ex vivo VHSV-exposed RBCs. Inhibition of autophagy degradation led to increased levels of VHSV in RBCs. We also detected p62 degradation at early stages post infection. We found up-regulation of MHCI, MHCII, CD83, and CD86 molecules at the protein level on rainbow trout RBCs after VHSV exposure. Therefore, we show for the first time to our knowledge that nucleated RBCs can display and up-regulate APCs cell markers and process viral antigens through autophagy.

2. Materials and Methods

2.1. Animals

Rainbow trout (*Oncorhynchus mykiss*) individuals of approximately 5 to 20 gr. were obtained from a commercial fish farm (Piszolla S.L., Cimballa Fish Farm, Zaragoza, Spain). Fish were maintained at the University Miguel Hernandez (UMH) facilities in a recirculating dechlorinated water system at a stocking density of 1 fish/3L and fed daily with a commercial diet (Skretting, Burgos, Spain). Water temperature was constantly monitored to maintain fish at 14 °C. Fish were acclimatized to laboratory conditions for 2 weeks before experimentation. Experimental protocols and methods of the experimental animals were reviewed and approved by the Animal Welfare Body and the Research Ethics Committee at the UMH (approval number 2014.205.E.OEP; 2016.221.E.OEP) and by the competent authority of the Regional Ministry of Presidency and Agriculture, Fisheries, Food and Water supply (approval number 2014/VSC/PEA/00205). All methods were carried out in accordance with the Spanish Royal Decree RD 53/2013 and EU Directive 2010/63/EU for the protection of animals used for research experimentation and other scientific purposes.

2.2. Cell Cultures and Virus

Rainbow trout were sacrificed by overexposure to tricaine methanesulfonate (Sigma-Aldrich, Madrid, Spain) at 0.3 g/L. Peripheral blood was sampled from the caudal vein using insulin syringes (NIPRO, Bridgewater, NJ, USA). Approximately 100 µL of blood was diluted in RPMI-1640 medium (Dutch modification) (Gibco, Thermo Fischer Scientific Inc., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Cultek, Madrid, Spain), 1 mM pyruvate (Gibco), 2 mM L-glutamine (Gibco), 50 µg/mL gentamicin (Gibco), 2 µg/mL fungizone (Gibco), and 100 U/mL penicillin/streptomycin (Sigma-Aldrich). Then, RBCs were purified by two consecutive density gradient centrifugations with Histopaque 1077 (7206g, Ficoll 1.007; Sigma-Aldrich). Finally, RBCs were washed twice with RPMI 2% FBS. An RBC purity of 99.99% was estimated by optical microscopy evaluation. Then, purified RBCs were cultured in the above indicated medium at a density of 10^7 cells/mL in cell culture flasks at 14 °C overnight.

For autophagy assays, RBCs were treated with niclosamide (Sigma-Aldrich) after three hours post-exposure (hpe) to VHSV and then incubated for the time and at the concentration indicated for each assay. Similarly, the proteasome inhibitor MG132 (Sigma-Aldrich) was added after three hpe to VHSV and then incubated for the time and at the concentration indicated for each assay.

Viral hemorrhagic septicemia virus (VHSV-07.71) [36] was purchased from the American Type Culture Collection (ATCC, VR-1388) and propagated in fathead minnow epithelioma papulosum cyprini EPC cells (ATCC, CRL-2872) at 14 °C, as previously reported [37].

2.3. Antibodies

To label VHSV, we used the mouse monoclonal 2C9 antibody against the N protein of VHSV (NVHSV) [38] produced at Dr Coll's laboratory. To label MHCI, mouse anti-MHCI against zebrafish MHCI (Ab-Mart, Shangai, China; Ref nº #X1-K4HVT2) was used (Supplementary Figure S1). Sequence alignment between zebrafish (UniprotKB Entry K4HVT2) and rainbow trout (NCBI Entry AAG53681.1) MHCI protein sequences, using NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov), resulted in 48% identity and 68% positives. To label LC3, rabbit anti-LC3A/B antibody (Cell Signaling Technology, Danvers, MA; Ref nº #4108) was used. To label p62, we used rabbit anti-p62/SQSTM1 antibody (www.antibodiesonline.com; Ref nº #ABIN2854836) (Supplementary Figure S2). This antibody shows reactivity with zebrafish. Sequence alignment between zebrafish (UniprotKB Entry F1Q5Z8) and rainbow trout (XP_021439759.1) p62/sequestosome 1 protein sequences, resulted in 61% identity and 70% positives. To label ubiquitin, rabbit anti-ubiquitin antibody (StressMarq, Victoria, Canada; Ref nº #SPC-119) was used. This antibody shows reactivity with rainbow trout. Mouse anti-MHCII, mouse anti-CD86, and rabbit anti-CD83 antibodies against respective rainbow trout molecules were produced at the laboratory of Dr Luis Mercado using synthetic epitopes from the indicated molecules [39]. Western blots of anti-MHCII, anti-CD86, and anti-CD83 antibodies in RBCs can be found in Supplementary Figure S3. A polyclonal antibody against VHSV G glycoprotein (GVHSV) produced in rabbit [40], kindly donated by Dr Niels Lorenzen to Dr Julio Coll, was used in the DuoLink proximity assay. Rabbit polyclonal antibody against human α-actin (Sigma-Aldrich, Nº #2066) was used for western blotting as a loading control. Secondary antibodies used are indicated in each assay.

2.4. Viral Exposure Assays

Ex vivo rainbow trout RBCs were exposed to VHSV at different multiplicities of infection (MOI), as indicated in each figure. After three hours of incubation at 14 °C, cells were washed with cold RPMI, then RPMI 2% FBS was added and the culture was incubated at 14 °C for the different times indicated in each assay. Virus was not removed in the time-course assays. MOI was calculated using the following formula:

$$\text{MOI} = \frac{\text{Viral titer}\left(\frac{\text{TCID}_{50}}{\text{mL}}\right)\cdot\text{Volume of infection (mL)}\cdot\text{Dilution}}{\text{N° of RBCs}}$$

2.5. Rainbow Trout Challenge with VHSV

Young rainbow trout individuals were infected by intramuscular injection of 50 µL RPMI 2% FBS medium with VHSV (10^8 $TCID_{50}$/mL). As a negative control, individuals were injected with 50 µL of sterile RPMI 2% FBS. Over the course of the challenge, individuals were maintained at 14 °C for the number of days indicated.

2.6. Proteasome Activity Assay

RBC proteasome activity was measured using Proteasome 20S Activity Assay Kit (Sigma-Aldrich). RBCs were exposed to VHSV for 24 h at the indicated MOI. After, approximately 2×10^5 cells in 90 µL RPMI were adhered to a transparent 96-well plate previously treated with poly-D lysine (Sigma-Aldrich) by centrifugation at 800 rpm for two minutes. Then, 100 µL of Proteasome Assay Loading Solution (prepared following manufacturer instructions) were added to each well. After five hours of incubation at room temperature with protection from light, fluorescence was measured using POLARstar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany) with an excitation wavelength of 490 nm and an emission wavelength of 525 nm.

2.7. RNA Isolation and cDNA Synthesis

The E.Z.N.A. Total RNA Kit (Omega Bio-Tek Inc., Norcross, GA, USA) was used for total RNA extraction in accordance with the manufacturer's instructions. To eliminate possible residual genomic DNA, the sample was treated using TURBO™ DNase (Ambion, Thermo Fischer Scientific Inc.) following the manufacturer's instructions. RNA was quantified with a NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

cDNA was synthesized from RNA using M-MLV reverse transcriptase (Invitrogen, Thermo Fischer Scientific Inc.) as previously described [41]. cDNA was stored at −20 °C.

2.8. Transcriptome Analysis

Ficoll-purified rainbow trout RBCs were exposed to VHSV as described above. After 4 and 72 hpe, VHSV-exposed (n = 16) and unexposed (n = 16) RBCs (10^6 cells per fish) were resuspended in a 1/10 dilution of 9.5 µL of 10× lysis buffer (Clontech, Takara Bio, Mountain View, CA, USA) and 0.5 µL of RNase Inhibitor (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA). Fish samples were grouped into 2 pools of 8 individuals for each condition (control and VHSV-exposed) and preserved at −80 °C until cDNA library construction. cDNA was directly produced from pooled lysed cells using SMART-Seq v4 Ultra Low Input RNA Kit (Clontech, Takara Bio) [31]. Sequence reads are available at SRA-NCBI accession SRP133501. RNA-Seq library preparation, sequencing, and mapping were carried out by STABVida Lda (Caparica, Portugal) as previously described [31].

2.9. Proteome Analysis

Ficoll-purified rainbow trout RBCs were exposed to VHSV as described above. At 72 hpe, VHSV-exposed (n = 16) and unexposed (n = 16) RBCs (8×10^6 cells per fish) were pelletized by centrifugation (1600 rpm), the supernatant was removed, and the cell pellet was washed three times with phosphate- buffered saline (PBS), digested, cleaned-up/desalted and grouped into 2 pools of 8 individuals for each condition (control and VHSV-exposed). Then, samples were subjected to liquid chromatography and mass spectrometry analysis (LC-MS) as previously described [31]. Log_2 peptide ratios followed a normal distribution that was fitted using least squares regression. Mean and standard deviation values derived from the Gaussian fit and were used to estimate *P* values and false discovery rates (FDR) at quantitation level. The confidence interval for protein identification was set to <95% ($P < 0.05$), and only peptides with an individual ion score above the 1% FDR threshold were considered correctly identified. Only proteins with at least two peptide spectrum matches (PSMs) were considered in the quantitation.

2.10. Pathway Enrichment Analysis

Using the transcriptomic and proteomic results, differentially expressed genes (DEGs) and proteins (DEPs) pathway enrichment analyses were performed using ClueGO [42], CluePedia [43], and Cytoscape [44]. The Gene Ontology (GO) Immune System Process, GO Biological Process, Reactome pathways, KEGG pathways, and Wikipathways databases were used. A *P* value ≤ 0.05 and Kappa score of 0.4 were used as threshold values. Genes and proteins were identified by sequence homology with *Homo sapiens* using Blast2GO version 4.1.9 (BioBam, Valencia, Spain) [45].

2.11. Semi-quantitative PCR

Semi-quantitative PCR was performed using the commercial kit GoTaq G2 DNA polymerase (Promega, Madison, WI, USA) and synthesized cDNA. PCR reactions were performed in a total volume of 12.5 µL using 10 µM for dNTPs (Invitrogen), 0.75 mM $MgCl_2$ (Promega), 1X GoTaq Green Buffer (Promega) and 1.25 U of GoTaq G2 DNA polymerase (Promega). Primer concentration was 50 nM for *cd83*, *mhcI*, and *mhcII* and 25 nM for *cd86*. A total of 12 ng of cDNA was used for each sample. Cycling conditions were 95 °C for 5 min; 35 cycles at 95 °C for 30 s, 60 °C or 62 °C (depending on the T_m of

primers) for 30 s, and 72 °C for 20 s; and 72 °C for 5 min. An Aeris (ESCO, Singapore, Singapore) thermal cycler was used for PCR. Primers sequences used are listed in Table 1. Samples were stored at −20 °C until analysis in agarose gel electrophoresis.

Table 1. List of primer sequences used for semi-quantitative PCR.

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Reference or Accession Number
mhcI	CCAGAGGATGTATGGTTGTGAG	TGGAGCGATCCATGTCTTTGTC	AF287490.1
mhcII	GTACTCCAGGTGGGAGTGGA	TGCAGCGCCTATGACTTCTA	AY273808.1
cd86	ATGTAACAGTGGCCTGTGA	CCACCCACTGCTGTTCACTA	FJ607781.1
cd83	GGAGCGTGAAGTGAACTTT	TCCTGGTTCTGCTCTCCTACA	AY263797.1
ef1α	TGGAGACTGGCACCCTGAAG	CCAACATTGTCACCAGGCATGG	[46]

2.12. Agarose Gel Electrophoresis

Each amplified DNA fragment generated by semi-quantitative PCR was separated via agarose gel (2%) (Cleaver Scientific, Warwickshire, UK) electrophoresis. Gel was prepared by diluting agarose in tris-borate-EDTA buffer (TBE) (45 mM TrisHCl, 0.45 M boric acid, 10 mM EDTA) (Merck, Ñuñoa, Chile) buffer with the pH adjusted to 8. To visualize DNA bands, 0.5 µL of GelRed (Biotium, Fremont, CA, USA) were added to 25 mL of TBE buffer/agarose, and 3 µL of each sample were loaded to the gel. Electrophoresis was done at 90V for 40 min using a PowerPac 300 power supply (Biorad, CA, USA). DNA bands were visualized using UV light in an Infinity 115 (Vilber Lourmart, Marné La Vallée, France) gel documentation system with the BioCapt software (Vilber Lourmart, Marné La Vallée, France). To determine the molecular weight, we used AccRuler 100 Bp Plus DNA RTU ladder (Maestrogen, Hsinchu City, Taiwan) which includes band sizes from 3000 bp to 100 bp.

2.13. Gene Expression by RT-qPCR

cDNA was synthetized as previously described. RT-qPCR was performed in 20 µL reactions using 12 ng of cDNA, 10 µL of TaqMan universal PCR master mix (Thermo Fischer Scientific), 900 nM final concentration of each primer (300 nM for NVHSV gene) and 300 nM of probe (150 nM for NVHSV gene) using the ABI PRISM 7300 System (Thermo Fischer Scientific). Cycling conditions were 50 °C for 2 min; 95 °C for 10 min; and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Gene expression was analyzed by the $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ method [47]. The eukaryotic 18S rRNA gene (Cat#4310893E, Thermo Fischer Scientific) was used as an endogenous control. Primer and probe sequences are listed in Table 2.

Table 2. List of primers and probes sequences used in quantitative RT-qPCR.

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Probe (5′–3′)	Reference or Accession Number
atg4b	GATCCTGTCCCTGTGATGATGA	CCCCTATTGGCTTCCCTTCT	ACCCCCCCCGGCGATTCTTC	XR_002473879
ulk1	CTTCTGCTGCTGGGTCTTCTG	GGTGACGGAAGAACTCCTCAAA	CGAAACCACAAGGACCGCATGGA	XR_002473462
becn1	GCGTGGGTGTCGTCTCAGTT	CAGGGAAGCAAGGAGAGCAT	ACCCTGGGTGTGCCCCTTGACC	NM_001124429
gabarap	CCTCATCCATCCATTTTTACCTCTT	ATTCAACCGAAATCCCCATCT	TCTGAATTTTATTTGCCTCCGGGTCTCC	NM_001165091
pik3c3	AGGCCAGCTGTGTGTGTTTCTA	GTTGCACATAGCGTTCCTGTTTA	TTTGCCCCCCCCGGATGATTGA	XM_021577851
cul3	GCAGCTTACGTTACAGCATCACA	TGGTGTTGGAGCCTGTTACCT	AACGCCACCTTCTACGGCCCAATC	XM_021587294.1
ikbkb	TGTTCCTGTTTGACCGTTCCT	CCGTCTGGACAAAGCGTATGT	CCTACGAGCCCCAGTTCACCCCC	XM_021621802.1
keap1	CCTCCACAAGCCCACCAA	AAGTATCCCCCTGCCGTGTA	CACGCCCAAAGTGCCCCAGC	XM_021556738.1
rab7	GTTGCGTGCTGGTGTTTGAC	ACTCGTCCCTCCAGCTGTCTAG	TGACCGCCCCCAACACCTTCAA	XM_021609589.1
sec13	GCAGTGATCCAGGCACAGAA	CTGGGACTAGGATAGATGGTAGAAGTG	ATTCCACTCCTCCTCCTACCCCCACA	XM_021610740.1
traf6	AGGACGCGGTGTGGAAGAG	CATGAATCTTGCTGTCCTCGTAAA	AGATGCACCAAAGCCAACACTGCCA	XM_021586866.1
mhcI	GACAGTCCGTCCCTCAGTGT	CTGGAAGGTTCCATCATCGT		[48]
mhcII	TGCCATGCTGATGTGCAG	GTCCCTCAGCCAGGTCACT	CGCCTATGACTTCTACCCCAAACAAAT	[49]
cd86	GGTCTGTGACCCTCCCCTGTA	CCCTCGTCTTATGGTAGCCATT		XR_002470439.1
cd83	TTGGCTGATGATTCTTTCGATATC	TGCTGCCAGGAGACACTTGT	TCCTGCCCAATGTAACGGCTGTTGA	[50]
NVHSV	GACTCAACGGGACAGGAATGA	GGGCAATGCCCAAGTTGTT	TGGGTTGTTCACCCAGGCCGC	[41]

2.14. Extracellular Immunofluorescence Staining

To stain the cell surface markers MHCI, MHCII, CD83, and CD86, RBCs were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich) and 0.008% glutaraldehyde (Sigma-Aldrich) diluted in RPMI medium for 20 min. Primary antibodies were diluted in PBS at 1/200 dilution for anti-MHCI, 1/200 for anti-MHCII, 1/100 for anti-CD83, and for 1/200 anti-CD86. Samples were incubated for 60 min. For flow cytometry, goat anti-rabbit IgG (H+L) CF™ 488 antibody (Sigma-Aldrich) was used for the secondary antibody for anti-CD83, and goat anti-mouse IgG (H+L) CF™ 488 antibody (Sigma-Aldrich) was used for anti-MHCI, anti-MHCII, and anti-CD86. Secondary antibodies were incubated for 30 min at 1/200 dilution. RBCs were washed with PBS after each antibody incubation. Flow cytometry analysis was done in a BD FACSCanto™ II (BD Biosciences) flow cytometer. Immunofluorescence (IF) images were taken with the INCell Analyzer 6000 cell imaging system (GE Healthcare, Little Chalfont, UK).

2.15. Intracellular Immunofluorescence Staining

RBCs were fixed with 4% PFA and 0.008% glutaraldehyde diluted in RPMI medium. RBCs were incubated with permeabilization buffer containing 0.05% saponin (Sigma-Aldrich) in PBS, for 15 min. Primary antibodies were used at 1/1000 dilution for 2C9 anti-NVHSV, 1/200 for anti-p62, and 1/100 for anti-ubiquitin in permeabilization buffer. Samples were incubated for 60 min at room temperature. Secondary antibodies were incubated for 30 min at 1/200 dilution in permeabilization buffer. RBCs were washed with permeabilization buffer after antibody incubations. Goat anti-rabbit IgG (H+L) CF™ 647 antibody and goat anti-mouse IgG (H+L) CF™ 488 antibody was used as secondary antibodies (Sigma-Aldrich). For anti-ubiquitin and anti-NVHSV double staining, goat anti-rabbit IgG (H+L) CF™ 488 antibody and goat anti-mouse IgG (H+L) CF™ 647 antibody was used as secondary antibodies. RBCs were maintained in 1% PFA in PBS. Nuclear staining was performed by staining RBCs with 1 μg/mL of 4'-6-408 Diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for five minutes.

For LC3 staining, RBCs were fixed using 4% PFA and 0.008% glutaraldehyde (Sigma-Aldrich) in PBS for 20 min and permeabilized with cold methanol (Panreac) for 15 min. LC3 antibody was diluted 1/100 in 0.3% Triton X-100 in PBS and incubated for two hours at room temperature for flow cytometry and overnight at 4 °C for immunofluorescence. Secondary antibody goat anti-rabbit IgG (H+L) CF™ 488 (Sigma-Aldrich) was diluted 1/200 in 0.3% Triton X-100 (Sigma-Aldrich) in PBS and incubated for 30 min for flow cytometry and 90 min for immunofluorescence, both at room temperature. RBCs were kept in 1% PFA in PBS before the analysis. Immunofluorescence images were taken in the INCell Analyzer 6000 cell imaging system (GE Healthcare).

2.16. Transmission Electron Microscopy (TEM)

Control and VHSV-exposed RBCs were fixed with glutaraldehyde at 2% in 0.1 M cacodylate buffer for three to four hours at room temperature. Post-fixation was performed with osmium tetroxide at 1% in 0.1 M cacodylate buffer for one hour at 4 °C. RBCs were centrifuged at 1600 rpm and washed with 0.1 M cacodylate buffer over 10 min three times after both steps. For the last wash, RBCs were kept at 4 °C overnight. The sample was applied to 3% agar and dehydrated using an increasing gradient of alcohol (30%, 50%, 70%, 96% and 100% during 10 min), acetone (two 10-min rounds), acetone/epon resin 1:1 (1 h), and epon resin (overnight with the Eppendorf tape open and then closed for four hours). Finally, a block with the sample was polymerized at 58 °C to 60 °C for 24 h. Images were taken using the electronic transmission microscope Jeol 1011 (JEOL, Inc. Peabody, MA, USA) from the UMH Institute of Bioengineering.

2.17. In situ Proximity Ligation Assay (PLA)

Superfrost microscope slides were cleaned using ethanol. Two areas of 1 cm^2 were delimited using a Dako pen (Agilent, Santa Clara, CA, USA) on each microscope slide, and Dako pen stain dried overnight. Ficoll-purified RBCs were washed three times, and approximately 2.5×10^5 RBCs were used

from unexposed or VHSV-exposed (MOI 10) RBCs, at 14 °C for 24 h. RBCs were added to each area in a volume of 125 µL of RPMI. RBCs were left to sediment for 15 min. Then, RPMI was carefully removed, and 100 µL fixation buffer consisting of RPMI with 4% PFA was added for 1 h at room temperature. RBCs were washed three times with PBS after removing the fixation buffer. Then, 70% ethanol was applied to the slides for 30 s. Slides dried on ice for one hour and then were stored at −20 °C.

Duolink In Situ–Fluorescence kit (Sigma-Aldrich) was used following the manufacturer's instructions to perform the PLA. Once slides dried, blocking solution was added to each area, and slides were incubated for one hour at 37ºC in a wet chamber. Blocking solution was removed, and a mixture containing the primary antibodies mouse anti-MHCI (1/200) or anti-MHCII (1/200) and polyclonal rabbit anti- GVHSV antibody [40] (kindly provided by Dr Neils Lorenzen to Dr Julio Coll) (1/300) were incubated overnight in a wet chamber at 4 °C. Alternatively, anti-MHCI or anti-MHCII were incubated together with rabbit serum (1/300) to detect nonspecific background signals. After incubation with the primary antibodies, RBCs were washed twice with wash buffer A for 5 min with slow agitation. Excess wash buffer A was removed, and the PLA Probes MINUS reagent was incubated at a 1/5 dilution for 1 h at 37 °C in the wet chamber. Then, ligation shock reagent and ligase were added to the RBCs after washing. Amplification reagents were added to the RBCs and then removed after 100 min of incubation. Slides were mounted with a cover slip using DuoLink In Situ Mounting Medium with DAPI and stored at −20 °C until analysis.

To quantify positive colocalization between MHCI or MHCII and GVHSV peptides in RBCs, we used a counting algorithm in the IN Cell Developer software (GE Healthcare). Briefly, RBC cytoplasm was delimited using a collar around the nucleus (labeled by DAPI) of a ~5 µm radius. Positive colocalization was noted by detection of granules inside the RBCs cytoplasm (settings were adjusted for a minimum brightness and granular size to be considered for colocalization between the two molecules).

2.18. Western Blot

RBCs pellets (10^7 cells) and head kidney tissue samples were resuspended in 100 µL PBS buffer with a protease inhibitor cocktail (Sigma-Aldrich). Cells were lysed by freezing and thawing samples three times. Tissues were disrupted using micropestles (Invitrogen). Cell debris was eliminated by centrifugation at 12,000 rpm for 10 min. Samples were loaded in a 12% polyacrylamide gel (Invitrogen), except for anti-ubiquitin which was at 16%, under reducing conditions. Electrophoresis was performed at 150 V for 100 min. Proteins in the gel were transferred to 0.4 µm pore size nitrocellulose membranes (BioRad, Madrid, Spain) for 120 min at 100 V in transfer buffer (2.5 mM Tris, 9 mM glycine, 20% methanol). Membranes were then blocked with 5% dry milk and 0.2% Tween-20 in PBS and incubated with rabbit polyclonal anti-ubiquitin, rabbit polyclonal anti-αactin (42 kDa), mouse monoclonal anti-MHCI (45 kDa), mouse polyclonal anti-MHCII (~34 kDa), mouse polyclonal anti-CD86 (~31 kDa), or rabbit polyclonal anti-CD83 (~24 kDa) in PBS containing 5% dry milk and 0.2% Tween-20 (blocking buffer) overnight at 4 °C. Membranes were washed three times for 10 min each with PBS Tween-20 0.2% buffer before incubation with GAR-Po (Sigma-Aldrich) or GAM-Po (Sigma-Aldrich) in blocking buffer for 60 min. Membranes were then washed three times with PBS Tween-20 0.2%. Peroxidase activity was detected using enhanced chemiluminescence (ECL) reagents (Amersham Biosciences, Buckinghamshire, UK) and exposure to X-ray. Protein lanes and bands were analyzed by densitometry using ImageJ software (version 1.51, National Institutes of Health, Bethesda, MD, USA). Lanes were selected using the rectangle tool of ImageJ software and the integrated density of the lane was measured. α-actin band densitometry was calculated by plotting the band density after selecting the bands with the rectangle tool.

2.19. Software and Statistics

All graphs show the mean and standard deviation of the data. *P* values associated with each graphic are represented by: *, *P* value < 0.05; **, *P* value < 0.01; ***, *P* value < 0.001; ****, *P* value < 0.0001. Graphpad Prism 6 (www.graphpad.com) (Graphpad Software Inc., San Diego, CA, USA) was used to prepare graphs and perform statistical calculations. Flow cytometry data were analyzed using Flowing Software v2.5.1 (http://flowingsoftware.btk.fi/) to obtain mean fluorescence intensity (MFI) values and Weasel v3.0.1 (https://frankbattye.com.au/Weasel/) to obtain graphical representation of histograms and dot plots.

3. Results

3.1. Transcriptomic Analysis Indicated Up-Regulation of Antigen-Processing-Related Molecules in Ex Vivo VHSV-Exposed Rainbow Trout RBCs

To identify major processes activated when rainbow trout RBCs are exposed to VHSV, a transcriptomic analysis using RNA-Seq and pathway enrichment evaluation were performed on VHSV-exposed RBCs at 4 and 72 hpe. Several up-regulated genes were classified into GO categories of ubiquitination and proteasome degradation and MHC class I antigen processing and presentation (Figure 1, Supplementary Table S1) at 4 hpe. Selected genes belonging to the ubiquitination and proteasome degradation category are listed in Table 3 (Supplementary Tables S1 and S2). Among these up-regulated genes are cullin 3 (*cul3*) and proteasome subunits α6 (*psma6*) and β5 (*psmb5*). Also related to the MHCI presentation pathway, our analysis identified calnexin (*canx*), GTPase activating protein SEC13 (*sec13*), and inhibitor of nuclear factor kappa B kinase (*ikbkb*). Ras-related rab 7 (*rab7*) and tumor necrosis factor (TNF) receptor-associated factor 6 (*traf6*) were analyzed by RT-qPCR as genes related to the MHCI presentation pathway. RT-qPCR validation of the genes identified in the transcriptomic analysis is shown in Supplementary Figure S4, where a tendency to up-regulation is observed at 4 hpe, although the RT-qPCR data do not strongly support the fold changes found by RNA-Seq. Moreover, we also identified up-regulation of some genes involved in autophagy, such as unc-51–like autophagy activating kinase 1 (*ulk1*), beclin 1 (*becn1*), and autophagy-related 9A (*atg9a*) (Table 4). In contrast, at 72 hpe, RBCs showed a global down-regulation (Supplementary Tables S1 and S2).

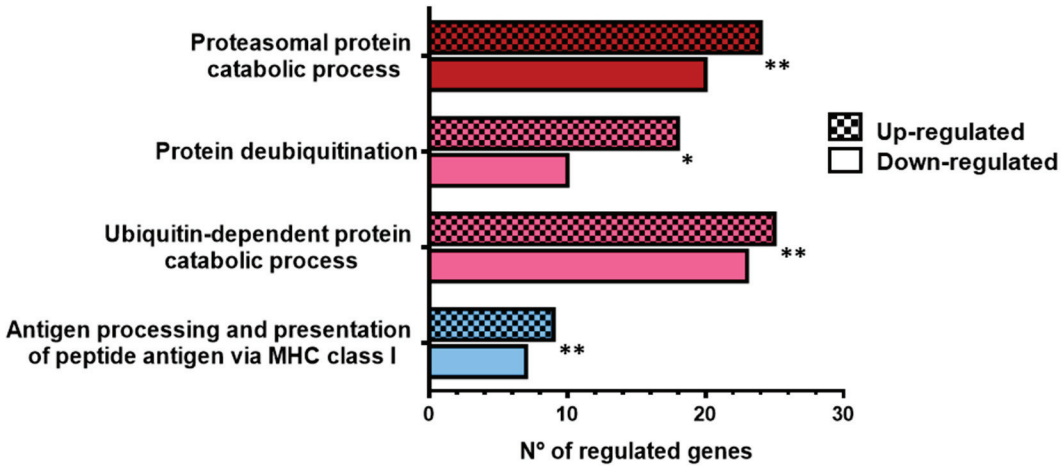


Figure 1. Transcriptomic analysis indicated up-regulation of antigen-processing-related molecules in ex vivo VHSV-exposed rainbow trout RBCs. Number of up-regulated and down-regulated genes related to proteasomal protein and catabolic process (GO:0010498), protein deubiquitination (GO:0016579), ubiquitin-dependent protein catabolic process (GO:0006511), antigen processing and presentation of peptide antigen via MHC class I (GO:0002474) (Supplementary Table S1), by RNA-Seq from ex vivo unexposed and VHSV-exposed RBCs at 4 hpe. Asterisks denote GO-term significance.

Table 3. Fold change of genes from the "class I MHC-mediated antigen processing and presentation" and "antigen processing: ubiquitination and proteasome degradation" pathways in the transcriptomic analysis of VHSV-exposed rainbow trout RBCs at 4 hpe. Gene expression values were calculated by normalization against unexposed RBCs. Gene *P* values were <0.001 and FDR *P* values < 0.05. Gene symbols correspond to homologue *Homo sapiens* genes identified by sequence homology using Blast2GO.

Antigen Processing: Ubiquitination and Proteasome Degradation		Class I MHC-Mediated Antigen Processing and Presentation	
Gene Symbol	**Log_2 Fold**	**Gene Symbol**	**Log_2 Fold**
cul3	4.77	*canx*	4.31
keap1	7.56	*sec13*	5.35
psma6	5.02	*ikbkb*	5.69
psmb5	3.72	*klhl13*	5.36

Table 4. Fold change of the autophagy-related genes *ulk1*, *becn1*, and *atg9a* obtained in the transcriptomic analysis of VHSV-exposed rainbow trout RBCs at 4 hpe. Gene expression values were calculated by normalization against uninfected RBCs. Gene *P* values were < 0.001 and FDR *P* values < 0.05.

Autophagy-Related Genes	
Gene Symbol	**Log_2 Fold**
ulk1	3.46
becn1	5.55
atg9a	3.69

3.2. Proteomic Analysis of VHSV-Exposed RBCs Showed Proteasome Down-Regulation, Increased Ubiquitination, and Regulation of Antigen Presentation-Related Molecules at 72 hpe

We analyzed the response of ex vivo RBCs to VHSV at 72 hpe using a proteomic analysis and pathway enrichment evaluation. Up-regulated proteins were overrepresented in antigen processing and presentation of peptide antigen via MHC class II (GO:0002495), and proteasome-mediated ubiquitin-dependent protein catabolic process (GO:0043161), while proteasome (KEGG:03050) and antigen-processing and presentation of exogenous peptide antigen (GO:0002478) were mostly down-regulated (Figure 2a). A list of all overrepresented terms and statistics is provided in Supplementary Table S3. Table 5 displays the fold change of proteins from these categories (Supplementary Table S4).

A Cytoscape pathway network of significantly overrepresented Immune System Process GO terms showed up-regulation in antigen processing and presentation of peptide antigen via MHC class II, cytoplasmic pattern recognition receptor signaling pathway, neutrophil degranulation, and leukocyte activation; however, it showed down-regulation of antigen presentation via MHC class I. (Figure 2b). In Supplementary Figure S5, we show a Venn diagram to compare the common products found in our omics studies.

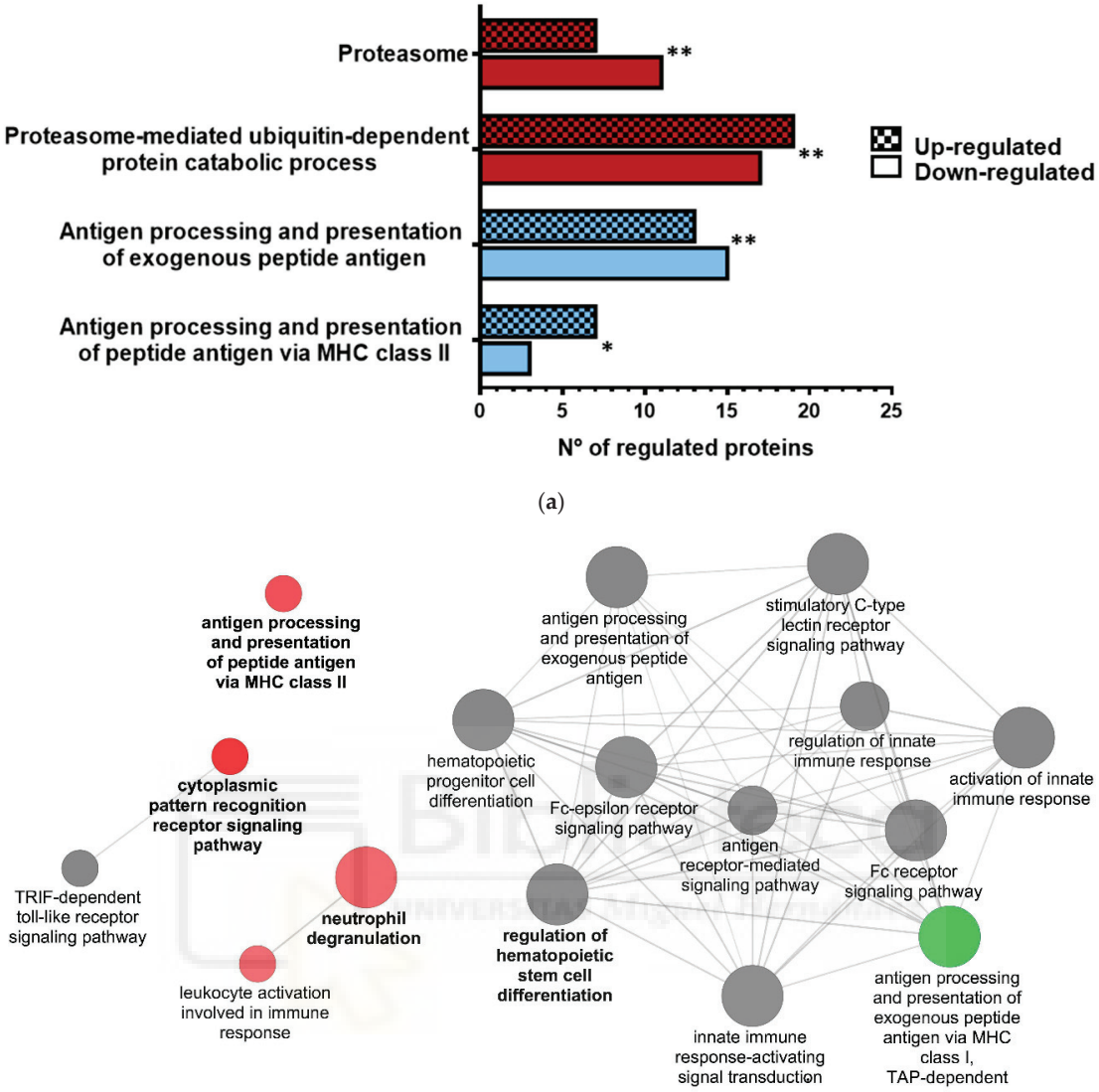


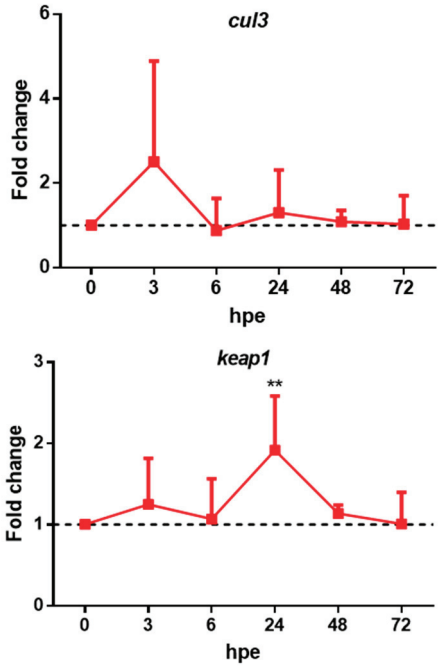
Figure 2. Proteomic analysis of VHSV-exposed RBCs showed proteasome down-regulation, increased ubiquitination, and regulation of molecules from antigen presentation pathways at 72 hpe. (**a**) Number of up-regulated and down-regulated proteins related to proteasome (KEGG:03050), proteasome-mediated ubiquitin-dependent protein catabolic process (GO:0043161), antigen-processing and presentation of exogenous peptide antigen (GO:0002478), and antigen processing and presentation of peptide antigen via MHC class II (GO:0002495), as identified by proteomic analysis from ex vivo unexposed and VHSV-exposed rainbow trout RBCs at 72 hpe (Supplementary Table S3). Asterisks denote GO-term significance. (**b**) Cytoscape pathway network of significantly overrepresented Immune System Process GO terms in VHSV-exposed RBCs at 72 hpe (Supplementary Table S3). Each node represents a GO-term from GO Immune System Process. Node size shows GO-term significance (*P* value): a smaller *P* value indicates larger node size. Edge (line) between nodes indicates the presence of common genes: a thicker line implies a larger overlap. The label of the most significant GO-term for each group is highlighted. Up-regulated pathways are coded as red, while down-regulated pathways are coded as green. Pathways with a similar number of up-regulated or down-regulated proteins are coded as gray. Asterisks denote statistical significance.

Table 5. List of up-regulated (left) and down-regulated (right) identified proteins from the "antigen processing and presentation of peptide antigen via MHC class II", "proteasome-mediated ubiquitin-dependent protein catabolic process" and "proteasome" pathways. Protein FDR *P* values were < 0.001. Protein symbols correspond to homologue *Homo sapiens* proteins identified by sequence homology using Blast2GO.

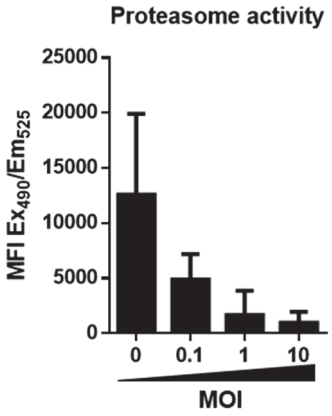
Antigen Processing and Presentation of Peptide Antigen via MHC Class II				Proteasome-mediated Ubiquitin-Dependent Protein Catabolic Process				Proteasome			
Upr. Protein	Log_2 Fold	Downr. Protein	Log_2 Fold	Upr. Protein	Log_2 Fold	Downr. Protein	Log_2 Fold	Upr. Protein	Log_2 Fold	Downr. Protein	Log_2 Fold
ACTR1B	3.37	CAPZB	−2.68	CD2AP	7.50	HSPA5	−6.23	PSMB3	4.44	PSMA1	−5.33
AP2S1	5.75	CLTC	−3.69	DDB1	3.32	PSMA1	−5.33	PSMB6	3.73	PSMA2	−5.43
CLTA	4.51	RAB7A	−4.69	GCLC	4.63	PSMA2	−5.43	PSMC2	2.98	PSMA3	−3.31
DNM2	5.32			HSPA1A	4.74	PSMA3	−3.31	PSMD13	2.26	PSMA4	−4.78
DYNC1H1	5.43			NPLOC4	1.68	PSMA4	−4.78	PSMD2	3.98	PSMA5	−6.15
KIF15	3.89			PLAA	5.08	PSMA5	−6.15	PSMD4	5.83	PSMA6	−6.49
PYCARD	3.28			PSMB3	4.44	PSMA6	−6.49	PSME1	5.73	PSMA8	−5.50
				PSMB6	3.73	PSMA8	−5.50			PSMB1	−4.45
				PSMC2	2.98	PSMB1	−4.45			PSMB2	−5.29
				PSMD13	2.26	PSMB2	−5.29			PSMB4	−3.69
				PSMD2	3.98	PSMB4	−3.69			PSME2	−3.44
				PSMD4	5.83	PSME2	−3.44				
				PSME1	5.73	RAD23B	−3.77				
				RACK1	3.95	UBC	−5.19				
				RAD23A	4.85	UBR2	−11.48				
				RPS27A	5.03	VCP	−2.86				
				UBB	4.39	WFS1	−10.33				
				USP19	8.29						
				YOD1	2.95						

3.3. VHSV Induced Ubiquitination But Impaired Proteasome Degradation in Ex Vivo VHSV-exposed Rainbow Trout RBCs

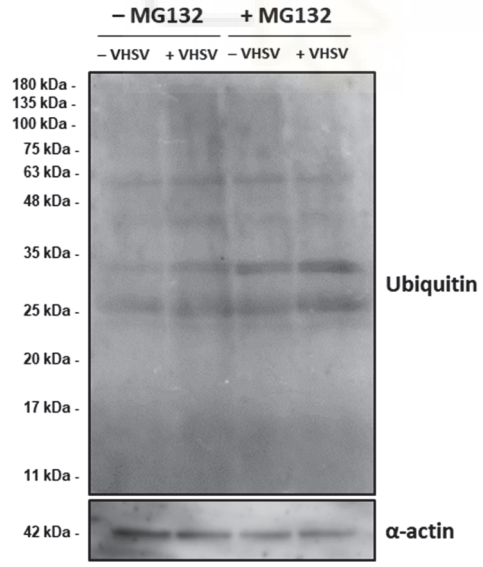
To validate the role of the UPS in the nonpermissive infection of rainbow trout RBCs by VHSV, we performed a time-course experiment analyzing the expression of two genes belonging to the ubiquitin E3 ligase complex: *cul3* and kelch-like ECH-associated protein 1 (*keap1*). The results showed increased expression of *cul3* at 3 hpe while *keap1* expression increased at 12 hpe (Figure 3a). We measured the activity of the 20S proteasomes using a commercial kit and observed a MOI-dependent decrease in 20S proteasome activity (Figure 3b). Then, we performed a western blot using an anti-ubiquitin antibody for unexposed and VHSV-exposed RBCs with or without the proteasome inhibitor MG132. Ubiquitination of proteins on VHSV-exposed RBCs increased in comparison with unexposed RBCs. A higher amount of ubiquitinated proteins was also found in RBCs treated with MG132 (Figure 3c,d). To test whether the proteasome is involved in the degradation of VHSV, we assessed the presence of NVHSV using 2C9 monoclonal antibody in VHSV-exposed RBCs treated with MG132. Flow cytometry results did not show an increase in intracellular NVHSV in VHSV-exposed RBCs treated with MG132 (Figure 3e). The population used for the flow cytometry analysis is depicted in Supplementary Figure S6. Double staining using 2C9 and anti-ubiquitin antibodies showed higher ubiquitin labeling in RBCs with VHSV (Figure 3f).



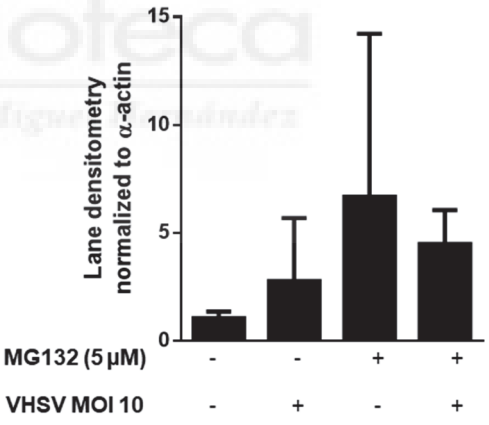
(a)



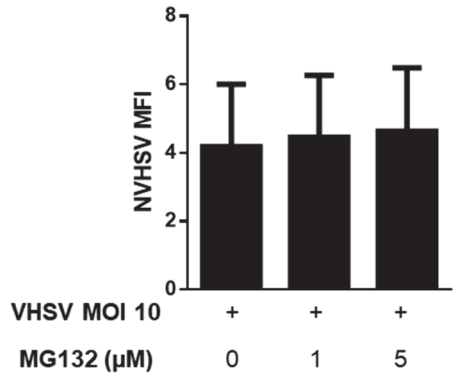
(b)



(c)

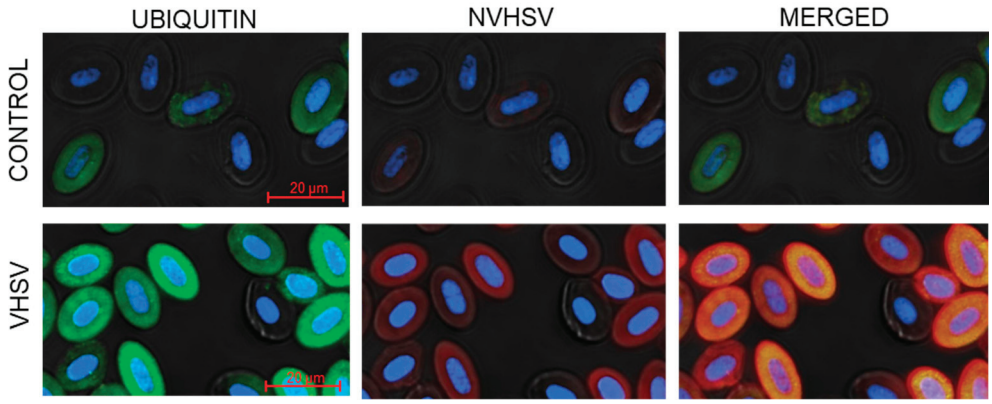


(d)



(e)

Figure 3. *Cont.*

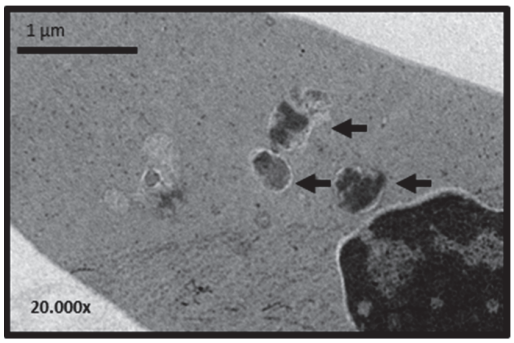


(**f**)

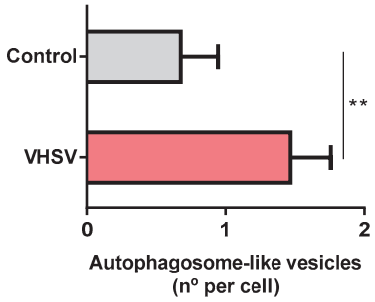
Figure 3. VHSV induced protein ubiquitination but impaired proteasome degradation in ex vivo VHSV-exposed rainbow trout RBCs. (**a**) Time-course expression of *cul3* and *keap1* at 0, 3, 6, 24, 48, and 72 hpe from VHSV-exposed (MOI 1) RBCs. Data represent mean ± SD (n = 5), relative to control cells (black dotted line). A two-way analysis of variance (ANOVA) with Sidak´s multiple comparisons test was performed to test statistical significance between unexposed and VHSV-exposed RBCs at each time point. (**b**) 20S proteasome activity measured by fluorogenic substrates in RBCs unexposed or exposed to VHSV at the indicated MOI at 24 hpe. Data represent mean ± SD (n = 3). Kruskal-Wallis with Dunn's multiple comparisons test was performed to test statistical significance between each condition and unexposed RBCs. (**c**) Western blot of ubiquitin of lysates from unexposed and VHSV-exposed (MOI 10) RBCs at 24 hpe, treated with or without MG132 (5 µM). α-actin was used as endogenous control. Results are representative of 2 independent experiments. (**d**) Integrated densitometry of ubiquitin lane content from unexposed and VHSV-exposed (MOI 10) RBCs at 24 hpe, treated with or without MG132 (5 µM). Values were normalized to α-actin. Data represent mean ± SD (n = 2). (**e**) Intracellular quantification by flow cytometry of NVHSV in VHSV-exposed (MOI 10) RBCs at 72 hpe, treated with or without MG132 (1 or 5 µM). Data represent mean ± SD (n = 4). Kruskal-Wallis with Dunn's multiple comparisons test was performed to test statistical significance between MG132 treated and non-treated RBCs. (**f**) Representative immunofluorescence of unexposed (control) and VHSV-exposed RBCs at MOI 100 and at 9 hpe stained with anti-ubiquitin (488 stain), 2C9 anti-NVHSV (647 stain), and DAPI for nuclei staining. Asterisks denote statistical significance.

3.4. VHSV Induced Autophagy in Ex Vivo VHSV-exposed Rainbow Trout RBCs

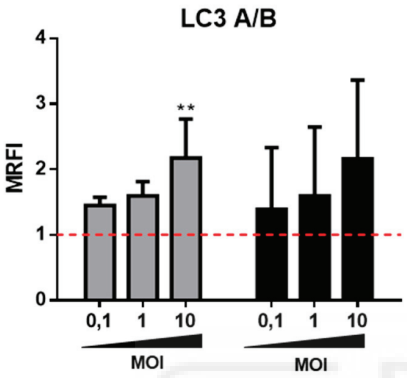
To determine whether VHSV induced autophagy in ex vivo rainbow trout RBCs, we exposed RBCs to VHSV at MOI 1 for 24 h. We identified the presence of autophagosome-like vesicles inside VHSV-exposed RBCs (Figure 4a) via TEM. We visually counted the number of autophagosome-like vesicles in unexposed RBCs and VHSV-exposed RBCs and noted a significant increase in VHSV-exposed RBCs (Figure 4b). The turnover of the autophagy protein LC3A/B was monitored by means of LC3A/B immunostaining, as previously described for rainbow trout cells [14,51]. LC3 immunostaining increased at higher MOIs in a dose-dependent manner up to 2-fold in comparison with unexposed RBCs at 24 and 72 hpe (Figure 4c). By immunofluorescence microscopy, we identified an increased number of LC3 dots in VHSV-exposed RBCs (Figure 4d). Moreover, we analyzed the ubiquitin-binding protein p62, which undergoes degradation during activation of autophagy [52], as it is an autophagosome cargo protein that targets other proteins for selective autophagy. To evaluate whether p62 undergoes degradation in the RBC response to VHSV, an intracellular staining using anti-p62 antibody was performed on unexposed and VHSV-exposed (MOI 10) RBCs at 6, 12, and 24 hpe. Decreased intracellular p62 levels were detected in VHSV-exposed RBCs at 6 hpe compared to control RBCs (Figure 4e,f). By 24 hpe, p62 levels recovered from the degradation observed at 6 hpe. Kinetics of expression of the autophagy-related genes *ulk1*, *becn1* and *gabarap* showed statistically significant up-regulation at 3 hpe (Supplementary Figure S7).


1 µm
20.000x

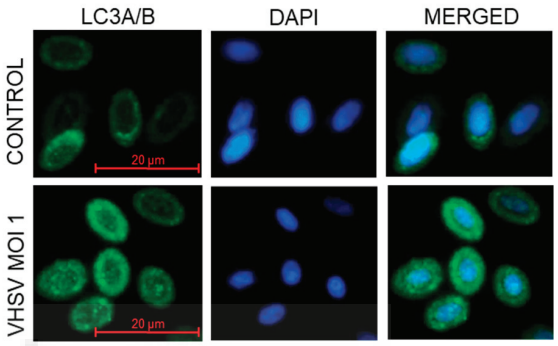
(a)


Control
VHSV
**
0
1
2
Autophagosome-like vesicles
(nº per cell)

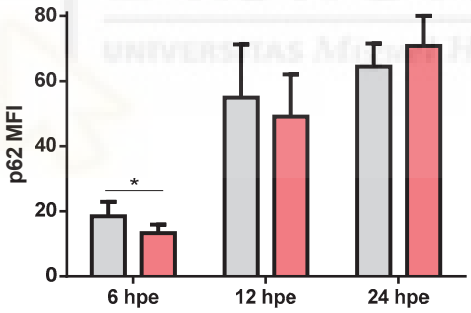
(b)


LC3 A/B
MRFI
**
0,1
1
10
MOI
0,1
1
10
MOI

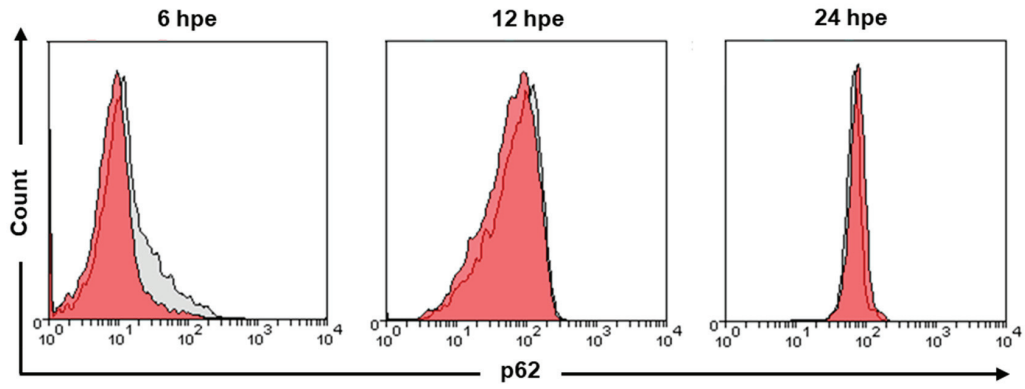
(c)


LC3A/B
DAPI
MERGED
CONTROL
VHSV MOI 1
20 µm
20 µm

(d)


p62 MFI
*
6 hpe
12 hpe
24 hpe

(e)

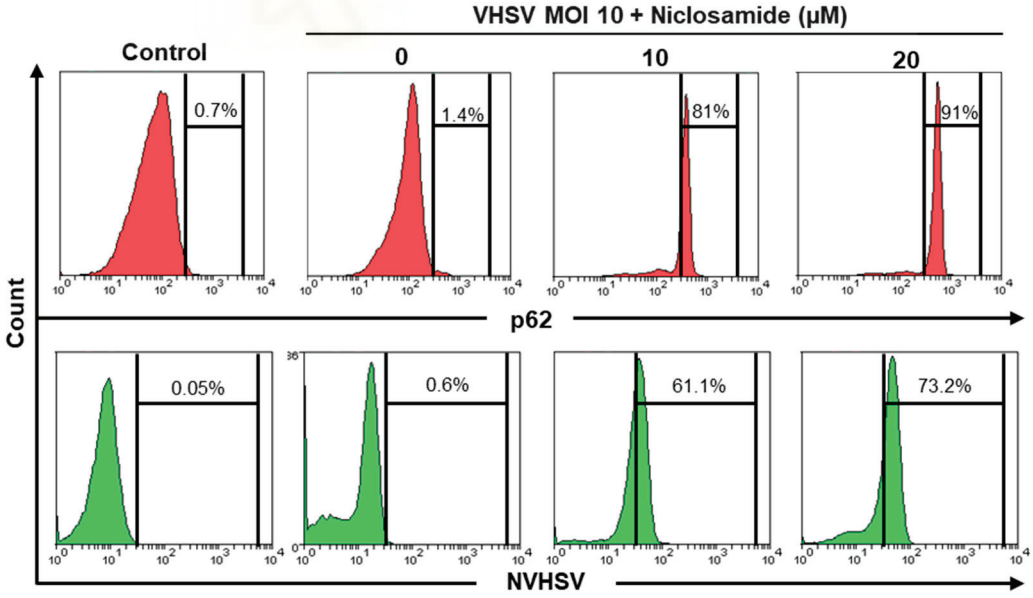

6 hpe
12 hpe
24 hpe
Count
p62

(f)

Figure 4. VHSV induced autophagy in ex vivo VHSV-exposed rainbow trout RBCs. (**a**) Representative transmission electron micrographs of VHSV-exposed RBCs, pointing out autophagosome-like vesicles (black arrows). (**b**) Count of autophagosome-like vesicles from transmission electron micrographs of unexposed and VHSV-exposed rainbow trout. Data represent the mean ± SD (n = 30). A Mann-Whitney test was performed to test statistical significance. (**c**) Autophagosome membrane protein LC3 expression levels in VHSV-exposed RBCs at 24 (gray bars) and 72 hpe (black bars) relative to unexposed RBCs (red line) evaluated by flow cytometry (n = 5). Data is represented as MRFI (Mean Relative Fluorescence Intensity) = fluorescence in VHSV-exposed RBCs/fluorescence in non-exposed RBCs. A Kruskal-Wallis with Dunn´s multiple comparisons test was performed to test statistical significance between each condition and unexposed RBCs. (**d**) Representative immunofluorescence of unexposed (control) and VHSV-exposed RBCs at MOI 1 and at 72 hpe stained with anti-LC3 (488) and DAPI for nuclei staining. (**e**) Mean fluorescence intensity of p62 protein expression in RBCs unexposed (gray bars) and exposed to VHSV at MOI 10 (red bars) after 6, 12, and 24 hpe. Data represent the mean ± SD (n = 5). A Mann-Whitney test was performed to test statistical significance between unexposed and VHSV-exposed RBCs at each time point. (**f**) Representative histograms of p62 in RBCs exposed to VHSV (MOI 10) at 6, 12, and 24 hpe: unexposed (gray histogram), VHSV-exposed (red histogram). Asterisks denote statistical significance.

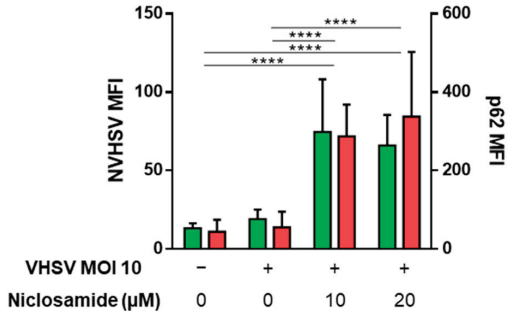
3.5. Niclosamide Increased p62 and Intracellular VHSV Levels in Ex Vivo VHSV-exposed RBCs

The drug niclosamide blocks autophagy degradation via lysosomal dysfunction [53,54]. Moreover, niclosamide has been previously used in the context of viral infections [55]. After exposing RBCs to VHSV MOI 10, RBCs were treated with niclosamide at 10 and 20 μM. Then, an intracellular stain using 2C9 and anti-p62 antibodies was done at 72 hpe. Flow cytometry results showed that VHSV-exposed RBCs treated with niclosamide at both tested concentrations had a higher percentage of NVHSV- and p62-positive cells compared to RBCs exposed to VHSV but not treated with niclosamide (Figure 5a). MFI of unexposed RBCs and VHSV-exposed (MOI 10) RBCs were similar, but both NVHSV and p62 MFI increased up to three-fold in the presence of niclosamide (Figure 5b).



(**a**)

Figure 5. *Cont.*

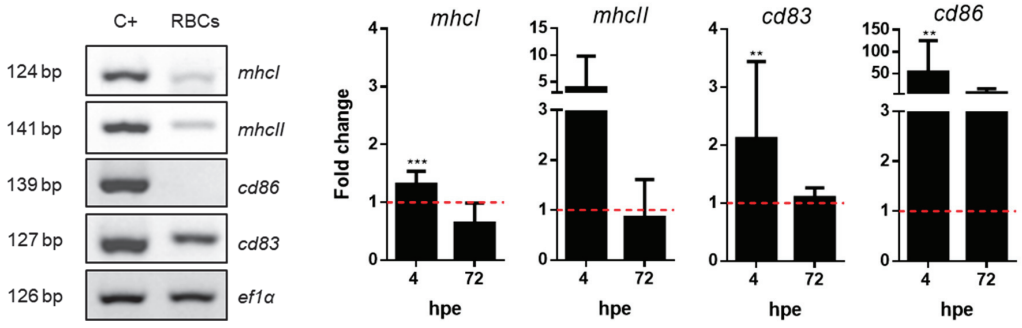


(**b**)

Figure 5. Niclosamide increased p62 and intracellular VHSV levels in ex vivo VHSV-exposed RBCs. (**a**) Representative histograms of NVHSV (green) and p62 (red) intracellular expression in RBCs unexposed (control) and VHSV-exposed (MOI 10) RBCs treated or not with niclosamide 10 or 20 μM and evaluated by flow cytometry at 72 hpe. Percentages represent the number of positive cells. Dimethyl sulfoxide (DMSO) was added to untreated RBCs to match culture conditions of treated cells (DMSO 0.04%). (**b**) MFI of intracellular NVHSV (green) and p62 (red) in unexposed (control) and VHSV-exposed (MOI 10) RBCs treated or not with niclosamide 10 or 20 μM and evaluated by flow cytometry at 72 hpe. Data represent mean ± SD (n = 6). A two-way ANOVA with Tukey´s multiple comparisons test was performed to test statistical significance. Asterisks denote statistical significance.

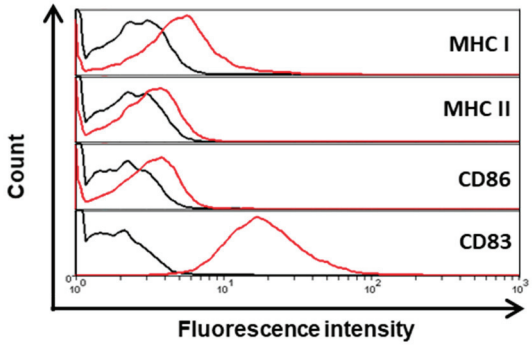
3.6. Rainbow Trout RBCs Up-Regulated MHCI, MHCII, CD86, and CD83 after VHSV Exposure

Because antigen presentation pathways were overrepresented in transcriptomic and proteomic analyses, we investigated whether RBCs expressed characteristic cell markers molecules of APCs. RNA was extracted from RBCs and then we performed RT-PCR. Semi-quantitative PCR was performed, and a mix of tissue samples from the head kidney, spleen, and gill was used as a positive control for APCs genes expression. Final products from semi-quantitative PCR were analyzed in agarose gel electrophoresis. mRNA transcripts from *mhcI*, *mhcII*, and *cd83* were detected in rainbow trout RBCs, whereas there was no *cd86* expression (Figure 6a). We then examined how VHSV modified the expression of these transcripts using quantitative RT-qPCR. We observed a slight increase in *mhcI* expression and a pronounced increase in *mhcII*, *cd83*, and *cd86* expression in VHSV-exposed RBCs at 4 hpe. Whereas, we only observed up-regulation of *cd86* at 72 hpe (although lower than levels at 4 hpe), while no up-regulation was observed at 72 hpe for the *mhcI*, *mhcII,* and *cd83* genes (Figure 6b). We confirmed the up-regulation of MHCI, MHCII, CD86, and CD83 at the protein level in VHSV-exposed RBCs 24 hpe (Figure 6c–e). Also, *cd83* gene expression was found to be up-regulated by transcriptomic analysis at 4 hpe (Log_2 fold: 4.87; Supplementary Table S2). In contrast, MHCI protein expression was found down-regulated by proteomic analysis at 72 hpe (Log_2 fold: −11.38; Supplementary Table S4).

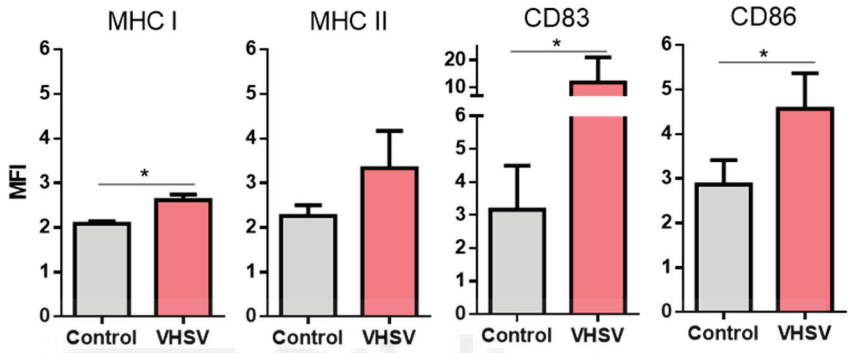


(**a**) (**b**)

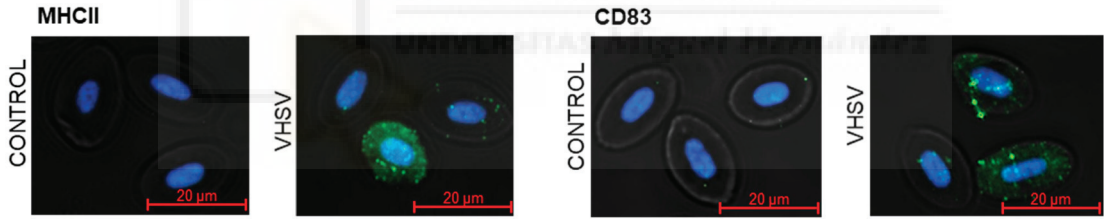
Figure 6. *Cont.*



(**c**)



(**d**)



(**e**)

Figure 6. Rainbow trout RBCs up-regulated MHCI, MHCII, CD83, and CD86 molecules after exposure to VHSV. (**a**) Specific transcript mRNA expression of *mhcI*, *mhcII*, *cd86*, and *cd83* genes from rainbow trout RBCs. A mix of gill, spleen, and head kidney tissues was used as a positive control of expression from the assayed cell markers (C+). The *ef1α* gene was used as an endogenous control. (**b**) Fold change in the expression of *mhcI*, *mhcII*, *cd86*, and *cd83* in rainbow trout RBCs at 4 and 72 hpe with VHSV MOI 1 in comparison to unexposed RBCs, by RT-qPCR. Data represent mean ± SD (n = 4). Dotted red line represents basal gene expression from unexposed RBCs. A Mann-Whitney test was performed to test statistical significance between VHSV-exposed and unexposed RBCs. (**c**) Representative histograms of MHCI, MHCII, CD86, and CD83 extracellular stain in unexposed RBCs (black) and VHSV-exposed RBCs (red) (MOI 10) at 24 hpe. (**d**) MFI of MHCI, MHCII, CD86, and CD83 extracellular stain in unexposed RBCs (gray bars) and VHSV-exposed RBCs (red bars) (MOI 10) at 24 hpe. Data represent mean ± SD (n = 4). A Mann-Whitney test was performed to test statistical significances between VHSV-exposed and unexposed RBCs. (**e**) Representative immunofluorescence images of MHCII and CD83 expression in control and VHSV-exposed RBCs at 24 hpe. Asterisks denote statistical significance.

3.7. VHSV Induced Autophagy and Antigen Presentation Genes Expression in RBCs from VHSV-challenged Rainbow Trout

We evaluated whether VHSV could induce both autophagy- and antigen-presentation-related genes in vivo by using RBCs from VHSV-challenged and mock-infected rainbow trout. We used tissue

samples from the spleen and head kidney, as well as total blood and Ficoll-purified RBC samples, from VHSV-challenged and mock-infected rainbow trout to quantify NVHSV gene transcripts by RT-qPCR. RBCs from challenged rainbow trout showed lower levels of NVHSV in comparison with total blood, spleen, and head kidney samples (Figure 7a). We also analyzed ubiquitination of proteins in RBCs from VHSV-challenged and mock-infected rainbow trout and we did not observe an increase in ubiquitinated proteins at 2 days post challenge (dpc) (Figure 7b,c), in contrast to ex vivo experiments. We analyzed the expression of a set of genes related to autophagy, E3 ubiquitin ligase component, and antigen presentation in RBCs from VHSV-challenged rainbow trout after 1 and 2 dpc by RT-qPCR. The expression of autophagy-related genes *gabarap* and *pik3c3* was significantly up-regulated at 1 dpc. However, only *pik3c3* gene expression was observed up-regulated at 2 dpc. On the other hand, *atg4b* and *becn1* genes were down-regulated at 1 and 2 dpc. *ulk1* gene expression was also down-regulated at 2 dpc (Figure 7d). For E3 ubiquitin ligase components, *cul3* and *keap1* expression was significantly increased at 1 and 2 dpc, respectively (Figure 7d). For antigen presentation-related genes, *mhcI* and *cd83* were highly up-regulated in RBCs from VHSV-challenged rainbow trout at 1 and 2 dpc, while *cd86* was significantly down-regulated at 1 dpc. *mhcII* gene expression showed a tendency to increase at 1 dpc, but not significantly (Figure 7d).

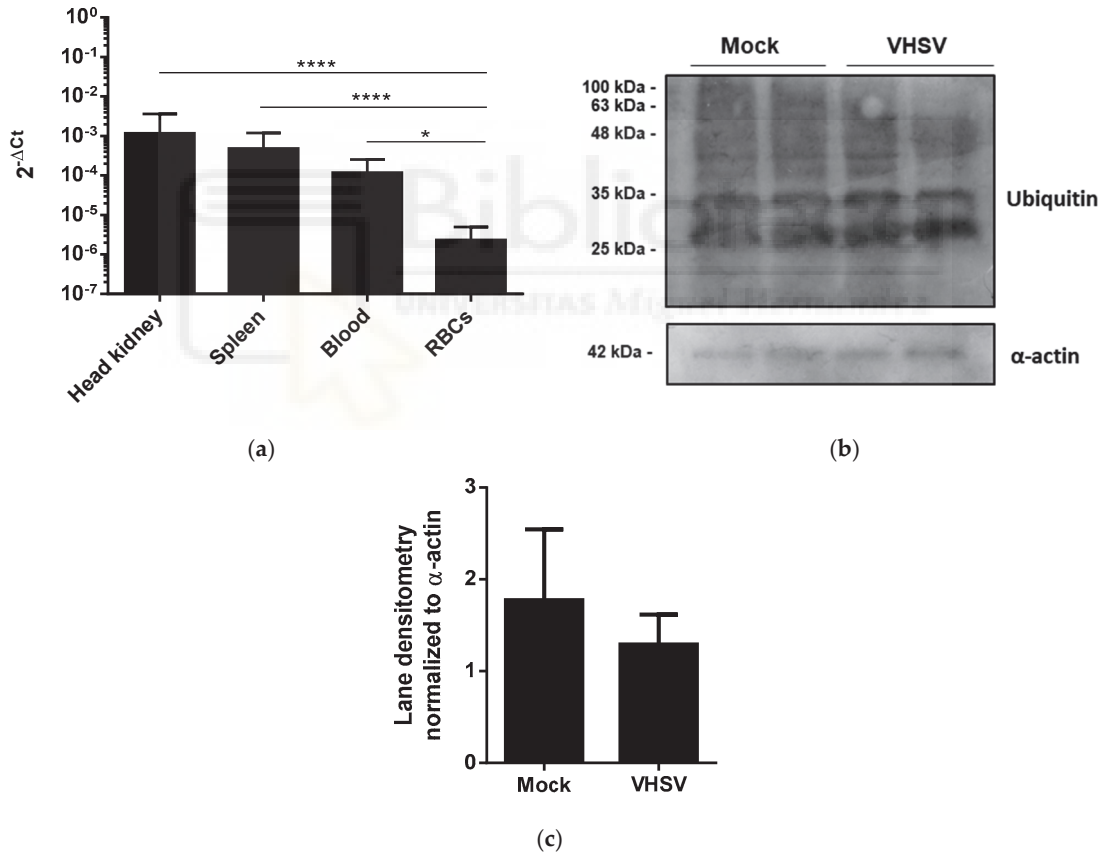
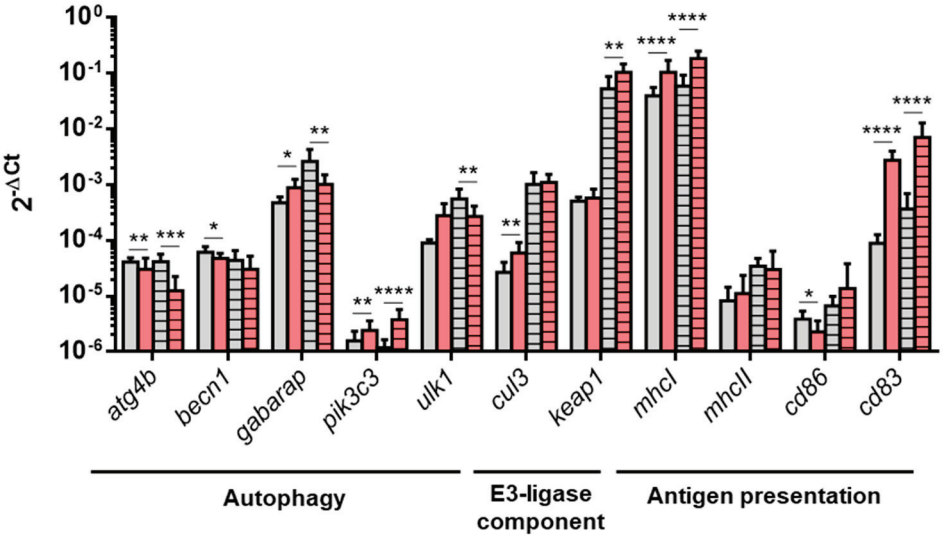


Figure 7. *Cont.*



(**d**)

Figure 7. VHSV induced autophagy, E3 ubiquitin ligase components, and antigen presentation genes expression in RBCs from VHSV-challenged rainbow trout. (**a**) Quantification of *NVHSV* in head kidney, spleen, blood, and purified RBC samples from challenged rainbow trout 2 dpc. Data represent mean ± SD (n = 7). A Kruskal-Wallis with Dunn´s multiple comparisons test was performed to test statistical significance. (**b**) Western blot of ubiquitin in RBCs from mock and VHSV-challenged rainbow trout at 2 dpc. α-actin was used as a loading control. Samples from 2 individuals were loaded for each condition. (**c**) Densitometry bar plot of ubiquitin lane protein content of RBCs from mock and VHSV-challenged rainbow trout after 2 dpc. Values were normalized to α-actin. Data represent mean ± SD (n = 2). Mann-Whitney test was used to test statistical differences. (**d**) Gene expression values of the autophagy-related genes *atg4b*, *ulk1*, *becn1*, *gabarap*, and *pik3c3*; E3 ligase component genes *cul3* and *keap1;* and antigen presentation genes *mhcI*, *mhcII*, *cd83*, and *cd86* measured by RT-qPCR in RBCs from mock (gray) and VHSV-challenged (red) rainbow trout at 1 dpc (no pattern) and 2 dpc (striped pattern). Data represent mean ± SD (n = 6). A Mann-Whitney test was performed to test statistical significances between RBCs from mock and VHSV-challenged rainbow trout. Asterisks denote statistical significance.

3.8. GVHSV Protein Peptides Colocalize with MHCI and MHCII in VHSV-Exposed Rainbow Trout RBCs

To establish a correlation between the presence of VHSV peptides from autophagy and MHCI and MHCII molecules (the expression of which was up-regulated after VHSV exposure), we performed a PLA between MHCI or MHCII and VHSV using the DuoLink kit. At 24 hpe, RBCs were stained using a rabbit polyclonal antibody against GVHSV and a mouse monoclonal antibody against MHCI or MHCII. We observed an increase in the percentage of positive cells in VHSV-exposed RBCs in contrast to unexposed RBCs (Figure 8a). A representative positive colocalization is shown in Figure 8b.

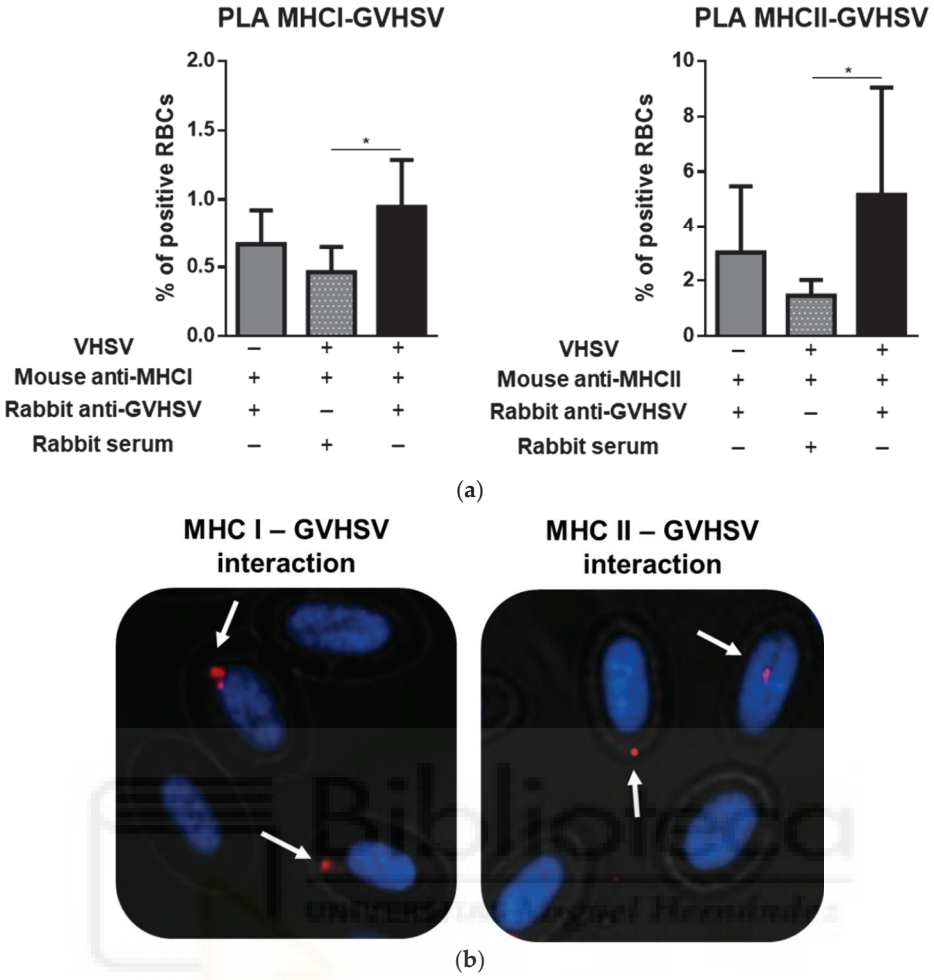


Figure 8. GVHSV protein peptides colocalize with MHCI and MHCII in VHSV-exposed rainbow trout RBCs. (**a**) Percentages of positive RBCs in MHCI – GVHSV and MHCII – GVHSV interaction under unexposed and VHSV-exposed conditions. Rabbit serum was used to test unspecific interaction with mouse anti-MHCI and anti-MHCII antibodies in VHSV-exposed RBCs. Data represent percentage of positives RBCs counted by IN Cell Developer software using an algorithm to detect fluorescent events in RBCs (n = 2 individuals, 8 fields were analyzed in each slide). A Kruskal-Wallis with Dunn´s multiple comparisons test was performed to test statistical significances between all the conditions. (**b**) Representative microscopy images of Duolink PLA for MHCI or MHCII and GVHSV in VHSV-exposed RBCs. Positive RBCs for the PLA are indicated with white arrows. Asterisks denote statistical significance.

4. Discussion

In this study, we have demonstrated that autophagy is implicated in the clearance of VHSV virions in nucleated rainbow trout RBCs, a cell whose main known function has been oxygen transportation. While previous studies have identified virus-related autophagy in teleost RBCs [16] and have localized the expression of MHCI molecules to the surface of nucleated RBCs [30], even in different vertebrate species [56], our results provide the first evidence of nucleated RBCs up-regulating APC markers in the context of a viral infection. Our findings suggest that RBCs could potentially play a new role in which autophagy is involved in viral protein degradation and the generated peptides are coupled to MHC molecules. A graphical summary of this process is shown in Figure 9.

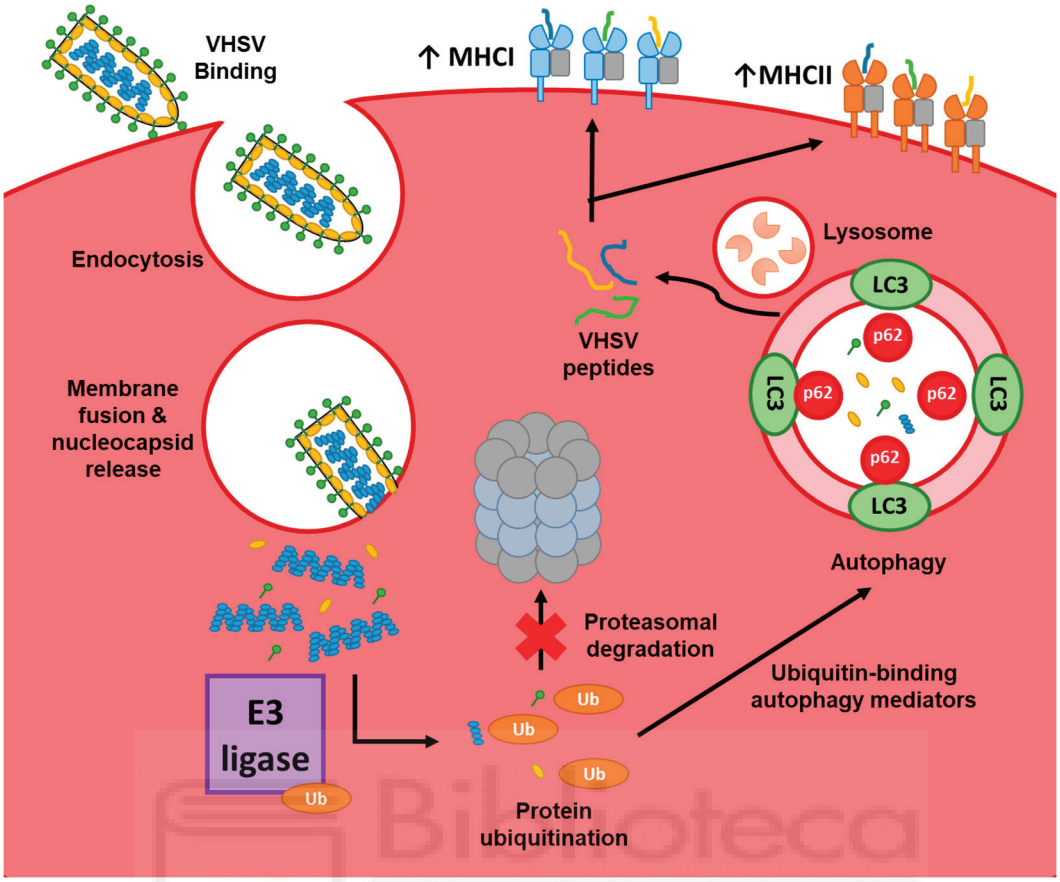


Figure 9. Proposed schematic representation of processes involved in VHSV degradation and antigen processing in rainbow trout nucleated RBCs. VHSV cell entry is mediated by endosome acidification, which leads to membrane fusion and thus release of the capsid. RBC transcription of autophagy genes and components of the E3 ubiquitin ligase then starts and intracellular proteins are ubiquitinated to be marked for degradation. The low proteasome activity induced as a consequence of the VHSV proteins presence leads to the accumulation of ubiquitinated proteins that are suggested to be degraded in the autophagosome. Finally, peptides from this process can be coupled into MHC molecules that are later transported to the membrane to potentially participate in the antigen presentation process.

Transcriptomic analysis of RBCs at four hours after VHSV exposure showed up-regulation of *cul3*, *keap1*, *psma6*, and *psmb5* genes from the antigen-processing category. *cul3* and *keap1* are components of the E3 ubiquitin ligase complex involved in the ubiquitination of proteins targeted for proteasome degradation [57], and *psma6* and *psmb5* are part of proteasome complexes. In the MHCI presentation pathway, our analysis identified *canx*, which is involved in the assembly of MHCI [58]; *sec13*, whose expression correlates with the expression of MHCI [58]; and *ikbkb*. These results correlated with the increase in ubiquitinated proteins induced by VHSV as detected by western blot. Different viruses have been reported to induce ubiquitination. This effect was observed with West Nile Virus; its capsid protein was ubiquitinated by Makorin ring finger 1 protein and later sent for proteasome degradation [59]. Ubiquitination was also reported for the core protein of human hepatitis C virus [60] and turnip yellow mosaic virus [61]. However, our results also showed lower proteasome activity, which could be due in part to the accumulation of ubiquitinated proteins in VHSV-exposed RBCs. Proteasome activity has been reported to favor the replication of different viruses [62,63], and it has been found to prevent viral replication [64]. In contrast, proteasome activity did not seem to play a role in VHSV degradation in our study.

Increased autophagy activity was demonstrated both at the transcriptional and translational levels in VHSV-exposed RBCs. Several studies have shown a protective role of autophagy against

different viruses, including dengue [65], sindbis [66], vesicular stomatitis virus (VSV) [67], and VHSV [14]. Our results demonstrated that VHSV exposure induced autophagy in rainbow trout RBCs; this prevented VHSV infection, as shown by p62 degradation and the results observed when VHSV-exposed RBCs were treated with niclosamide, which led to the accumulation of both p62 and VHSV. p62 accumulation suggested that autophagosome degradation was blocked in RBCs. We also observed an increase in intracellular VHSV. Therefore, autophagy may be a mechanism involved in VHSV degradation. We previously reported that the N:G gene expression ratio in RBCs exposed to VHSV was lower than commonly reported ratio levels [7], indicating that VHSV replication in RBCs was inhibited early after VHSV exposure. VHSV starts replication of the N gene within the first 6 hpe in RBCs in the permissive cell line RTG2 [7], so all processes aiming to inhibit VHSV infection in RBCs should occur during this time. Our results correlated with this report, since there was an early transcriptional response of autophagy-related genes together with p62 degradation at 6 hpe. p62 has been described to be the link between autophagy and the UPS [68,69], since autophagosome degradation of ubiquitinated proteins has been already reported [70], and p62 itself undergoes degradation upon autophagy activation. Moreover, some studies have reported a direct interaction between p62 and different viruses [66] or bacteria [71]. Our results showed a decrease in p62 levels that later recovered [72], suggesting that p62 may act as an adaptor protein that targeted VHSV for autophagic degradation, although this cannot be confirmed because we have not observed interaction between p62 and VHSV proteins. In this sense, other ubiquitin-binding autophagy mediators such as NRB1, NDP52 and optineurin [73] could be evaluated in the future.

RBCs exhibited an APC-like profile with MHCII, CD86, and CD83 endogenous expression. CD86 and CD83 are known costimulatory cell surface markers of APC maturation [74] and are involved in the regulation of different immune processes, such as lymphocytes proliferation and activation [75]. The presence of MHCII with the costimulatory molecules CD83 and CD86 suggests a more professionalized APC profile for RBCs, since MHCI has been reported to be expressed in almost all nucleated cells [76]. Our results showed modulation in the expression of MHCI, MHCII, CD83, and CD86 proteins when RBCs were exposed to VHSV. Antigen presentation via MHCI is normally associated with peptides derived from UPS, but recent reports have shown a contribution of autophagy to antigen presentation via MHCI molecules [77]. On the other hand, autophagy is the main source of peptides for MHCII molecules [78]. Moreover, we showed that antigen presentation via MHCI and MHCII potentially could be functional, because peptides from GVHSV colocalized with MHC molecules. Recently, it has been reported that different cell types called atypical APCs, such as neutrophils [79] or lymph node stromal cells [80] could be involved in antigen presentation, supporting the hypothesis that these atypical APCs could play an important role in various immune processes apart from antigen presentation [34]. However, to properly classify teleost RBCs as a typical APC, studies are needed to test their ability to activate naïve T cells, as this is main difference between atypical and typical APCs [34].

The results obtained from ex vivo RBCs culture experiments were partially corroborated under an in vivo scenario. RBCs from VHSV-challenged rainbow trout showed lower *NVHSV* transcript load compared to other tissues, similar to VHSV halted infection in ex vivo RBCs cultures [7]. We observed the expression of autophagy genes early after VHSV challenge, similar to the kinetics observed in ex vivo RBCs exposed to VHSV. In addition, we observed up-regulation of *cul3* at 1 dpc followed by *keap1* up-regulation at 2 dpc, just as we observed in ex vivo time-course experiments. In vivo results showed up-regulation of *mhcI*, *mhcII*, *cd83*, and *cd86* in RBCs from challenged rainbow trout, which correlated with their increased expression observed in ex vivo RBCs exposed to VHSV. In contrast, lack of ubiquitination was observed in RBCs from VHSV-challenged rainbow trout.

In summary, after VHSV cell entry into RBCs, the transcription of autophagy genes and components of the E3 ubiquitin ligase started. The low proteasome activity that was induced as a consequence of the presence of VHSV led to the accumulation of ubiquitinated proteins. Finally, peptides from this process could be coupled into intracellular MHC molecules that would be later transported to the

membrane to potentially participate in the antigen presentation process. Further studies are being performed to fully describe the potential functional APC role in nucleated teleost RBCs to ascertain how MHC molecules participate or are implicated in the presentation of degraded viral antigens in nucleated RBCs. Given that RBCs are the most abundant cell type in the blood, this new knowledge will shed light on the design of novel vaccine targets. Potential applications of these results could imply that RBCs, which can be transfected and induce immune gene expression [32], are target of new strategies for vaccination.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4409/8/5/386/s1. Figure S1: Anti-MHCI zebrafish antibody labeling in rainbow trout. (a) Western blot of anti-MHCI in rainbow trout RTS11 monocyte/macrophage-like cell line and Ficoll-purified red blood cells (RBCs). Black arrow indicates a protein band around 45 kDa marker. (b) Immunofluorescence of anti-MHCI in rainbow trout RBCs. Figure S2: Anti-p62/SQSTM1 zebrafish antibody labeling in rainbow trout. (a) Western blot of anti-p62/SQSTM1 in rainbow trout RBCs. Black arrow indicates a protein band under 63 kDa marker. (b) Immunofluorescence of anti-p62 in VHSV-exposed rainbow trout RBCs. White arrow indicates vesicle-like dots. Figure S3: Protein expression of MHCII, CD83, and CD86 in rainbow trout head kidney (HK) and RBCs, by means of western blot. HK samples were used as positive control of expression. Figure S4. Validation of RNA-Seq study by RT-qPCR representing fold change of *cul3*, *psmb5*, *keap1*, *sec13*, *ikbkb*, *rab7*, and *traf6* genes in the transcriptomic analysis of VHSV-exposed RBCs at 4 and 72 hpe. Gene expression values were calculated with normalization to unexposed RBCs. Data represent the mean ± SD (n = 4). Mann-Whitney test was used to test statistical significances between VHSV-exposed and unexposed RBCs. Figure S5: Venn diagram of the common omics products identified by transcriptomics at 4 and 72 h post-exposure and proteomics at 72 h post-exposure. Figure S6: Gated population of RBCs used for flow cytometry analysis. Figure S7. Time-course expression of the autophagy-related genes *beclin1*, *ulk1*, and *gabarap* at 0, 3, 6, 24, 48, and 72 hpe from unexposed and VHSV-exposed (MOI 1) RBCs. Data represent the mean ± SD (n = 5) relative to control cells (black dotted line). A Two-way ANOVA with Sidak´s multiple comparisons test was performed to test statistical significance between unexposed and VHSV-exposed RBCs at each time point. Table S1: GO Terms identified in VHSV-exposed RBCs after 4 and 72 h post-exposure by RNA-Seq. Table S2: List of significantly regulated genes after 4 and 72 h post-exposure in VHSV-exposed RBCs. All the listed genes have an FDR lower than 0.05. Table S3: GO Terms identified in VHSV-exposed RBCs after 72 h post-exposure by proteomic sequencing. Table S4: List of significantly regulated proteins after 72 h post-exposure in VHSV-exposed RBCs. All the listed proteins have an FDR lower than 0.001.

Author Contributions: Conceptualization, I.N. and M.d.M.O.-V.; Methodology, I.N., S.P.-M., V.C., M.C.M., S.C., and M.d.M.O.-V.; Validation, I.N., S.P.-M., V.C., and R.R.-P.; Formal analysis, I.N., R.R.-P., M.C.M., S.C., and M.d.M.O.-V.; Resources, B.M.-L. and L.M.; Data curation, I.N.; Writing—original draft preparation, I.N. and M.d.M.O.-V.; Writing—review and editing, M.d.M.O.-V., J.C. and L.P.; Supervision, M.d.M.O.-V.; Project administration, M.d.M.O.-V.; Funding acquisition, M.d.M.O.-V.

Funding: This work was supported by the European Research Council (ERC Starting Grant GA639249).

Acknowledgments: The authors are thankful to Remedios Torres and Efren Lucas for their technical assistance. The proteomic analysis was performed in the Proteomics Facility of The Spanish National Center for Biotechnology (CNB-CSIC) belonging to ProteoRed, PRB3-ISCIII, supported by grant PT17/0019. We would like to thank Paulo Almeida (STABVida) for technical support. The authors are also thankful to the constructive comments and corrections from the reviewers.

Conflicts of Interest: The authors declare that the research was carried out in the absence of any personal, professional, or financial relationships that could potentially be construed as a conflict of interest.

References

1. Workenhe, S.T.; Kibenge, M.J.; Wright, G.M.; Wadowska, D.W.; Groman, D.B.; Kibenge, F.S. Infectious salmon anaemia virus replication and induction of alpha interferon in atlantic salmon erythrocytes. *Virol. J.* **2008**, *5*, 36. [CrossRef]
2. Finstad, O.W.; Dahle, M.K.; Lindholm, T.H.; Nyman, I.B.; Lovoll, M.; Wallace, C.; Olsen, C.M.; Storset, A.K.; Rimstad, E. Piscine orthoreovirus (prv) infects atlantic salmon erythrocytes. *Vet. Res.* **2014**, *45*, 35. [CrossRef] [PubMed]
3. Wessel, O.; Olsen, C.M.; Rimstad, E.; Dahle, M.K. Piscine orthoreovirus (prv) replicates in atlantic salmon (salmo salar l.) erythrocytes ex vivo. *Vet. Res.* **2015**, *46*, 26. [CrossRef]
4. Wessel, O.; Krasnov, A.; Timmerhaus, G.; Rimstad, E.; Dahle, M.K. Antiviral responses and biological concequences of piscine orthoreovirus infection in salmonid erythrocytes. *Front. Immunol.* **2018**, *9*, 3182. [CrossRef] [PubMed]

5. Dahle, M.K.; Jorgensen, J.B. Antiviral defense in salmonids - mission made possible? *Fish. Shellfish Immunol.* **2019**, *87*, 421–437. [CrossRef] [PubMed]
6. Dahle, M.K.; Wessel, O.; Timmerhaus, G.; Nyman, I.B.; Jorgensen, S.M.; Rimstad, E.; Krasnov, A. Transcriptome analyses of atlantic salmon (salmo salar l.) erythrocytes infected with piscine orthoreovirus (prv). *Fish. Shellfish Immunol.* **2015**, *45*, 780–790. [CrossRef] [PubMed]
7. Nombela, I.; Puente-Marin, S.; Chico, V.; Villena, A.J.; Carracedo, B.; Ciordia, S.; Mena, M.C.; Mercado, L.; Perez, L.; Coll, J.; et al. Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of vhs virus. *F1000Research* **2017**, *6*, 1958. [CrossRef] [PubMed]
8. Nombela, I.; Carrion, A.; Puente-Marin, S.; Chico, V.; Mercado, L.; Perez, L.; Coll, J.; Ortega-Villaizan, M.d.M. Infectious pancreatic necrosis virus triggers antiviral immune response in rainbow trout red blood cells, despite not being infective. *F1000Research* **2017**, *6*, 1968. [CrossRef] [PubMed]
9. Chico, V.; Salvador-Mira, M.; Nombela, I.; Puente-Marin, S.; Ciordia, S.; Mena, M.C.; Perez, L.; Coll, J.; Guzman, F.; Encinar, J.A.; et al. Ifit5 participates in the antiviral mechanisms of rainbow trout red blood cells. *Front. Immunol.* **2019**, *10*, 613. [CrossRef]
10. Jackson, W.T. Viruses and the autophagy pathway. *Virology* **2015**, *479–480*, 450–456. [CrossRef]
11. Chiramel, A.I.; Brady, N.R.; Bartenschlager, R. Divergent roles of autophagy in virus infection. *Cells* **2013**, *2*, 83–104. [CrossRef]
12. Lee, H.K.; Lund, J.M.; Ramanathan, B.; Mizushima, N.; Iwasaki, A. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. *Science* **2007**, *315*, 1398–1401. [CrossRef]
13. Gorbea, C.; Makar, K.A.; Pauschinger, M.; Pratt, G.; Bersola, J.L.; Varela, J.; David, R.M.; Banks, L.; Huang, C.H.; Li, H.; et al. A role for toll-like receptor 3 variants in host susceptibility to enteroviral myocarditis and dilated cardiomyopathy. *J. Biol. Chem.* **2010**, *285*, 23208–23223. [CrossRef]
14. Garcia-Valtanen, P.; Ortega-Villaizan Mdel, M.; Martinez-Lopez, A.; Medina-Gali, R.; Perez, L.; Mackenzie, S.; Figueras, A.; Coll, J.M.; Estepa, A. Autophagy-inducing peptides from mammalian vsv and fish vhsv rhabdoviral g glycoproteins (g) as models for the development of new therapeutic molecules. *Autophagy* **2014**, *10*, 1666–1680. [CrossRef]
15. Ortega-Villaizan, M.; Chico, V.; Martinez-Lopez, A.; Garcia-Valtanen, P.; Coll, J.M.; Estepa, A. Development of new therapeutical/adjuvant molecules by pepscan mapping of autophagy and ifn inducing determinants of rhabdoviral g proteins. *Mol. Immunol.* **2016**, *70*, 118–124. [CrossRef] [PubMed]
16. Pereiro, P.; Romero, A.; Diaz-Rosales, P.; Estepa, A.; Figueras, A.; Novoa, B. Nucleated teleost erythrocytes play an nk-lysin- and autophagy-dependent role in antiviral immunity. *Front. Immunol.* **2017**, *8*, 1458. [CrossRef] [PubMed]
17. Shaid, S.; Brandts, C.H.; Serve, H.; Dikic, I. Ubiquitination and selective autophagy. *Cell Death Differ.* **2013**, *20*, 21–30. [CrossRef]
18. Deretic, V. Autophagy: An emerging immunological paradigm. *J. Immunol.* **2012**, *189*, 15–20. [CrossRef] [PubMed]
19. Lin, X.; Li, S.; Zhao, Y.; Ma, X.; Zhang, K.; He, X.; Wang, Z. Interaction domains of p62: A bridge between p62 and selective autophagy. *DNA Cell Biol.* **2013**, *32*, 220–227. [CrossRef] [PubMed]
20. Weissman, A.M. Themes and variations on ubiquitylation. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 169–178. [CrossRef] [PubMed]
21. Neelam, S.; Kakhniashvili, D.G.; Wilkens, S.; Levene, S.D.; Goodman, S.R. Functional 20s proteasomes in mature human red blood cells. *Exp. Biol. Med. (Maywood)* **2011**, *236*, 580–591. [CrossRef]
22. Luo, H. Interplay between the virus and the ubiquitin-proteasome system: Molecular mechanism of viral pathogenesis. *Curr. Opin. Virol.* **2016**, *17*, 1–10. [CrossRef] [PubMed]
23. Gaczynska, M.; Rock, K.L.; Goldberg, A.L. Role of proteasomes in antigen presentation. *Enzyme Protein* **1993**, *47*, 354–369. [CrossRef] [PubMed]
24. Gannage, M.; Munz, C. MHC presentation via autophagy and how viruses escape from it. *Semin. Immunopathol.* **2010**, *32*, 373–381. [CrossRef] [PubMed]
25. English, L.; Chemali, M.; Duron, J.; Rondeau, C.; Laplante, A.; Gingras, D.; Alexander, D.; Leib, D.; Norbury, C.; Lippe, R.; et al. Autophagy enhances the presentation of endogenous viral antigens on mhc class i molecules during hsv-1 infection. *Nat. Immunol.* **2009**, *10*, 480–487. [CrossRef]
26. Paludan, C.; Schmid, D.; Landthaler, M.; Vockerodt, M.; Kube, D.; Tuschl, T.; Munz, C. Endogenous mhc class ii processing of a viral nuclear antigen after autophagy. *Science* **2005**, *307*, 593–596. [CrossRef] [PubMed]

27. Lugo-Villarino, G.; Balla, K.M.; Stachura, D.L.; Banuelos, K.; Werneck, M.B.; Traver, D. Identification of dendritic antigen-presenting cells in the zebrafish. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 15850–15855. [CrossRef] [PubMed]
28. Bassity, E.; Clark, T.G. Functional identification of dendritic cells in the teleost model, rainbow trout (oncorhynchus mykiss). *PLoS ONE* **2012**, *7*, e33196. [CrossRef] [PubMed]
29. Sarder, M.R.; Fischer, U.; Dijkstra, J.M.; Kiryu, I.; Yoshiura, Y.; Azuma, T.; Kollner, B.; Ototake, M. The mhc class i linkage group is a major determinant in the in vivo rejection of allogeneic erythrocytes in rainbow trout (oncorhynchus mykiss). *Immunogenetics* **2003**, *55*, 315–324. [CrossRef]
30. Dijkstra, J.M.; Kollner, B.; Aoyagi, K.; Sawamoto, Y.; Kuroda, A.; Ototake, M.; Nakanishi, T.; Fischer, U. The rainbow trout classical mhc class i molecule onmy-uba*501 is expressed in similar cell types as mammalian classical mhc class i molecules. *Fish. Shellfish Immunol.* **2003**, *14*, 1–23. [CrossRef] [PubMed]
31. Puente-Marin, S.; Nombela, I.; Ciordia, S.; Mena, M.C.; Chico, V.; Coll, J.; Ortega-Villaizan, M.D.M. In silico functional networks identified in fish nucleated red blood cells by means of transcriptomic and proteomic profiling. *Genes (Basel)* **2018**, *9*, 202. [CrossRef]
32. Puente-Marin, S.; Nombela, I.; Chico, V.; Ciordia, S.; Mena, M.C.; Coll, J.; Mercado, L.; Ortega-Villaizan, M.D.M. Rainbow trout erythrocytes ex vivo transfection with a DNA vaccine encoding vhsv glycoprotein g induces an antiviral immune response. *Front. Immunol.* **2018**, *9*, 2477. [CrossRef] [PubMed]
33. St Paul, M.; Paolucci, S.; Barjesteh, N.; Wood, R.D.; Sharif, S. Chicken erythrocytes respond to toll-like receptor ligands by up-regulating cytokine transcripts. *Res. Vet. Sci.* **2013**, *95*, 87–91. [CrossRef]
34. Kambayashi, T.; Laufer, T.M. Atypical mhc class ii-expressing antigen-presenting cells: Can anything replace a dendritic cell? *Nat. Rev. Immunol.* **2014**, *14*, 719–730. [CrossRef] [PubMed]
35. Dasari, V.; Rehan, S.; Tey, S.K.; Smyth, M.J.; Smith, C.; Khanna, R. Autophagy and proteasome interconnect to coordinate cross-presentation through mhc class i pathway in b cells. *Immunol. Cell Biol.* **2016**, *94*, 964–974. [CrossRef] [PubMed]
36. LeBerre, M.; De Kinkelin, P.; Metzger, A. Identification sérologique des rhabdovirus des salmonidés. *Bull. Off. Int Epizoot* **1977**, *87*, 391–393.
37. Basurco, B.; Coll, J. Spanish isolates and reference strains of viral haemorrhagic septicaemia virus shown similar protein size patterns. *Bull. Eur. Ass. Fish. Pathol.* **1989**, *9*, 92–95.
38. Sanz, F.; Basurco, B.; Babin, M.; Dominguez, J.; Coll, J. Monoclonal antibodies against the structural proteins of viral haemorrhagic septicaemia virus isolates. *J. Fish. Dis.* **1993**, *16*, 53–63. [CrossRef]
39. Morales-Lange, B.; Ramirez, F.; Guzman, F.; Wong, V.; Imarai, M.; Lagos, L.; Øverland, M.; Boltana, S.; Mercado, L. Interferon gamma induces the overexpression of antigen presenting cell surface markers in spleen leukocytes from salmo salar. Unpublished manuscript.
40. Lorenzen, N.; Lapatra, S.E. Immunity to rhabdoviruses in rainbow trout: The antibody response. *Fish. Shellfish Immunol.* **1999**, *9*, 345–360. [CrossRef]
41. Chico, V.; Gomez, N.; Estepa, A.; Perez, L. Rapid detection and quantitation of viral hemorrhagic septicemia virus in experimentally challenged rainbow trout by real-time rt-pcr. *J. Virol. Methods* **2006**, *132*, 154–159. [CrossRef] [PubMed]
42. Bindea, G.; Mlecnik, B.; Hackl, H.; Charoentong, P.; Tosolini, M.; Kirilovsky, A.; Fridman, W.H.; Pages, F.; Trajanoski, Z.; Galon, J. Cluego: A cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* **2009**, *25*, 1091–1093. [CrossRef] [PubMed]
43. Bindea, G.; Galon, J.; Mlecnik, B. Cluepedia cytoscape plugin: Pathway insights using integrated experimental and in silico data. *Bioinformatics* **2013**, *29*, 661–663. [CrossRef]
44. Shannon, P.; Markiel, A.; Ozier, O.; Baliga, N.S.; Wang, J.T.; Ramage, D.; Amin, N.; Schwikowski, B.; Ideker, T. Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res.* **2003**, *13*, 2498–2504. [CrossRef] [PubMed]
45. Gotz, S.; Garcia-Gomez, J.M.; Terol, J.; Williams, T.D.; Nagaraj, S.H.; Nueda, M.J.; Robles, M.; Talon, M.; Dopazo, J.; Conesa, A. High-throughput functional annotation and data mining with the blast2go suite. *Nucleic Acids Res.* **2008**, *36*, 3420–3435. [CrossRef] [PubMed]
46. Alvarez, C.A.; Ramirez-Cepeda, F.; Santana, P.; Torres, E.; Cortes, J.; Guzman, F.; Schmitt, P.; Mercado, L. Insights into the diversity of nod-like receptors: Identification and expression analysis of nlrc3, nlrc5 and nlrx1 in rainbow trout. *Mol. Immunol.* **2017**, *87*, 102–113. [CrossRef] [PubMed]

47. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. *Methods* **2001**, *25*, 402–408. [CrossRef]
48. Chaves-Pozo, E.; Montero, J.; Cuesta, A.; Tafalla, C. Viral hemorrhagic septicemia and infectious pancreatic necrosis viruses replicate differently in rainbow trout gonad and induce different chemokine transcription profiles. *Dev. Comp. Immunol.* **2010**, *34*, 648–658. [CrossRef]
49. Jorgensen, T.R.; Raida, M.K.; Kania, P.W.; Buchmann, K. Response of rainbow trout (oncorhynchus mykiss) in skin and fin tissue during infection with a variant of gyrodactylus salaris (monogenea: Gyrodactylidae). *Folia Parasitol. (Praha)* **2009**, *56*, 251–258. [CrossRef] [PubMed]
50. Ortega-Villaizan, M.; Martinez-Lopez, A.; Garcia-Valtanen, P.; Chico, V.; Perez, L.; Coll, J.M.; Estepa, A. Ex vivo transfection of trout pronephros leukocytes, a model for cell culture screening of fish DNA vaccine candidates. *Vaccine* **2012**, *30*, 5983–5990. [CrossRef]
51. Klionsky, D.J.; Abdelmohsen, K.; Abe, A.; Abedin, M.J.; Abeliovich, H.; Acevedo Arozena, A.; Adachi, H.; Adams, C.M.; Adams, P.D.; Adeli, K.; et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* **2016**, *12*, 1–222. [CrossRef]
52. Bjorkoy, G.; Lamark, T.; Brech, A.; Outzen, H.; Perander, M.; Overvatn, A.; Stenmark, H.; Johansen, T. P62/sqstm1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J. Cell Biol.* **2005**, *171*, 603–614. [CrossRef] [PubMed]
53. Li, M.; Khambu, B.; Zhang, H.; Kang, J.H.; Chen, X.; Chen, D.; Vollmer, L.; Liu, P.Q.; Vogt, A.; Yin, X.M. Suppression of lysosome function induces autophagy via a feedback down-regulation of mtor complex 1 (mtorc1) activity. *J. Biol. Chem.* **2013**, *288*, 35769–35780. [CrossRef] [PubMed]
54. Gao, Y.; Liang, A.; Kang, J.-H.; Liu, P.; Yin, X.-m.; Li, M. Niclosamide blocks autophagy via lysosomal dysfunction (663.18). *FASEB J.* **2014**, *28*, 663.18.
55. Kao, J.C.; HuangFu, W.C.; Tsai, T.T.; Ho, M.R.; Jhan, M.K.; Shen, T.J.; Tseng, P.C.; Wang, Y.T.; Lin, C.F. The antiparasitic drug niclosamide inhibits dengue virus infection by interfering with endosomal acidification independent of mtor. *PLoS Negl. Trop. Dis.* **2018**, *12*, e0006715. [CrossRef] [PubMed]
56. Nombela, I.; Ortega-Villaizan, M.D.M. Nucleated red blood cells: Immune cell mediators of the antiviral response. *PLoS Pathog.* **2018**, *14*, e1006910. [CrossRef]
57. Cullinan, S.B.; Gordan, J.D.; Jin, J.; Harper, J.W.; Diehl, J.A. The keap1-btb protein is an adaptor that bridges nrf2 to a cul3-based e3 ligase: Oxidative stress sensing by a cul3-keap1 ligase. *Mol. Cell. Biol.* **2004**, *24*, 8477–8486. [CrossRef] [PubMed]
58. Diedrich, G.; Bangia, N.; Pan, M.; Cresswell, P. A role for calnexin in the assembly of the mhc class i loading complex in the endoplasmic reticulum. *J. Immunol.* **2001**, *166*, 1703–1709. [CrossRef]
59. Ko, A.; Lee, E.W.; Yeh, J.Y.; Yang, M.R.; Oh, W.; Moon, J.S.; Song, J. Mkrn1 induces degradation of west nile virus capsid protein by functioning as an e3 ligase. *J. Virol.* **2010**, *84*, 426–436. [CrossRef] [PubMed]
60. Suzuki, R.; Moriishi, K.; Fukuda, K.; Shirakura, M.; Ishii, K.; Shoji, I.; Wakita, T.; Miyamura, T.; Matsuura, Y.; Suzuki, T. Proteasomal turnover of hepatitis c virus core protein is regulated by two distinct mechanisms: A ubiquitin-dependent mechanism and a ubiquitin-independent but pa28gamma-dependent mechanism. *J. Virol.* **2009**, *83*, 2389–2392. [CrossRef]
61. Drugeon, G.; Jupin, I. Stability in vitro of the 69k movement protein of turnip yellow mosaic virus is regulated by the ubiquitin-mediated proteasome pathway. *J. Gen. Virol.* **2002**, *83*, 3187–3197. [CrossRef] [PubMed]
62. Gilfoy, F.; Fayzulin, R.; Mason, P.W. West nile virus genome amplification requires the functional activities of the proteasome. *Virology* **2009**, *385*, 74–84. [CrossRef] [PubMed]
63. Kaspari, M.; Tavalai, N.; Stamminger, T.; Zimmermann, A.; Schilf, R.; Bogner, E. Proteasome inhibitor mg132 blocks viral DNA replication and assembly of human cytomegalovirus. *FEBS Lett.* **2008**, *582*, 666–672. [CrossRef]
64. Reichel, C.; Beachy, R.N. Degradation of tobacco mosaic virus movement protein by the 26s proteasome. *J. Virol.* **2000**, *74*, 3330–3337. [CrossRef] [PubMed]
65. Datan, E.; Roy, S.G.; Germain, G.; Zali, N.; McLean, J.E.; Golshan, G.; Harbajan, S.; Lockshin, R.A.; Zakeri, Z. Dengue-induced autophagy, virus replication and protection from cell death require er stress (perk) pathway activation. *Cell Death Dis.* **2016**, *7*, e2127. [CrossRef]
66. Orvedahl, A.; MacPherson, S.; Sumpter, R., Jr.; Talloczy, Z.; Zou, Z.; Levine, B. Autophagy protects against sindbis virus infection of the central nervous system. *Cell Host Microbe* **2010**, *7*, 115–127. [CrossRef]

67. Shelly, S.; Lukinova, N.; Bambina, S.; Berman, A.; Cherry, S. Autophagy is an essential component of drosophila immunity against vesicular stomatitis virus. *Immunity* **2009**, *30*, 588–598. [CrossRef] [PubMed]
68. Cohen-Kaplan, V.; Ciechanover, A.; Livneh, I. P62 at the crossroad of the ubiquitin-proteasome system and autophagy. *Oncotarget* **2016**, *7*, 83833–83834. [CrossRef] [PubMed]
69. Liu, W.J.; Ye, L.; Huang, W.F.; Guo, L.J.; Xu, Z.G.; Wu, H.L.; Yang, C.; Liu, H.F. P62 links the autophagy pathway and the ubiqutin-proteasome system upon ubiquitinated protein degradation. *Cell Mol. Biol. Lett* **2016**, *21*, 29. [CrossRef] [PubMed]
70. Zaffagnini, G.; Savova, A.; Danieli, A.; Romanov, J.; Tremel, S.; Ebner, M.; Peterbauer, T.; Sztacho, M.; Trapannone, R.; Tarafder, A.K.; et al. P62 filaments capture and present ubiquitinated cargos for autophagy. *EMBO J.* **2018**, *37*. [CrossRef] [PubMed]
71. Zheng, Y.T.; Shahnazari, S.; Brech, A.; Lamark, T.; Johansen, T.; Brumell, J.H. The adaptor protein p62/sqstm1 targets invading bacteria to the autophagy pathway. *J. Immunol.* **2009**, *183*, 5909–5916. [CrossRef] [PubMed]
72. Sahani, M.H.; Itakura, E.; Mizushima, N. Expression of the autophagy substrate sqstm1/p62 is restored during prolonged starvation depending on transcriptional upregulation and autophagy-derived amino acids. *Autophagy* **2014**, *10*, 431–441. [CrossRef] [PubMed]
73. Lippai, M.; Low, P. The role of the selective adaptor p62 and ubiquitin-like proteins in autophagy. *Biomed. Res. Int.* **2014**, *2014*, 832704. [CrossRef] [PubMed]
74. Berchtold, S.; Muhl-Zurbes, P.; Heufler, C.; Winklehner, P.; Schuler, G.; Steinkasserer, A. Cloning, recombinant expression and biochemical characterization of the murine cd83 molecule which is specifically upregulated during dendritic cell maturation. *FEBS Lett.* **1999**, *461*, 211–216. [CrossRef]
75. Breloer, M.; Fleischer, B. Cd83 regulates lymphocyte maturation, activation and homeostasis. *Trends Immunol.* **2008**, *29*, 186–194. [CrossRef]
76. Ferm, M.T.; Gronberg, A. Human mhc class i antigens are associated with a 90-kda cell surface protein. *Scand J. Immunol.* **1991**, *34*, 221–227. [CrossRef] [PubMed]
77. Van Kaer, L.; Parekh, V.V.; Postoak, J.L.; Wu, L. Role of autophagy in mhc class i-restricted antigen presentation. *Mol. Immunol.* **2017**. [CrossRef] [PubMed]
78. Dengjel, J.; Schoor, O.; Fischer, R.; Reich, M.; Kraus, M.; Muller, M.; Kreymborg, K.; Altenberend, F.; Brandenburg, J.; Kalbacher, H.; et al. Autophagy promotes mhc class ii presentation of peptides from intracellular source proteins. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 7922–7927. [CrossRef] [PubMed]
79. Cassatella, M.A. Human mature neutrophils as atypical apc. *Blood* **2017**, *129*, 1895–1896. [CrossRef]
80. Hirosue, S.; Dubrot, J. Modes of antigen presentation by lymph node stromal cells and their immunological implications. *Front. Immunol.* **2015**, *6*, 446. [CrossRef] [PubMed]

PUBLICACIÓN 4

TÍTULO: Integrated transcriptomic and proteomic analysis of red blood cells from rainbow trout challenged with VHSV point towards novel immunomodulant targets

AUTORES: Ivan Nombela, Marina Lopez-Lorigados, Maria Elizabeth Salvador, Sara Puente-Marin, Verónica Chico, Sergio Ciordia, M. C. Mena, Luis Mercado, Julio Coll, Luis Perez y Maria del Mar Ortega-Villaizan

REVISTA: *Vaccines*

DOI: En revisión. Pendiente de publicación.

[Vaccines] Manuscript ID: vaccines-532136 - Submission Received

susy@mdpi.com en nombre de Editorial Office [vaccines@mdpi.com]

Enviado: miércoles, 05 de junio de 2019 11:32
Para: Ortega-Villaizan Romo, Maria Del Mar
CC: Nombela Diaz, Ivan; Marina Lopez-Lorigados [marina.lopez03@alu.umh.es]; Maria Elizabhet Salvador-Mira [maria.salvador04@goumh.umh.es]; Puente Marin, Sara; Chico Gras, Veronica; Sergio Ciordia [sciordia@cnb.csic.es]; Maria Carmen Mena [mcmena@cnb.csic.es]; Luis Mercado [luis.mercado@pucv.cl]; Julio Coll [juliocoll@inia.es]; Perez Garcia-Estañ, Luis; Ortega-Villaizan Romo, Maria Del Mar

Dear Dr. Ortega-Villaizan,

Thank you very much for uploading the following manuscript to the MDPI submission system. One of our editors will be in touch with you soon.

Journal name: Vaccines
Manuscript ID: vaccines-532136
Type of manuscript: Article
Title: Integrated transcriptomic and proteomic analysis of red blood cells from rainbow trout challenged with VHSV point towards novel immunomodulant targets
Authors: Ivan Nombela, Marina Lopez-Lorigados, Maria Elizabhet Salvador-Mira, Sara Puente-Marin, Veronica Chico, Sergio Ciordia, Maria Carmen Mena, Luis Mercado, Julio Coll, Luis Perez, Maria del Mar Ortega-Villaizan *
Received: 5 June 2019
E-mails: inombela@umh.es, marina.lopez03@alu.umh.es, maria.salvador04@goumh.umh.es, spuente@umh.es, vchico@umh.es, sciordia@cnb.csic.es, mcmena@cnb.csic.es, luis.mercado@pucv.cl, juliocoll@inia.es, luis.perez@umh.es, mortega-villaizan@umh.es

You can follow progress of your manuscript at the following link (login required):
https://susy.mdpi.com/user/manuscripts/review_info/89140e33f6e5a1f17308dd1c226342b8

The following points were confirmed during submission:

1. Vaccines is an open access journal with publishing fees of 650 CHF for an accepted paper (see https://www.mdpi.com/about/apc/ for details). This manuscript, if accepted, will be published under an open access Creative Commons CC BY license (https://creativecommons.org/licenses/by/4.0/), and I agree to pay the Article Processing Charges as described on the journal webpage (https://www.mdpi.com/journal/vaccines/apc). See https://www.mdpi.com/about/openaccess for more information about open access publishing.

Please note that you may be entitled to a discount if you have previously received a discount code or if your institute is participating in the MDPI Institutional Open Access Program (IOAP), for more information see https://www.mdpi.com/about/ioap. If you have been granted any other special discounts for your submission, please contact the Vaccines editorial office.

2. I understand that:

a. If previously published material is reproduced in my manuscript, I will provide proof that I have obtained the necessary copyright permission. (Please refer to the Rights & Permissions website: https://www.mdpi.com/authors/rights).

b. My manuscript is submitted on the understanding that it has not been published in or submitted to another peer-reviewed journal. Exceptions to this rule are papers containing material disclosed at conferences. I confirm that I will inform the journal editorial office if this is the case for my manuscript. I confirm that all authors are familiar with and agree with submission of the contents of the manuscript. The journal editorial office reserves the right to contact all authors to confirm this in case of doubt. I will provide email addresses for all authors and an institutional e-mail address for at least one of the co-authors, and specify the name, address and e-mail for invoicing purposes.

If you have any questions, please do not hesitate to contact the Vaccines editorial office at vaccines@mdpi.com

Kind regards,

Vaccines Editorial Office
St. Alban-Anlage 66, 4052 Basel, Switzerland
E-Mail: vaccines@mdpi.com
Tel. +41 61 683 77 34
Fax: +41 61 302 89 18

*** This is an automatically generated email ***

Integrated transcriptomic and proteomic analysis of red blood cells from rainbow trout challenged with VHSV point towards novel immunomodulant targets

Ivan Nombela[1], Marina Lopez-Lorigados[1], Maria Elizabeth Salvador-Mira[1], Sara Puente-Marin[1], Veronica Chico[1], Sergio Ciordia[2], Maria Carmen Mena[2], Luis Mercado[3], Julio Coll[4], Luis Perez[1], Maria del Mar Ortega-Villaizan[1,*]

[1] Instituto de Biologia Molecular y Celular, Universidad Miguel Hernández (IBMC-UMH), 03202 Elche (Spain)
[1] Instituto de Investigación, Desarrollo e Innovación en Biotecnología Sanitaria de Elche (IDiBE-UMH), 03202 Elche, Spain
[2] Unidad de Proteómica, Centro Nacional de Biotecnología (CNB- CSIC), 28049 Madrid, Spain
[3] Instituto de Biología, Pontificia Universidad Católica de Valparaiso, 2373223 Valparaiso, Chile
[4] Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), 28040 Madrid, Spain

***** Correspondence: mortega-villaizan@umh.es

Abstract: Teleost red blood cells (RBCs) are nucleated and therefore can propagate cellular responses to exogenous stimuli. RBCs can mount an immune response against a variety of fish viruses, including viral septicemia hemorrhagic virus (VHSV), which is one of the most prevalent fish viruses resulting in aquaculture losses. In this work, RBCs from blood and head kidney samples of rainbow trout challenged with VHSV were analyzed via transcriptomics and proteomics analyses. We detected overrepresentation of differentially expressed genes (DEGs) related to type I interferon response and signaling in RBCs from head kidney and of complement activation in RBCs from blood. Antigen processing and presentation of peptide antigen was overrepresented in RBCs from both organs. DEGs shared by both organs showed an opposite expression profile. In summary, this work demonstrated that teleost RBCs can modulate the immune response during an *in vivo* viral infection, which implicates RBCs as cell targets for the development of novel immunomodulants.

Keywords: erythrocytes, red blood cells, transcriptome, proteome, interferon, complement, VHSV, rhabdoviruses, Mx, IFIT5, β-defensin 1, antigen presentation

1. INTRODUCTION

Teleost red blood cells (RBCs) have garnered interest in recent years due to the fact that, in contrast to mammalian RBCs, they are nucleated and possess organelles within the cytoplasm [1] and contain the intracellular machinery necessary to develop a response to pathogens [2].

Teleost nucleated RBCs have been recently defined as immune cell mediators of the antiviral response [2, 3]. Several immune functions have been associated with RBCs, such as pattern-recognition receptor expression [2, 4-6], interferon signaling pathway responses [5-10], antigen presentation via major histocompatibility complex (MHC) class

I (MHCI) or class II (MHCII) [5, 7, 9, 11, 12], inflammatory cytokines and chemokines [5, 7, 9, 13], and even expression of immunoglobulin M in a differentiated state [14].

Different viral pathogens are known to infect teleost RBCs. Among them, infectious salmonid anemia virus (ISAV) is able to cause endocytosis and hemagglutination on RBCs [15] and can induce replication and production of type I interferon [10]. In an *in vivo* context, ISAV can adhere to the membrane of RBCs and eventually endothelial cells [16]. Piscine orthoreovirus (PRV) also infects

RBCs. Previous studies have shown infectivity in Atlantic salmon RBCs under both *ex vivo* and *in vivo* conditions [17, 18]. Similar to ISAV, PRV can increase the expression of type I interferon and its related genes in Atlantic salmon RBCs [9, 17]. Moreover, a transcriptomic analysis of RBCs from PRV-challenged Atlantic salmon showed upregulation of genes related to antigen presentation via MHCI and interferon-regulated genes with antiviral activity [9].

On the other hand, infectious pancreatic necrosis virus (IPNV), despite not being infective in rainbow trout RBCs, increased the *ex vivo* expression of type I interferon and interferon-stimulated genes (eg, Mx) [6]. Similarly, viral hemorrhagic septicemia virus (VHSV) infection has been reported to be halted in rainbow trout RBCs in conjunction with an RBC antiviral response [8, 13]. Moreover, a recent study has shown that RBCs display antigen-processing mechanisms, such as autophagy and proteasome activity, as detected by a transcriptomics, proteomics, and functional analysis of RBCs exposed *ex vivo* to VHSV [11]. RBCs have been reported to modulate the expression of MHCI and MHCII, cluster of differentiation 83 (CD83), and cluster of differentiation 86 (CD86) molecules when they are exposed to VHSV [11] both *ex vivo* and *in vivo*. Separately, rock bream RBCs have been reported to generate a response to challenge with rock bream iridovirus (RBIV), a virus that can replicate inside rock bream RBCs [19]. This response, which was evaluated via proteomic analysis, was mainly characterized by the upregulation of apoptosis- and MHCI–related pathways and the downregulation of ISG15 antiviral mechanisms [19].

To further investigate the immune response elicited by rainbow trout RBCs against VHSV, we evaluated the global RBC immune response using transcriptomic analyses of RBCs from single-cell sorted peripheral blood and head kidney, the major hematopoietic organ in fish [20], as well as a proteomic analysis of RBCs from peripheral blood, of rainbow trout challenged with VHSV. We detected upregulation of genes in peripheral blood RBCs (PB-RBCs) of VHSV-challenged rainbow trout in several immune-related overrepresented GO Term categories, including activation of complement system,

granulocyte activation, neutrophil chemotaxis, immunoglobulin-mediated humoral response, erythrocyte differentiation, B-cell receptor signaling pathway, stimulatory C-type lectin receptor signaling pathway, cytoplasmic pattern recognition receptor signaling, and antigen processing and presentation of exogenous peptide. From the proteomic analysis, we mainly highlight proteins with known immune functions such as NOD-like receptor with CARD domain 3 (NLRC3), NLR family pyrin domain-containing 12 (NLRP12) inflammasome, and interferon-induced protein with tetratricopeptide repeat 5 (IFIT5). On the other hand, RBCs from head kidney (HK-RBCs) of VHSV-challenged individuals showed upregulated genes related to the type I interferon and antigen processing and presentation of endogenous peptide antigen via MHC-I pathways. Antiviral effectors stimulated by type I interferon, such as Mx and IFIT5, were highly expressed in PB-RBCs from VHSV-challenged rainbow at both the transcript and protein levels. In summary, RBCs can develop an immune response during *in vivo* VHSV infection of rainbow trout, despite their RBCs are not infected by VHSV. Our results implicate RBCs as potential cell targets for the development of novel immunomodulants for aquaculture-farmed species.

2. MATERIALS AND METHODS

2.1. Animals

Rainbow trout (Oncorhynchus mykiss) individuals of approximately 5 to 6 cm were obtained from a commercial fish farm (Piszolla S.L., Cimballa Fish Farm, Zaragoza, Spain). Fish were maintained at the University Miguel Hernandez (UMH) facilities in a recirculating, dechlorinated water system and fed daily with a commercial diet (Skretting, Burgos, Spain). Water temperature was constantly monitored to maintain fish at 14°C. Fish were acclimatized to laboratory conditions for 2 weeks before experimentation.

2.2. Rainbow trout challenge with VHSV

Young rainbow trout individuals were infected by intramuscular injection of 50 µL RPMI-1640 medium (Dutch modification) (Gibco, Thermo Fischer Scientific Inc., Carlsbad, CA) supplemented with 2% FBS (Cultek, Madrid, Spain), 1 mM pyruvate (Gibco), 2 mM L-glutamine (Gibco), 50 µg/mL gentamicin (Gibco), 2 µg/mL fungizone (Gibco), and 100 U/mL penicillin/streptomycin (Sigma-Aldrich) with VHSV (108 tissue culture infectious dose 50% (TCID50)/mL). As a negative infection control, individuals were injected with 50 µL sterile RPMI 2% FBS. Over the course of the challenge, individuals were maintained at 14°C for the number of days indicated in each assay. Viral hemorrhagic septicemia virus (VHSV-07.71) was purchased from the American Type Culture

Collection (ATCC, VR-1388) and propagated in fathead minnow epithelioma papulosum cyprini (EPC) cells [21] at 14°C, as previously reported [22].

2.3. Blood extraction and staining

Rainbow trout were sacrificed by overexposure to tricaine methanesulfonate (Sigma-Aldrich) at 300 mg/L. Peripheral blood was sampled from the caudal vein using insulin syringes (NIPRO Bridgewater, NJ). Approximately 100 µL blood was diluted in RPMI 10% FBS. Blood cells were dyed using SYTO RNASelect (Thermo Fischer Scientific Inc.) diluted 1/1000 in RPMI 10% FBS for 20 min at room temperature. The diluted SYTO RNASelect was removed by centrifugation of the suspension at 1600 rpm, and cells were resuspended in RPMI for cell sorting.

2.4. Cell sorting

RBCs from head kidney and peripheral blood were sorted using the BD FACSJazz cell sorter (BD Biosciences, Madrid, Spain). The single-cell sorting workflow is represented in Figure 1A. Samples were previously labeled with SYTO RNASelect. HK-RBCs (102 cells per individual) were sorted from the previously selected population (circle in Figure 1B) in a forward-scattering (FSC)/side-scattering (SSC) dot plot, which is part of the P2 population in the fluorescence histogram, using a 1.0 drop single-cell mask in the BD FACSJazz software to maximize purity. PB-RBCs (106 cells per individual) were sorted using a 2.0 drop enrich mask to maximize yield. Sample purity was confirmed by optical microscopy using the IN Cell Analyzer 6000 cell imaging system (GE Healthcare, Little Chalfont, UK). A representative image of the RBCs that were single-cell sorted from HK is shown in Figure 1C.

(**A**)

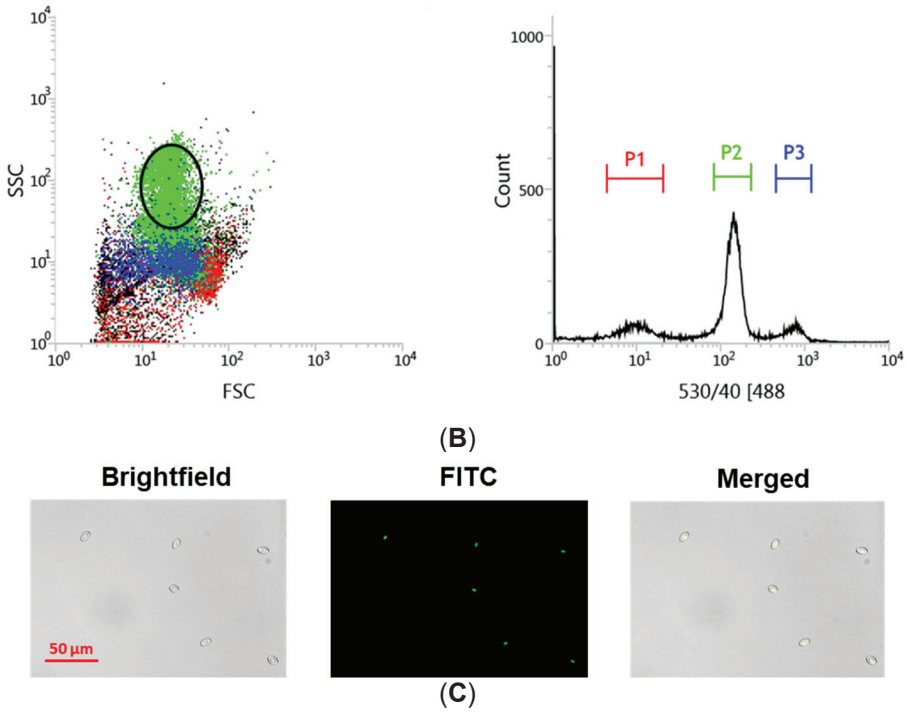


Figure 1. Overview of the single-cell sorting methodology. (**A**) Workflow of single-cell sorting using the BD FACSJazz cell sorter. (**B**) Double gating population selection for RBCs sorting based on: i) fluorescence intensity, where the P2 (green) population corresponds to RBCs, and ii) FSC/SSC, where the selected circled population corresponds to RBCs. (**C**) Representative bright-field and fluorescein (FITC) microscopy images of single-cell sorted RBCs from HK, taken with 10× magnification.

2.5. Transcriptome analysis

RBCs from 16 individuals were grouped into 2 pools of 8 individuals for each condition (mock- and VHSV-challenged). RBCs were preserved in a 1/10 dilution of 9.5 µL of 10× lysis buffer (Clontech, Takara Bio, Mountain View, CA, USA) and 0.5 µL of RNase Inhibitor (Invitrogen, ThermoFisher Scientific Inc.) at -80°C until cDNA library construction. Then, cDNA was produced directly from pooled lysed cells using SMART-Seq v4 Ultra Low Input RNA Kit (Clontech, Takara Bio). Sequence reads are available at SRA-NCBI accession SRP133501. RNA-Seq library preparation and sequencing were carried out by STABVida Lda. (Caparica, Portugal) as previously described [12].

2.6. Proteome analysis

Ficoll-purified RBCs from rainbow trout were exposed to VHSV as described above. At 2 days post-challenge, VHSV-exposed (n=16) and mock (n=16) RBCs (8·106 cells per fish) were pelletized by centrifugation (1600 rpm) and the supernatant was removed. The cell pellet was washed 3 times with PBS, and then it was digested, cleaned-up/desalted, and pooled into 2 pools of 8 individuals for each condition (mock- and VHSV-challenged).

Then, samples were subjected to liquid chromatography and mass spectrometry analysis (LC-MS) as previously described [12]. Log2 peptide ratios followed a normal distribution that was fitted using least squares regression. Mean and standard deviation values derived from the Gaussian fit were used to estimate P values and false discovery rates (FDR) at a quantitative level. The confidence interval for protein identification was set to <95% (P-value <0.05), and only peptides with an individual ion score above the 1% FDR threshold were considered correctly identified. Only proteins with ≥2 peptide spectrum matches (PSMs) were considered in the quantitation.

2.7. Pathway enrichment analysis

For transcriptomic and proteomic analyses, pathway enrichment analysis was performed for DEGs and differentially expressed proteins (DEPs) using ClueGO v2.3.5 [23], CluePedia v1.5.3 [24], and Cytoscape v3.7.0 [25]. The GO Immune System Process and GO Biological Process databases (updated on February 23, 2017) were used. Genes and proteins selected for functional pathway analysis had a FDR ≤0.05. Genes and proteins were identified by sequence homology with Homo sapiens using Blast2GO version 4.1.9 [26]. Genes and proteins only identified in fish are indicated in cursive.

2.8. RNA isolation and cDNA synthesis

The E.Z.N.A. Total RNA Kit (Omega Bio-Tek Inc., Norcross, GA) was used for total RNA extraction in accordance with the manufacturer's instructions. To eliminate possible residual genomic DNA, a DNAse treatment of the sample was done using TURBO DNase (Ambion, Thermo Fischer Scientific Inc.) following the manufacturer's instructions. RNA quantification was done with a NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE).

cDNA was synthesized from RNA using M-MLV reverse transcriptase (Invitrogen, Thermo Fischer Scientific Inc.) as previously described [27]. cDNA was stored at -20°C until used.

2.9. Quantitative PCR

Quantitative reverse transcription PCR (RT-qPCR) was performed in 20-µL reactions using 24 ng cDNA, 10 µL TaqMan universal PCR master mix (Thermo Fischer Scientific), and 900 nM final concentration of each primer (300 nM for NVHSV gene) using the CFX96 System (BioRad, Irvine, CA). Cycling conditions were 50°C for 2 min; 95°C for 10 min; and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Gene expression was analyzed by the 2-ΔCt method [28]. The ef1a gene was used as an endogenous control. Primer sequences are listed in Table 1.

Table 1. Primer sequences used in quantitative PCR.

Gene	Forward	Reverse	Probe	Reference or Accession number
c4bpa	TGAGAATGGCGTTAGGATTGAA	GTTGCACTTATACGTCACAAAAGACTT		XM_021564396.1
cd55	CGCTCAAATTAACCTGCAAAAA	GTGCCTTCCTTAAACTCATATGTCAA		XM_021609679.1
cd59	CGGAGCCACATCCATTGG	TTACTGCATACACCACCACATCACT		NM_001124497.1
dhx58	GCTCTCCACTTGCGTCAGTACA	GACCCTAAAGGCATCCACCAT		XM_021624832.1
ef1a	ACCCTCCTCTTGGTCGTTTC	TGATGACACCAACAGCAACA	GCTGTGCGTGACATGAGGCA	[29]
gbp1	TGGTTCCGCTCTAGGTTTCTTC	AGCCTAAAACCCAAAAGAGCAA		XM_021579826.1
ifi35	CTGGTGCCCTGTCAAGTAGAGA	TTCTTGGGCAGGTTGGAAAC		XM_021558400.1
ifih1	GAGCCCGTCCAAAGTGAAGTT	AGTGAGGTGTTTTCTCTTTGAATGAA		NM_001195179.1
ifit5	CCCTCAATGACTCTGACAAGCA	CCCTGCCCTCATCTTTCTTCT	CCAGCTTCGGCCTGTTTCTGTTCCA	[7]
irak1	CAGACAGACCAACGCTCACAA	GCAGATCGCACCCACATG		XM_021567162.1
mavs	GAGGGCAGAGTGGAACAAACA	TCAGAGCTGGTAGAAGGAATTGGT		NM_001195181.1
mx1-3	TGAAGCCCAGGATGAAATGG	TGGCAGGTCGATGAGTGTGA	ACCTCATCAGCCTAGAGATTGGCTCCCC	[30]
nlrc5	CTGCTATGTGCCGCCAATT	CCAGTGTAGGCCAAGGATCAC		XM_021570046.1
nlrx1	CCTGCTTTTTACCTTCCTATTGCT	CACCTCCCCTCCAAAGTTGA		XM_021581927.1
nod2	GAGAGACAGGAGTTGACGATTCTG	TTGTCTGACTTCTTCGAGATCATCA		NM_001201555.1
NVHSV	GACTCAACGGGACAGGAATGA	GGGCAATGCCCAAGTTGTT	TGGGTTGTTCACCCAGGCCGC	[27]
stat1	GCCGAGAACATCCCTGAGAA	GCTTACTCGCCAACTCCATTG		XM_021596980.1
traf3	GGGCTTCAGGGACCACTTC	ACCAGCTTGCAGGACTCACA		NM_001124615.1

2.10. Antibodies

The rabbit polyclonal antibodies against rainbow trout β-defensin 1 (BD1) [13] and Mx3 [31] were produced in the laboratory of Prof. Amparo Estepa. Mouse polyclonal antibody against rainbow trout IFIT5 was produced in the laboratory of Prof. Luis Mercado [8].

Rabbit polyclonal antibody against human α-actin (Sigma-Aldrich, Cat. #2066) was used for western blotting as a loading control.

2.11. Western blotting

RBCs pellets were resuspended at a concentration of 10^8 RBCs/mL in PBS buffer with a protease inhibitor cocktail (Sigma-Aldrich). Cells were lysed by freezing and thawing samples 3 times. Cell debris was eliminated by centrifugation at 12,000 rpm for 10 min. Then, 50 µg of each sample were loaded on a 12% (for Mx3) and 18% (for BD1) polyacrylamide gel under reducing conditions. Electrophoresis was performed at 150 V for 90 min. Proteins in the gel were transferred to 0.4-µm pore size nitrocellulose membranes (BioRad) for 120 min at 100 V in transfer buffer (2.5 mM Tris, 9 mM glycine, 20% methanol). Membranes were then blocked in PBS containing 5% dry milk and 0.1% Tween-20 (PMT buffer) and incubated with primary antibodies in PMT buffer (5% milk for Mx3 and IFIT5 antibodies and 0.5% for BD1 antibody) overnight at 4°C. Membranes were then washed 3 times for 10 min each with PBS 0.1%Tween-20 buffer and then incubated with secondary antibody GAR-Po (Sigma-Aldrich) or GAM-Po (Sigma-Aldrich) in PMT buffer (5% milk for Mx3 and IFIT5 antibodies and 0.5% for BD1 antibody) for 60 min. Membranes were then washed 3 times with PBS 0.2% Tween-20. Peroxidase activity was detected using ECL chemiluminescence reagents (Amersham Biosciences, Buckinghamshire, UK) and revealed using a ChemiDoc XRS+ system (BioRad). Protein band images were processed using Image Lab software v6.0.1 (BioRad).

2.12. Ethics statement

Experimental procedures on experimental animals were reviewed and approved by the Animal Welfare Body and the Research Ethics Committee at the UMH (approval number 2014.205.E.OEP; 2016.221.E.OEP) and by the competent authority of the Regional Ministry of Presidency and Agriculture, Fisheries, Food and Water supply (approval number 2014/VSC/PEA/00205). All procedures were carried out in accordance with the Spanish Royal Decree RD 53/2013 and EU Directive 2010/63/EU for the protection of animals used for research experimentation and other scientific purposes.

2.13. Software and statistics

All figures and graphics show the mean and standard deviation of the data. P values associated with each graphic are represented in the figure legends: *, P-value <0.05; **, P-value <0.01. Graphpad Prism 6 (www.graphpad.com) (Graphpad Software Inc., San Diego, CA) was used to prepare graphs and perform statistical calculations. ImageJ software (version 1.51, National Institutes of Health, Bethesda, MD, USA) was used for

western blot band densitometry. Venny 2.1 (www.bioinfogp.cnb.csic.es/tools/venny) was used for Venn diagram construction. Clustering of gene expression was performed using ClustVis (https://biit.cs.ut.ee/clustvis/)[32].

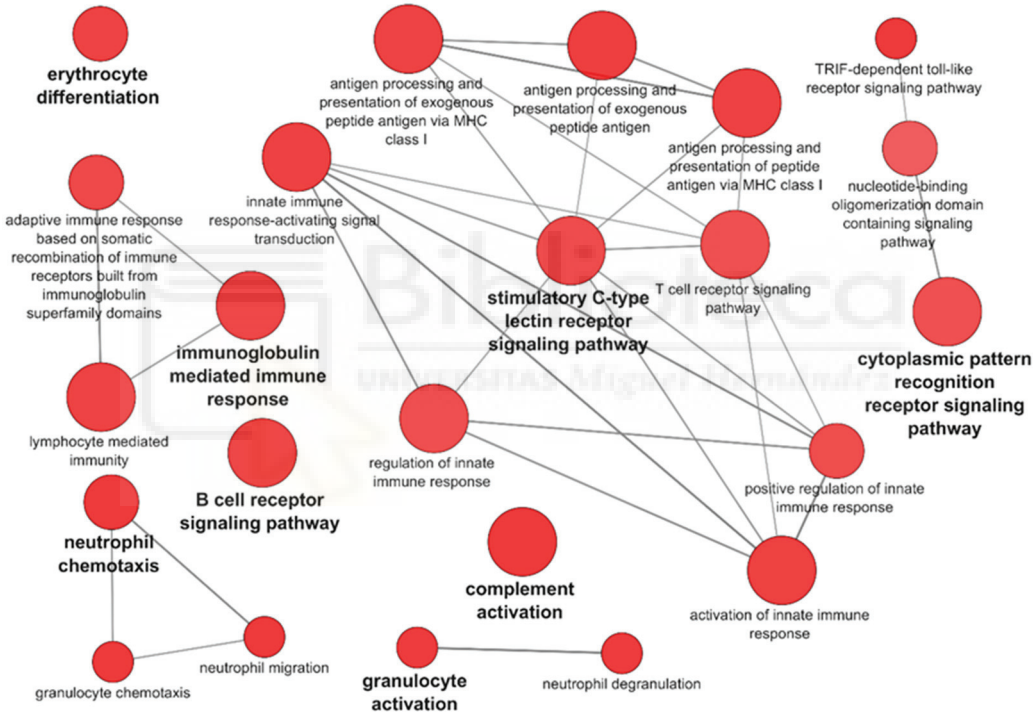
3. RESULTS

3.1. Transcriptomic analysis of PB-RBCs and HK-RBCs from VHSV-challenged rainbow trout reveals upregulation of genes related to the complement system and the interferon pathway, respectively.

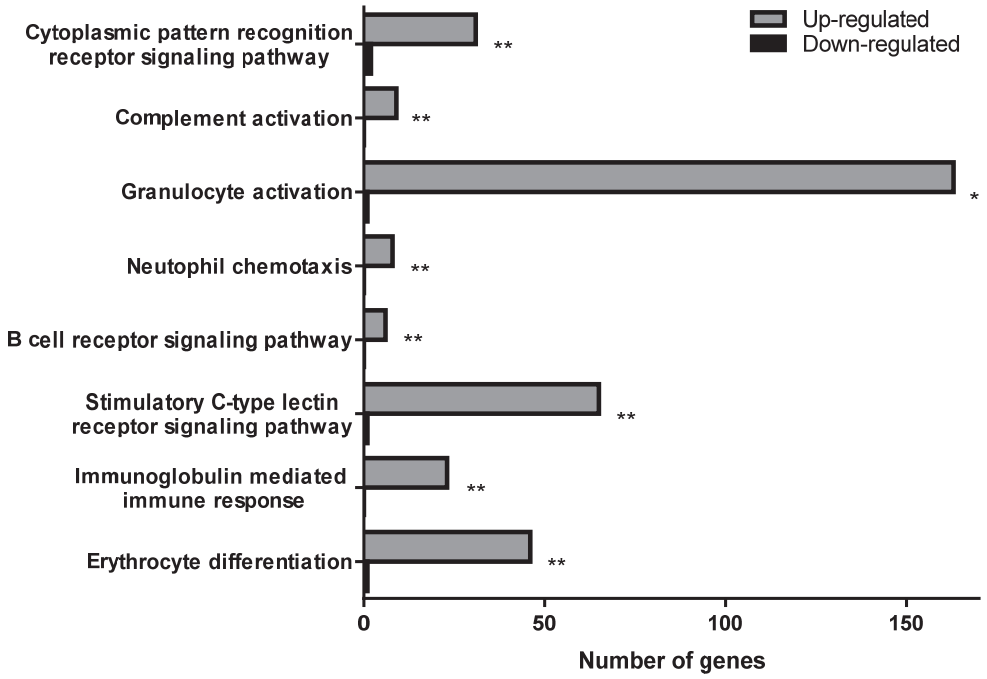
The transcriptomic analysis comparing RBCs from mock- and VHSV-challenged rainbow trout revealed differential regulation of 4196 (4137 upregulated and 59 downregulated) genes for PB-RBCs and 1578 (841 upregulated and 737 downregulated) for HK-RBCs. A list with all DEGs in PB-RBCs and HK-RBCs is shown in Supplementary Tables S1 and S2, respectively. Using Cytoscape software and the GO Immune System Process database, we identified the following majorly overrepresented pathways: i) activation of complement system, ii) granulocyte activation, iii) neutrophil chemotaxis, iv) immunoglobulin-mediated humoral response, v) erythrocyte differentiation, vi) B-cell receptor signaling pathway, vii) stimulatory C-type lectin receptor signaling pathway, and viii) cytoplasmic pattern recognition receptor signaling (Figure 2A,B) (Supplementary Table S3) in PB-RBCs from VHSV-challenged rainbow trout. The following pathways were also overrepresented to a lesser degree: i) antigen processing and presentation of exogenous peptide antigen and ii) TRIF-dependent Toll-like receptor signaling pathway. Among genes related to complement activation, we highlight the following: complement component 4 binding protein alpha (*c4bpa*), with a log2 fold change (FC) of 7.17; cluster of differentiation 55 (*cd55*), also known as complement decay-accelerating factor, with a log2FC of 4.03; and cluster of differentiation 59 (*cd59*) with a log2FC of 8.75. Among the genes related to cytoplasmic pattern recognition receptor signaling, we highlight DExH-box helicase 58 (*dhx58*) (log2FC of 8.69), interferon-induced with helicase C domain 1 (*ifih1*) (log2FC of 8.20), interleukin 1 receptor associated kinase 1 (*irak1*) (log2FC of 5.12), NLR family member X1 (*nlrx1*) (log2FC of 3.81), nucleotide-binding oligomerization domain-containing protein 2 (*nod2*) (log2FC of 11.54), and mitochondrial antiviral signaling protein (*mavs*) (log2FC of 3.32). In addition, we highlight genes related to the TRIF-dependent Toll-like receptor signaling pathway, including TNF receptor-associated factor 3 (*traf3*) (log2FC of 4.84), and genes related to antigen presentation, including cd83 (log2FC of 8.19), *mhcI* (log2FC of 11.04), and *mhcII* (log2FC of 4.93).

On the other hand, HK-RBCs from VHSV-challenged rainbow trout showed high upregulation of genes related to type I interferon signaling and antigen processing and

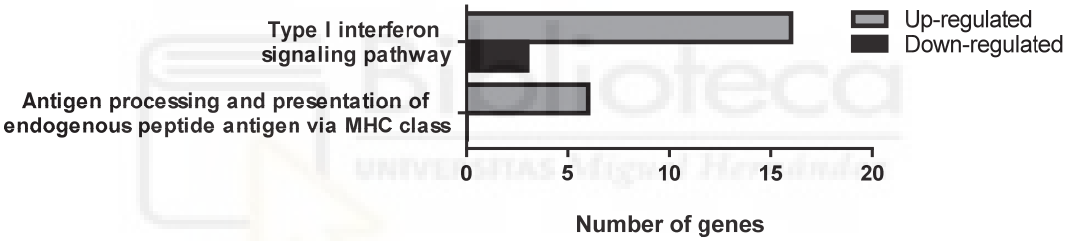
presentation of endogenous peptide antigen via MHC class I pathways (Figure 2C) (Supplementary Table S4). Among the genes within the type I interferon signaling pathway, we highlight upregulation in the NOD-like receptor family CARD domain-containing 5 (*nlrc5*) with a log2FC of 11.79; guanilate binding protein 2 (*gbp2*) with a log2FC of 5.76; signal transducing activating factor (*stat1*) with a log2FC of 4.45; MX dynamin like GTPase 1 (*mx1*) with a log2FC of 6.76; *ifit5* with a log2FC of 4.85; and interferon induced protein 35 (*ifi35*) with a log2FC of 5.12. Genes identified in the GO Immune System Process terms from HK-RBCs of VHSV-challenged rainbow trout interacted strongly, as revealed by a protein-protein interaction (PPI) network analysis (Figure 2D). The PPI network also corroborated the overrepresentation of type I interferon signaling pathway and antigen processing and presentation pathways.



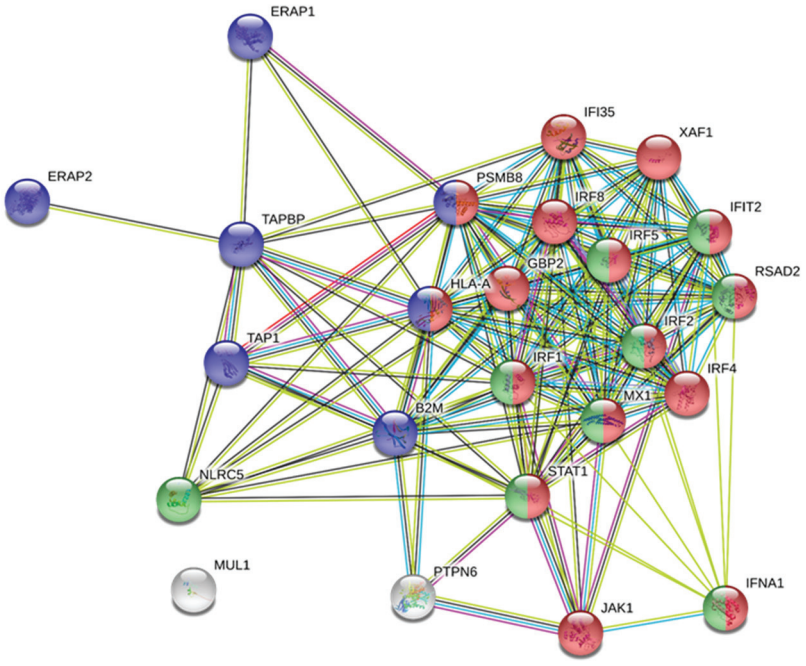
(**A**)


Up-regulated
Down-regulated
Cytoplasmic pattern recognition receptor signaling pathway
Complement activation
Granulocyte activation
Neutophil chemotaxis
B cell receptor signaling pathway
Stimulatory C-type lectin receptor signaling pathway
Immunoglobulin mediated immune response
Erythrocyte differentiation
**
**
*
**
**
**
**
0
50
100
150
Number of genes

(**B**)


Up-regulated
Down-regulated
Type I interferon signaling pathway
Antigen processing and presentation of endogenous peptide antigen via MHC class
0
5
10
15
20
Number of genes

(**C**)

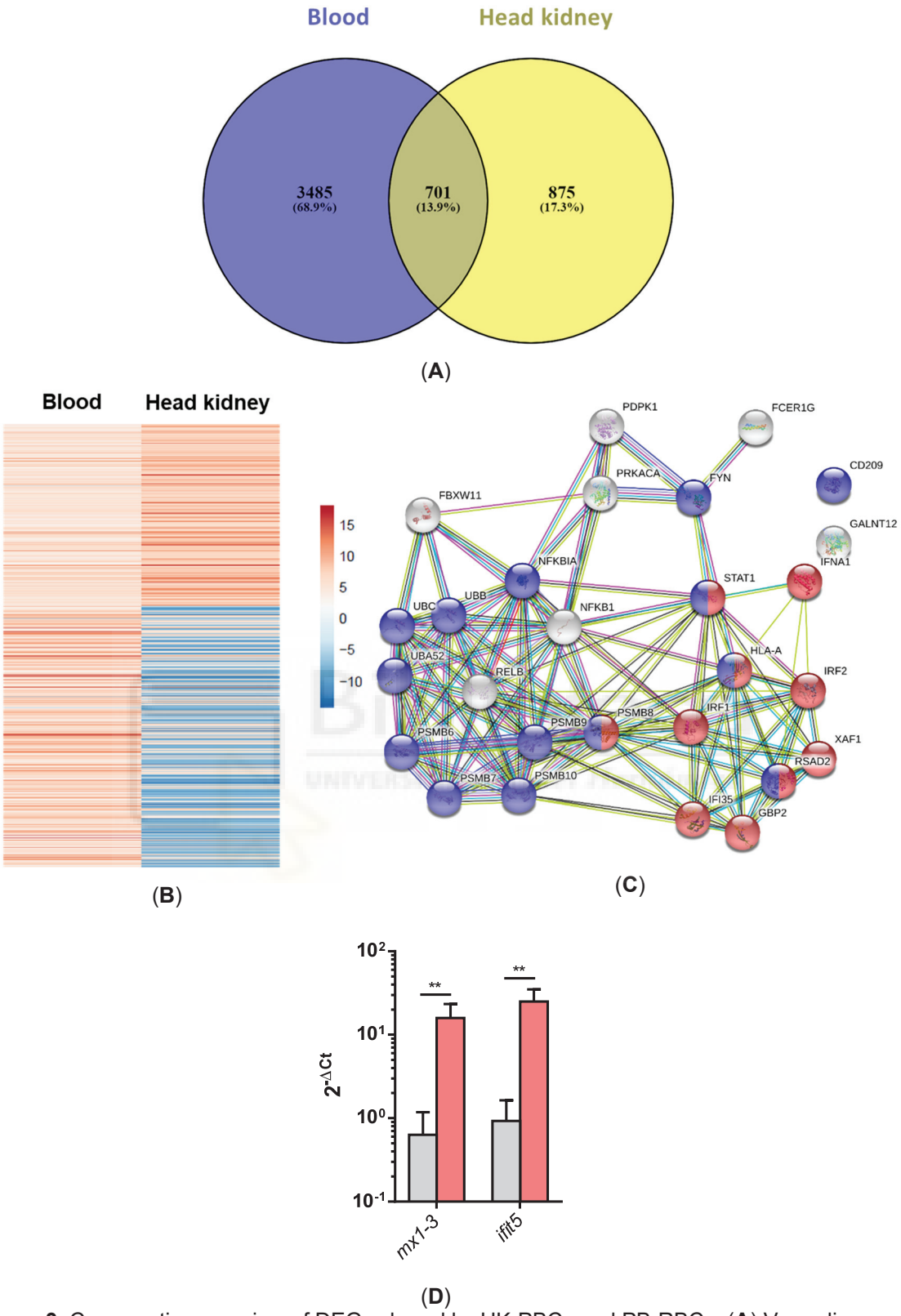

ERAP1
ERAP2
TAPBP
TAP1
NLRC5
MUL1
PSMB8
HLA-A
B2M
PTPN6
IFI35
XAF1
IRF8
IRF5
GBP2
IRF1
STAT1
JAK1
IFIT2
RSAD2
IRF2
IRF4
MX1
IFNA1

(**D**)

277

Figure 2. GO Immune System process categories overrepresented in the transcriptomic analysis of PB-RBCs and HK-RBCs from VHSV-challenged rainbow trout. (**A**) Pathway network analysis under the GO Immune System Process database from PB-RBCs of VHSV-challenged rainbow trout. Gene enrichment analysis was performed selecting GO Immune System Process terms with *P*-value <0.05, GO Term fusion, and GO Tree interval of 3-8. Red indicates upregulated pathway expression. (**B**) The number of upregulated and downregulated genes in each represented GO Immune System Process category from VHSV-challenged rainbow trout PB-RBCs. Asterisks denote GO term significance. (**C**) Number of upregulated and downregulated genes in each overrepresented GO Immune System Process category from VHSV-challenged rainbow trout HK-RBCs with the following analysis parameters: *P*-value <0.1, GO Term fusion, and GO Tree interval of 3-8. (**D**) PPI networks of DEGs identified in the GO Immune System Process terms of HK-RBCs from VHSV-challenged rainbow trout constructed using STRING software (*P*-value <10-16). Nodes represent proteins, while edges denote the interactions between 2 proteins. Different line colors represent the types of evidence used in predicting the associations: gene fusion (red), gene neighborhood (green), co-expression (black), gene co-occurrence (blue), experimentally determined (purple), from curated databases (teal), text-mining (yellow), or protein homology (lilac). Red nodes denote proteins implicated in the type I interferon signaling pathway (GO:0060337), blue nodes denote proteins implicated in antigen processing and presentation (GO:0019882), and green nodes denote proteins implicated in defense response to virus (GO:0051607).

A Venn diagram of the DEGs for PB-RBCs and HK-RBCs (Figure 3A) showed 701 genes common to both samples (13.9% of the total). Supplementary Table S5 shows common and exclusive DEGs for each organ. Interestingly, DEGs common between PB-RBCs and HK-RBCs that were mainly upregulated in peripheral blood appeared to be downregulated in head kidney and vice versa (Figure 3B). Overrepresented pathways included the type I interferon and stimulatory C-type lectin receptor signaling pathways (Supplementary Table S6). DEGs identified in the GO Immune System Process terms interacted strongly as revealed by a PPI network analysis (Figure 3C). The PPI network also corroborated the overrepresentation of the type I interferon signaling pathway. Gene expression of mx1-3 and ifit5 was further validated by RT-qPCR in PB-RBCs from VHSV-challenged rainbow trout at 2 days post challenge (dpc) (Figure 3D), which showed statistically significant upregulation. Analysis of DEGs exclusive to PB-RBCs showed overrepresentation of i) antigen processing and presentation of exogenous peptide antigen via MHCI, TAP-dependent, ii) stimulatory C-type lectin receptor signaling pathway, iii) immunoglobulin-mediated immune response, and iv) cytoplasmic pattern recognition receptor signaling pathway (Figure 4) (Supplementary Table S7). No significant upregulated or downregulated pathways were detected for DEGs exclusive to HK-RBCs from VHSV-challenged rainbow trout.



316 **Figure 3**. Comparative overview of DEGs shared by HK-PBCs and PB-RBCs. (**A**) Venn diagram
317 of DEGs in RBCs from each organ. The middle region shows genes expressed in RBCs from
318 both organs. (**B**) Clustering of gene expression of common DEGs in HK-RBCs and PB-RBCs was
319 performed using ClustVis. The dataset was inserted into matrix category. Parameters for
320 clusterization included no data transformation and no row scaling, and the PCA method used was

SVD with imputation. Red indicates higher expression and blue represents lower expression. (**C**) PPI networks of the DEGs shared between PB-RBCs and HK-RBCs from VHSV-challenged rainbow trout identified with GO Immune System Process terms and constructed using STRING software (*P*-value <10-16). Nodes represent proteins, while edges denote interaction between 2 proteins. Different line colors represent the types of evidence used in predicting the associations: gene fusion (red), gene neighborhood (green), co-expression (black), gene co-occurrence (blue), experimentally determined (purple), from curated databases (teal), text-mining (yellow), or protein homology (lilac). Red nodes denote proteins implicated in the type I interferon signaling pathway (GO:0060337) and blue nodes denote proteins implicated in viral process (GO:0016032). (**D**) Expression of interferon-stimulated genes *mx1-3* and *ifit5* in PB-RBCs from VHSV-challenged rainbow trout (red bars) compared to mock-challenged (gray bars) as control at 2 dpc. Data represent mean ± SD (n=6). A Mann-Whitney test was performed to test statistical significance between PB-RBCs from both groups. Asterisks denote statistical significance. **, *P*-value <0.01

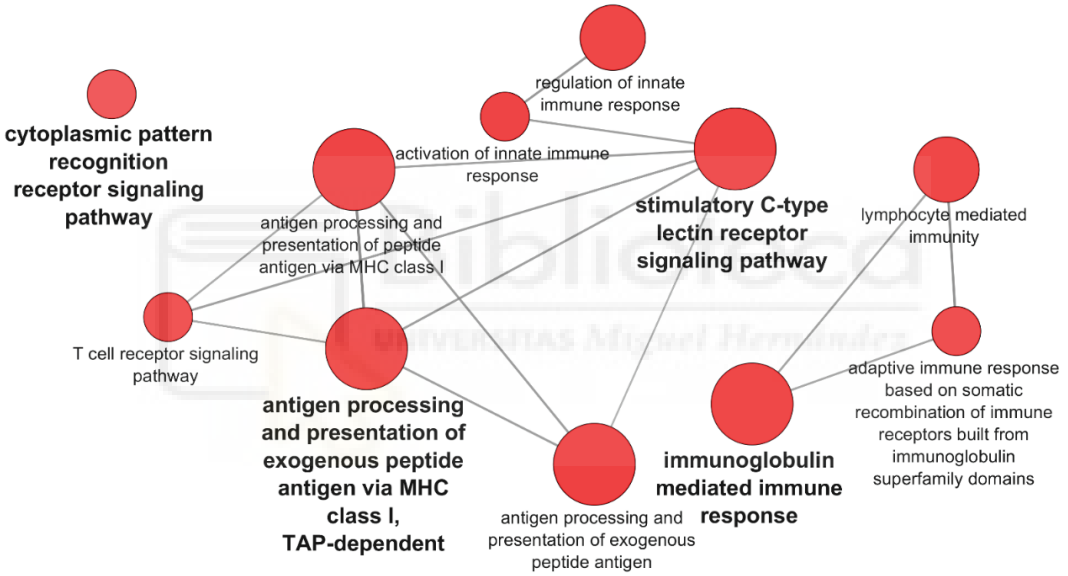


Figure 4. Pathways overrepresented in DEGs exclusively in PB-RBCs from VHSV-challenged rainbow trout. Gene enrichment analysis was performed using GO Immune System Process terms with P-value <0.05, GO Term fusion, and GO Tree interval of 3-8.

3.2. Upregulated DEGs from overrepresented pathways were validated by RT-qPCR.

An RT-qPCR analysis was performed to validate pathways overrepresented in PB-RBCs or HK-RBCs from VHSV-challenged individuals. For the complement activation pathway, we analyzed the expression of *c4bpa*, *cd55*, and *cd59*. The TRIF-dependent Toll-like receptor signaling pathway was validated with*traf3*. For the cytoplasmic pattern recognition receptor signaling pathway, we chose *dhx58*, *ifih1*, *irak1*, *nlrx1*, *nod2*, and *mavs*. From the type I interferon signaling pathway, we analyzed *nlrc5*, *stat1*, guanylate binding protein 1 (*gbp1*), which is a rainbow trout homolog of human *gbp2*, and *ifi35*. The results showed statistically significant upregulation in PB-RBCs from VHSV-challenged

rainbow trout in all tested genes, except *cd55*, *gbp1*, *mavs*, and *nlrx1,* which were upregulated but not statistically significant (Figure 5). *NVHSV* gene transcripts were barely detectable by RT-qPCR (Cts ranging from 32 to undetected). However, NVHSV gene or protein was not detected in transcriptomic or proteomic analyses, respectively.

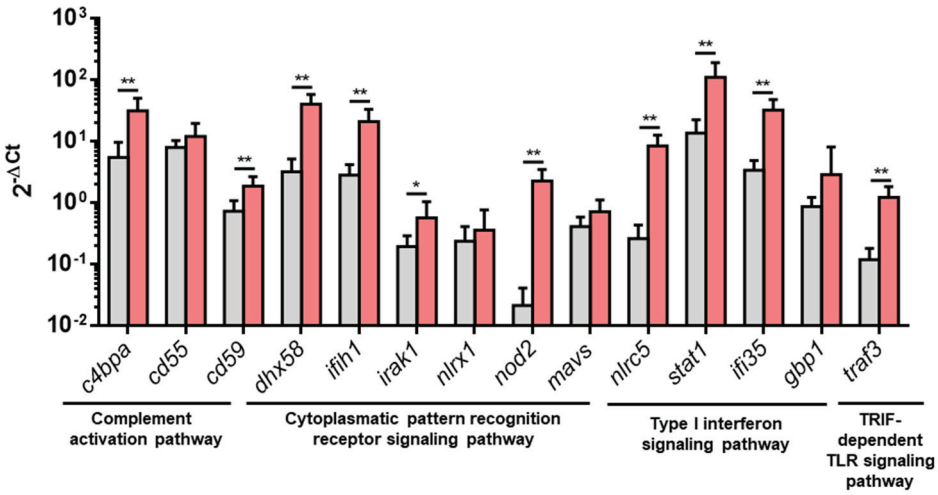
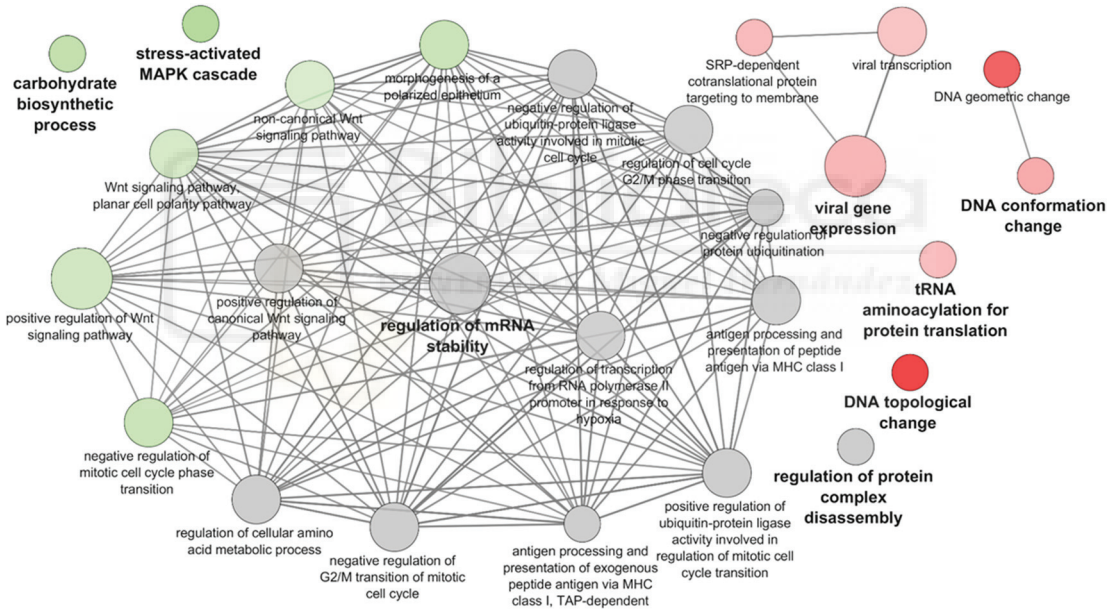


Figure 5. Upregulated DEGs from overrepresented pathways validated by RT-qPCR. Gene expression of PB-RBCs from VHSV-challenged rainbow trout (red bars) compared to the control, mock-challenged fish (gray bars) at 2 dpc. Data represent mean ± SD (n=6). A Mann-Whitney test was performed to test statistical significance between PB-RBCs from both groups. Asterisks denote statistical significance. *, *P*-value <0.05; **, *P*-value <0.01.

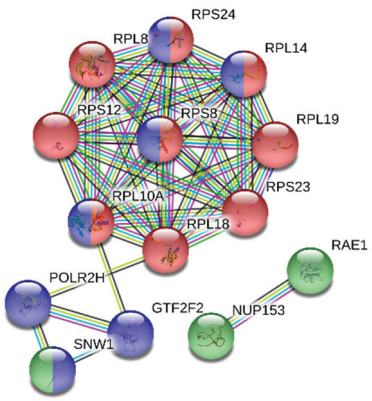
3.3. Proteomic analysis of PB-RBCs from VHSV-challenged rainbow trout shows upregulation and interaction of proteins involved in the immune response.

PB-RBCs were collected and purified from mock- and VHSV-challenged rainbow trout for proteome analysis. In total, 380 DEPs were detected, 194 of which were upregulated and 186 of which were downregulated. The list of DEPs can be found in Supplementary Table S8. The functional pathway evaluation of DEPs was performed using the Cytoscape ClueGo platform and the GO Immune System Process database. Overrepresented pathways are listed in Supplementary Table S9. Among these processes, we identified the positive regulation of processes related to viral gene expression and transcription, and categories related to DNA topological change were found to be upregulated as well (Figure 6A). On the other hand, the overrepresented Wnt signaling, carbohydrate biosynthetic process, and stress-activated MAPK cascade (Figure 6A) pathways appeared to be downregulated. Regulation of mRNA stability and antigen processing and presentation of peptide antigen via MHCI pathways appeared to be overrepresented but nonspecifically regulated.

A PPI network using STRING software was performed with the DEPs identified in the viral gene expression and transcription pathways. High PPI interaction was observed between ribosomal proteins L (RPL) 19, 18, and 8 and ribosomal protein S (RPS) 8 and 12 (Figure 6B). Other proteins, such as eukaryotic translation initiation factor 3 subunit L (EIF3L), RNA polymerase II subunit H (POLR2H), and general transcription factor IIF subunit 2 (GTF2F2), interacted strongly with RPL and RPS proteins (Figure 6B). We would like to highlight the presence and high level of interaction between the nuclear pore complex protein 153 (NUP153) and mRNA export factor RAE1. Among the upregulated DEPs, it is also noteworthy to point out the presence of proteins with known immune functions in viral infections, such as NLRC3 (log2FC of 2.79), the NOD-like receptor GBP1 (log2FC of 3.71), IFIT5 (log2FC of 4.60), IFI35 (log2FC of 2.50), radical SAM domain-containing 2 (RSAD2) (log2FC of 2.08), and GTPase, very large interferon inducible pseudogene 1 (GVINP1) (log2FC of 3.23).



(**A**)



(**B**)

Figure 6. Functional protein association networks of DEPs in PB-RBCs from VHSV-challenged individuals. (**A**) Functional pathway analysis was performed using the GO Immune System Process and GO Biological Process databases with *P*-value <0.05, GO Term fusion, and GO Tree interval of 3-8. Red indicates upregulated pathways, green indicates downregulated pathways, and gray indicates nonspecific regulation. (**B**) PPI network of proteins associated with the viral transcription GO Term (GO:0019083) using STRING software (*P*-value <9.99·10-15). Red nodes denote proteins implicated in translation initiation (GO:0006413). Blue nodes denote proteins implicated in RNA processing (GO:0006396). Green nodes denote proteins implicated in viral process (GO:0016032). Nodes represent proteins, while edges denote interactions between 2 proteins. Different line colors represent the types of evidence used in predicting the associations: gene fusion (red), gene neighborhood (green), co-expression (black), gene co-occurrence (blue), experimentally determined (purple), from curated databases (teal), text-mining (yellow), or protein homology (lilac).

3.4. Antiviral effectors were upregulated in VHSV-challenged rainbow trout RBCs.

Several antiviral effectors, including cholesterol 25-hydroxylase (*ch25h*), *gvinp1*, *mx*, *ifi35*, *rsad2* (also known as viperin), *ifit5*, interferon-induced transmembrane protein 3 (*ifitm3*), tripartite motif (*trim*) gene family, and SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase 1 (*samhd1*) were identified in PB-RBCs and HK-RBCs from VHSV-challenged rainbow trout and are listed in Table 2 with corresponding log2FC. We identified antiviral effectors genes specific to teleost species in PB-RBCs and HK-RBCs, including grass carp reovirus induced gene 2 (*gig2h*), with a log2FC of 10.84 and 5.41 in PB-RBCs and HK-RBCs, respectively, and VHSV-induced gene 2 (*vig2*), with a log2FC of 6.82 in PB-RBCs.

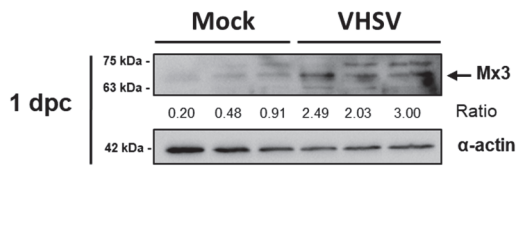
Table 2. Summary of interferon-stimulated genes (ISGs)/antiviral effectors orthologs identified in transcriptomic analyses.

Name	Symbol	Log_2FC PB-RBCs	Log_2FC HK-RBCs	References[1]
Cholesterol 25-hydroxylase	*ch25h*	-	8.68	[33]
GTPase, very large interferon inducible pseudogene 1	*gvinp1*	8.54	-	[34]
Interferon induced protein 35	*ifi35*	8.38	5.12	[35]
Interferon induced protein with tetratricopeptide repeats 5	*ifit5*	14.58	4.85	[8, 36]
Interferon-induced transmembrane protein 3	*ifitm3*	5.00	-	[37, 38]
Interferon-induced GTP-binding protein Mx	*mx1*	10.95	6.76	[39]
Radical SAM domain-containing protein 2	*rsad2*	11.61	5.47	[40, 41]

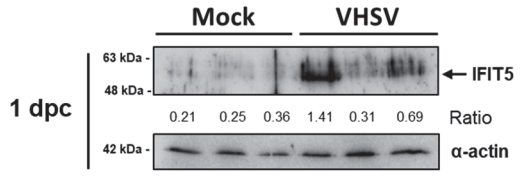
SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase 1	*samhd1*	7.74	-	[42]
Tripartite motif family 16	*trim16*	8.80	3.95	[43]
Tripartite motif family 21	*trim21*	6.65	-	[44]
Tripartite motif family 25	*trim25*	9.76	7.90	[45-47]
Tripartite motif family 39	*trim39*	7.01	8.46	[48]
Tripartite motif family 47	*trim47*	3.75	6.15	[49]

[1] Related to previous reports about the implication of the respective ISGs as antiviral effectors.

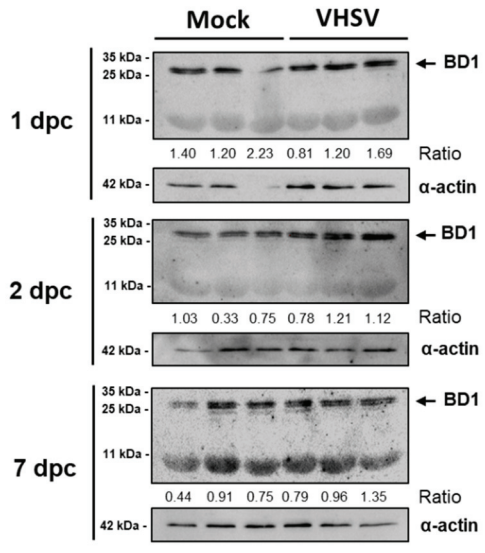
The expression of the antiviral proteins Mx3 and IFIT5 was validated by western blotting. These proteins were upregulated in PB-RBCs from VHSV-challenged rainbow trout. Mx3 protein was increased in PB-RBCs from VHSV-challenged individuals at 1 dpc in comparison with mock-challenged fish, as shown by the calculated ratios (Figure 7A). Similarly, IFIT5 was overexpressed at 1 dpc in VHSV-challenged individuals (Figure 7B), following a similar individual expression level trend to Mx3. We did not observe protein expression changes for Mx3 and IFIT5 at 2 and 7 dpc, by western blotting (data not shown). We also evaluated the expression of BD1, an antimicrobial peptide known to be induced by interferon [50] and also considered to be antiviral effector [51]. The BD1 tetrameric form (~28 kDa) expression was higher in PB-RBCs from VHSV-challenged individuals at 2 and 7 dpc, but monomeric BD1 expression (7.1 kDa) did not appear to be altered by VHSV infection (Figure 7C). Basal expression of these proteins was detected in the mock condition.



(**A**)



(**B**)



(**C**)

Figure 7. Protein kinetics of interferon-stimulated proteins (**A**) Mx3 (70.8 kDa) [52], (**B**) IFIT5 (51 kDa) [8], and (**C**) BD1 (monomeric, 7.1 kDa [13], tetrameric, ~28 kDa) in PB-RBCs from mock- and VHSV-challenged rainbow trout at 1, 2, and 7 dpc. Black arrow indicates the band selected for densitometry. Ratio represents expression normalized to α-actin expression.

4. DISCUSSION

Previous reports have shown that RBCs exposed *ex vivo* to VHSV mainly induced a moderate antiviral response and type I interferon downregulation at early stages after viral exposure and a cellular shut-off at later stages after viral exposure [13]. Autophagy activation, antigen processing, and upregulation of MHCI, MHCII, CD86, and CD83 antigen-presenting cell markers have been also reported at early timepoints after *ex vivo* VHSV exposure [11]. In the present study, transcriptomic analysis of PB-RBCs and HK-RBCs from VHSV-challenged individuals showed overrepresentation of processes related to type I interferon signaling, antigen processing, and presentation of peptide antigen. Cytoplasmic pattern recognition receptor signaling and TRIF-dependent Toll like receptor signaling, both related to RNA virus sensing and posterior immune and inflammatory response signaling [53], were detected in PB-RBCs from VHSV-challenged individuals. Among identified cytoplasmic pattern recognition receptors, NOD2 is an intracellular pattern recognition receptor that can interact with MAVS to activate type I interferon signaling in response to RNA viruses [54]. Similarly, NLRX1 has been defined as a regulator of antiviral mitochondrial activity [55]. DHX58, which binds double-stranded RNA (dsRNA), can interact with retinoic acid-inducible gene I (RIG-I) [56]. The helicase activity of DHX58 has been found to have a key role in RIG-I signaling [57]. Melanoma differentiation-associated protein 5 (MDA5), which is encoded by *ifih1* genes, is another receptor with helicase activity, has been reported to bind viral dsRNA [58]. Among the adaptors, we can find MAVS, involved in RIG-I signaling of RNA viruses [59]. TRIF signaling is known to be required for the production of TLR-mediated type I interferon [60]. TRIF adaptor has been reported to be a key component of TLR3 double-stranded RNA sensing/signaling cascade [61]. TRIF could therefore be implicated in the signaling cascade of TLRs of RBCs in response to VHSV infection, finally leading to type I interferon production and secretion. We found upregulation of NLRP3 in PB-RBCs from VHSV-challenged individuals. NLRP3 has been described as a versatile inflammasome protein that can be activated by a huge number of molecules [62], including RNA viruses [63]. Other pathways exclusively overrepresented in the transcriptomic analysis of PB-RBCs, including stimulatory c-type lectin receptor signaling, B cell receptor signaling, immunoglobulin-mediated immune response, and erythrocyte differentiation, have not been further described in this manuscript but are part of our ongoing research.

The PB-RBC transcriptome analysis showed upregulation of molecules related to complement activation or regulation, such as complement receptor 1 (*cr1*), which binds C3b/C4b complement proteins; complement 4 binding protein alpha (*c4bpa*), which is involved in C4b assembly [64]; and the *cd55* and *cd59* genes, which are involved in negative regulation of complement activation to prevent RBC lysis [65]. In the 1950s, Nelson described the immunoadherence phenomenon as the binding of antibody-opsonized substrates with C3b, activation of the classical pathway of the complement system, and binding to the surface of RBCs [66, 67]. However, the role of rainbow trout RBCs in the immunoadherence/complement response against viral infections is still being studied by our research group.

Molecules implicated in antigen processing and presentation have been previously reported to be upregulated in rainbow trout PB-RBCs exposed to VHSV or from VHSV-challenged individuals [11]. In this study, we also observed overrepresentation/upregulation of antigen presentation molecules in PB-RBCs and HK-RBCs implicated in antigen processing and presentation of exogenous and endogenous peptide antigen, respectively. Genes *cd83*, *mhcI* and *mhcII* appeared highly upregulated in transcriptomic analysis of PB-RBCs from VHSV-challenged rainbow trout.

The proteomic analysis mainly identified proteins related to viral transcription. We identified numerous members of the ribosomal S/L proteins that have been previously implicated in viral infections [68] and that closely interact. Viruses may have evolved stimulation mechanisms that synthesize ribosomal proteins to facilitate translation of their viral components. Moreover, the PPI analysis showed interaction of the RPL/S with EIF3L, POLR2H, and GTF2F2, which also implicated viral replication [69-71]. However, we previously described that VHSV replication appeared to stop in rainbow trout RBCs *ex vivo* early after exposure [13]. In addition, PB-RBCs from VHSV-challenged rainbow trout only showed low levels of *NVHSV* gene transcripts. Therefore, because viral replication processes appeared to be upregulated, other mechanisms triggered inside RBCs may be interrupting viral replication, such as autophagy as shown in RBCs exposed to VHSV *ex vivo* [11] or the vast number of antiviral effector ISGs found to be upregulated in RBCs in the present study (Table 2). The integrated 'omic' analyses revealed that in response to VHSV, PB-RBCs upregulated genes and proteins that interact with viruses at different stages, such as viral entry or replication, thus acting as antiviral effectors. Such genes/proteins include IFIT5, Mx, GBP1, GVINP1, RSAD2, IFITM3, IFI35, several TRIM proteins, CH25H, and SAMHD1 (supported by the references detailed in Table 2). In this sense, Mx, RSAD2, GVINP1, TRIM4, and GIG2 have been previously reported to be induced in RBCs from PRV infected Atlantic salmon

individuals [9], but it is important to highlight that PRV infects RBCs while VHSV cannot actively replicate inside rainbow trout RBCs [13]. Moreover, GIG2 and VIG2 are molecules known to be induced by RNA virus [72].

The Mx and IFIT5 proteins, as well as the BD1 antimicrobial peptide, are expressed in response to type I interferon [39, 50, 73]. In the present work, we demonstrated that PB-RBCs from VHSV-challenged rainbow trout increased the levels of these proteins in transcriptomic and proteomic analyses. Mx is a family of GTPases with antiviral activity [39, 74]. High basal expression levels of Mx have been found in rainbow trout RBCs [6, 13], and it has been suggested that these high basal levels of Mx may have contributed to the halted VHSV infection in RBCs [2, 13]. This has been suggested for other immune cell types as well, such as the rainbow trout spleen monocyte/macrophage (RTS11) cell line [75]. Further, it has been reported that the expression of Mx protein is augmented in response to IPNV exposure [6].

IFIT family proteins present motifs that can bind viral components to prevent or inhibit viral replication [73]. Recently, we detected a correlation between high IFIT5 expression levels in rainbow trout RBCs and a decline in VHSV replication early after VHSV exposure. Moreover, *ifit5* gene silencing increased VHSV replication in rainbow trout RBCs [8]. Therefore, these previous results in relation to Mx and IFIT5 proteins in RBCs *ex vivo* scenarios are corroborated by the present *in vivo* study.

The BD1 antimicrobial peptide has been previously reported to be induced by type I interferon [50], and *ex vivo* exposure of rainbow trout RBCs to VHSV increased BD1 expression [13]. Our results identified increased expression of BD1 bands of ~28 kDa by western blotting in VHSV-challenged PB-RBCs. This band size corresponds to a BD1 tetramer, as the monomeric protein is 7.1 kDa [13]. In this regard, it has been previously suggested that β-defensins could form an oligomer to exert antimicrobial activities [76].

In conclusion, we show how RBCs respond to VHSV infection *in vivo* by increasing the expression of genes and proteins related to viral RNA sensing, type I interferon response, ISG antiviral effectors, antigen processing and presentation of peptide antigen, and complement activation. RBCs are the major cell type in the blood, and understanding their contribution to the antiviral response can allow their use in the development of new prophylactic or therapeutic strategies for viral infections of aquacultured species.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1

Supplementary Table S1. Complete list of DEGs identified in the transcriptomic analysis of PB-RBCs from mock- and VHSV-challenged rainbow trout.

Supplementary Table S2. Complete list of DEGs identified in the transcriptomic analysis of HK-RBCs from VHSV-challenged rainbow trout.

Supplementary Table S3. List of GO Immune System Process terms identified in the transcriptomic analysis of PB-RBCs from VHSV-challenged rainbow trout using P <0.05, GO Term fusion, and GO Tree interval 3-8.

Supplementary Table S4. List of GO Immune System Process terms identified in the transcriptomic analysis of HK-RBCs from VHSV-challenged rainbow trout using P <0.1, GO Term fusion, and GO Tree interval 3-8.

Supplementary Table S5. Complete list of common DEGs from both PB-RBCs and HK-RBCs identified in the transcriptomic analysis from mock- and VHSV-challenged rainbow trout. THE Second sheet lists DEGs found exclusively in PB-RBCs identified in the transcriptomic analysis of mock- and VHSV-challenged rainbow trout.

Supplementary Table S6. List of GO Immune System Process terms identified in the transcriptomic analysis of common DEGs from both PB-RBCs and HK-RBCs from VHSV-challenged rainbow trout using P-value <0.05, GO Term fusion and GO Tree interval 3-8.

Supplementary Table S7. List of GO Immune System Process terms identified in the transcriptomic analysis of DEGs found exclusively in PB-RBCs from VHSV-challenged rainbow trout using P <0.05, GO Term fusion, and GO Tree interval 3-8.

Supplementary Table S8. Complete list of DEPs identified in PB-RBCs from VHSV-challenged rainbow trout. Listed proteins represent those with P-value <0.05, >2 PSMs, and a fold-change greater or less than 1.5.

Supplementary Table S9. List of GO Immune System Process and GO Biological Process terms identified in the proteomic analysis of PB-RBCs from VHSV-challenged rainbow trout using P-value <0.05, GO Term fusion, and GO Tree interval 3-8.

Author Contributions: Conceptualization, I.N. and M.O.; Methodology, I.N., S.P., M.O., S.C., and M.M; Validation, S.P., V.C. and M.S.; Formal analysis, I.N. , M.L., V.C., M.S., S.C., and M.M; Investigation, I.N., M.L., and M.O; Resources, L.M.; Data curation, I.N. and M.L.; Writing—original draft preparation, I.N. and M.O.; Writing—review and editing, I.N., L.P., J.C., and M.O.; Visualization, I.N., and M.O.; Supervision, M.O.; Project administration, M.O.; Funding acquisition, M.O.

Funding: This work was supported by the European Research Council (ERC Starting Grant GA639249).

Acknowledgments: The authors are thankful to Remedios Torres and Efren Lucas for their technical assistance. The proteomic analysis was performed in the Proteomics Facility of the Spanish National Center for Biotechnology (CNB-CSIC) belonging to ProteoRed, PRB3-ISCIII, supported by grant PT17/0019. We would like to thank Paulo Almeida (STABVida) for technical support.

Conflicts of Interest: The authors declare that the research was carried out in the absence of any personal, professional, or financial relationships that could potentially be construed as a conflict of interest.

References

1. Glomski, C.A., J. Tamburlin, and M. Chainani, *The phylogenetic odyssey of the erythrocyte. III. Fish, the lower vertebrate experience.* Histol Histopathol, 1992. **7**(3): p. 501-28.

2. Nombela, I. and M.D.M. Ortega-Villaizan, *Nucleated red blood cells: Immune cell mediators of the antiviral response.* PLoS Pathog, 2018. **14**(4): p. e1006910.

3. Dahle, M.K. and J.B. Jorgensen, *Antiviral defense in salmonids - Mission made possible?* Fish Shellfish Immunol, 2019. **87**: p. 421-437.

4. Morera, D., et al., *RNA-Seq reveals an integrated immune response in nucleated erythrocytes.* PLoS One, 2011. **6**(10): p. e26998.

5. Puente-Marin, S., et al., *Fish Red Blood Cells Modulate Immune Genes in Response to Bacterial Inclusion Bodies Made of TNFα and a G-VHSV Fragment.* Frontiers in Immunology, 2019. **10**(1055).

6. Nombela, I., et al., *Infectious pancreatic necrosis virus triggers antiviral immune response in rainbow trout red blood cells, despite not being infective.* F1000Res, 2017. **6**: p. 1968.

7. Puente-Marin, S., et al., *Rainbow Trout Erythrocytes ex vivo Transfection With a DNA Vaccine Encoding VHSV Glycoprotein G Induces an Antiviral Immune Response.* Front Immunol, 2018. **9**: p. 2477.

8. Chico, V., et al., *IFIT5 Participates in the Antiviral Mechanisms of Rainbow Trout Red Blood Cells.* Front Immunol, 2019. **10**: p. 613.

9. Dahle, M.K., et al., *Transcriptome analyses of Atlantic salmon (Salmo salar L.) erythrocytes infected with piscine orthoreovirus (PRV).* Fish Shellfish Immunol, 2015. **45**(2): p. 780-90.

10. Workenhe, S.T., et al., *Infectious salmon anaemia virus replication and induction of alpha interferon in Atlantic salmon erythrocytes.* Virol J, 2008. **5**: p. 36.

11. Nombela, I., 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).

12. Puente-Marin, S., et al., *In Silico Functional Networks Identified in Fish Nucleated Red Blood Cells by Means of Transcriptomic and Proteomic Profiling.* Genes (Basel), 2018. **9**(4).

13. Nombela, I., et al., *Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus.* F1000Res, 2017. **6**: p. 1958.

14. Chico, V., et al., *Shape-Shifted Red Blood Cells: A Novel Red Blood Cell Stage?* Cells, 2018. **7**(4).

15. Workenhe, S.T., et al., *Demonstration of infectious salmon anaemia virus (ISAV) endocytosis in erythrocytes of Atlantic salmon.* Virol J, 2007. **4**: p. 13.

16. Aamelfot, M., et al., *Expression of the infectious salmon anemia virus receptor on atlantic salmon endothelial cells correlates with the cell tropism of the virus.* J Virol, 2012. **86**(19): p. 10571-8.

17. Wessel, O., et al., *Piscine orthoreovirus (PRV) replicates in Atlantic salmon (Salmo salar L.) erythrocytes ex vivo.* Vet Res, 2015. **46**: p. 26.

18. Finstad, O.W., et al., *Piscine orthoreovirus (PRV) infects Atlantic salmon erythrocytes.* Vet Res, 2014. **45**: p. 35.

19. Jung, M.H., et al., *The Megalocytivirus RBIV Induces Apoptosis and MHC Class I Presentation in Rock Bream (Oplegnathus fasciatus) Red Blood Cells.* Front Immunol, 2019. **10**: p. 160.

20. Press, C.M. and Ø. Evensen, *The morphology of the immune system in teleost fishes.* Fish & Shellfish Immunology, 1999. **9**(4): p. 309-318.

21. Winton, J., et al., *Current lineages of the epithelioma papulosum cyprini (EPC) cell line are contaminated with fathead minnow, Pimephales promelas, cells.* J Fish Dis, 2010. **33**(8): p. 701-4.

22. Basurco, B.C., J., *Spanish isolates and reference strains of viral haemorrhagic septicaemia virus show similar protein size patterns.* Bull Eur Ass Fish Pathol., 1989. **9**(4): p. 92 - 5.

23. Bindea, G., et al., *ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks.* Bioinformatics, 2009. **25**(8): p. 1091-3.

24. Bindea, G., J. Galon, and B. Mlecnik, *CluePedia Cytoscape plugin: pathway insights using integrated experimental and in silico data.* Bioinformatics, 2013. **29**(5): p. 661-3.

25. Shannon, P., et al., *Cytoscape: a software environment for integrated models of biomolecular interaction networks.* Genome Res, 2003. **13**(11): p. 2498-504.

26. Gotz, S., et al., *High-throughput functional annotation and data mining with the Blast2GO suite.* Nucleic Acids Res, 2008. **36**(10): p. 3420-35.

27. Chico, V., et al., *Rapid detection and quantitation of viral hemorrhagic septicemia virus in experimentally challenged rainbow trout by real-time RT-PCR.* J Virol Methods, 2006. **132**(1-2): p. 154-9.

28. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.

29. Raida, M.K. and K. Buchmann, *Temperature-dependent expression of immune-relevant genes in rainbow trout following Yersinia ruckeri vaccination.* Dis Aquat Organ, 2007. **77**(1): p. 41-52.

30. Ortega-Villaizan, M., et al., *In vitro analysis of the factors contributing to the antiviral state induced by a plasmid encoding the viral haemorrhagic septicaemia virus glycoprotein G in transfected trout cells.* Vaccine, 2011. **29**(4): p. 737-43.

31. Chico, V., et al., *Pepscan mapping of viral hemorrhagic septicemia virus glycoprotein G major lineal determinants implicated in triggering host cell antiviral responses mediated by type I interferon.* J Virol, 2010. **84**(14): p. 7140-50.

32. Metsalu, T. and J. Vilo, *ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap.* Nucleic Acids Res, 2015. **43**(W1): p. W566-70.

33. Liu, S.Y., et al., *Interferon-inducible cholesterol-25-hydroxylase broadly inhibits viral entry by production of 25-hydroxycholesterol.* Immunity, 2013. **38**(1): p. 92-105.

34. Kim, B.H., et al., *IFN-inducible GTPases in host cell defense.* Cell Host Microbe, 2012. **12**(4): p. 432-44.

35. Tan, J., et al., *IFP35 is involved in the antiviral function of interferon by association with the viral tas transactivator of bovine foamy virus.* J Virol, 2008. **82**(9): p. 4275-83.

36. Zhang, B., et al., *IFIT5 potentiates anti-viral response through enhancing innate immune signaling pathways.* Acta Biochim Biophys Sin (Shanghai), 2013. **45**(10): p. 867-74.

37. Narayana, S.K., et al., *The Interferon-induced Transmembrane Proteins, IFITM1, IFITM2, and IFITM3 Inhibit Hepatitis C Virus Entry.* J Biol Chem, 2015. **290**(43): p. 25946-59.

38. Mudhasani, R., et al., *IFITM-2 and IFITM-3 but not IFITM-1 restrict Rift Valley fever virus.* J Virol, 2013. **87**(15): p. 8451-64.

39. Haller, O., P. Staeheli, and G. Kochs, *Interferon-induced Mx proteins in antiviral host defense.* Biochimie, 2007. **89**(6-7): p. 812-8.

40. Helbig, K.J. and M.R. Beard, *The role of viperin in the innate antiviral response.* J Mol Biol, 2014. **426**(6): p. 1210-9.

41. Gizzi, A.S., et al., *A naturally occurring antiviral ribonucleotide encoded by the human genome.* Nature, 2018. **558**(7711): p. 610-614.

663 42. Li, M., et al., *Roles of SAMHD1 in antiviral defense, autoimmunity and cancer.* Rev Med Virol,
664 2017. **27**(4).

665 43. Yu, Y., et al., *Fish TRIM16L exerts negative regulation on antiviral immune response against*
666 *grouper iridoviruses.* Fish Shellfish Immunol, 2016. **59**: p. 256-267.

667 44. Vaysburd, M., et al., *Intracellular antibody receptor TRIM21 prevents fatal viral infection.* Proc
668 Natl Acad Sci U S A, 2013. **110**(30): p. 12397-401.

669 45. Gack, M.U., et al., *TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated*
670 *antiviral activity.* Nature, 2007. **446**(7138): p. 916-920.

671 46. Gack, M.U., et al., *Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade*
672 *recognition by the host viral RNA sensor RIG-I.* Cell Host Microbe, 2009. **5**(5): p. 439-49.

673 47. Manokaran, G., et al., *Dengue subgenomic RNA binds TRIM25 to inhibit interferon expression*
674 *for epidemiological fitness.* Science, 2015. **350**(6257): p. 217-21.

675 48. Wang, W., et al., *Fish TRIM39 regulates cell cycle progression and exerts its antiviral function*
676 *against iridovirus and nodavirus.* Fish Shellfish Immunol, 2016. **50**: p. 1-10.

677 49. Wang, Y., et al., *Characterization and biological function analysis of the TRIM47 gene from*
678 *common carp (Cyprinus carpio).* Gene, 2017. **627**: p. 188-193.

679 50. Corleis, B., et al., *Early type I Interferon response induces upregulation of human beta-*
680 *defensin 1 during acute HIV-1 infection.* PLoS One, 2017. **12**(3): p. e0173161.

681 51. Ryan, L.K. and G. Diamond, *Modulation of Human beta-Defensin-1 Production by Viruses.*
682 Viruses, 2017. **9**(6).

683 52. Trobridge, G.D., P.P. Chiou, and J.A. Leong, *Cloning of the rainbow trout (Oncorhynchus*
684 *mykiss) Mx2 and Mx3 cDNAs and characterization of trout Mx protein expression in salmon cells.*
685 J Virol, 1997. **71**(7): p. 5304-11.

686 53. Said, E.A., et al., *Viruses Seen by Our Cells: The Role of Viral RNA Sensors.* J Immunol Res,
687 2018. **2018**: p. 9480497.

688 54. Sabbah, A., et al., *Activation of innate immune antiviral responses by Nod2.* Nat Immunol,
689 2009. **10**(10): p. 1073-80.

690 55. Moore, C.B., et al., *NLRX1 is a regulator of mitochondrial antiviral immunity.* Nature, 2008.
691 **451**(7178): p. 573-7. 56. Saito, T., et al., *Regulation of innate antiviral defenses through a shared*
692 *repressor domain in RIG-I and LGP2.* Proc Natl Acad Sci U S A, 2007. **104**(2): p. 582-7.

693 57. Satoh, T., et al., *LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral*
694 *responses.* Proc Natl Acad Sci U S A, 2010. **107**(4): p. 1512-7.

58. Rice, G.I., et al., *Gain-of-function mutations in IFIH1 cause a spectrum of human disease phenotypes associated with upregulated type I interferon signaling.* Nat Genet, 2014. **46**(5): p. 503-509.

59. Vazquez, C. and S.M. Horner, *MAVS Coordination of Antiviral Innate Immunity.* J Virol, 2015. **89**(14): p. 6974-7.

60. Ullah, M.O., et al., *TRIF-dependent TLR signaling, its functions in host defense and inflammation, and its potential as a therapeutic target.* J Leukoc Biol, 2016. **100**(1): p. 27-45.

61. Kawai, T. and S. Akira, *The roles of TLRs, RLRs and NLRs in pathogen recognition.* Int Immunol, 2009. **21**(4): p. 317-37.

62. Abderrazak, A., et al., *NLRP3 inflammasome: from a danger signal sensor to a regulatory node of oxidative stress and inflammatory diseases.* Redox Biol, 2015. **4**: p. 296-307.

63. da Costa, L.S., et al., *RNA viruses promote activation of the NLRP3 inflammasome through cytopathogenic effect-induced potassium efflux.* Cell Death Dis, 2019. **10**(5): p. 346.

64. Arenzana, N., S. Rodriguez de Cordoba, and J. Rey-Campos, *Expression of the human gene coding for the alpha-chain of C4b-binding protein, C4BPA, is controlled by an HNF1-dependent hepatic-specific promoter.* Biochem J, 1995. **308 (Pt 2)**: p. 613-21.

65. Ruiz-Arguelles, A. and L. Llorente, *The role of complement regulatory proteins (CD55 and CD59) in the pathogenesis of autoimmune hemocytopenias.* Autoimmun Rev, 2007. **6**(3): p. 155-61.

66. Nelson, R.A., Jr., *The immune-adherence phenomenon; an immunologically specific reaction between microorganisms and erythrocytes leading to enhanced phagocytosis.* Science, 1953. **118**(3077): p. 733-7.

67. Nelson, R.A., Jr., *The immune-adherence phenomenon; a hypothetical role of erythrocytes in defence against bacteria and viruses.* Proc R Soc Med, 1956. **49**(1): p. 55-8.

68. Mizuno, C.M., et al., *Numerous cultivated and uncultivated viruses encode ribosomal proteins.* Nat Commun, 2019. **10**(1): p. 752.

69. Dubois, J., O. Terrier, and M. Rosa-Calatrava, *Influenza viruses and mRNA splicing: doing more with less.* MBio, 2014. **5**(3): p. e00070-14.

70. Morais, A., et al., *The eukaryotic translation initiation factor 3 subunit L protein interacts with Flavivirus NS5 and may modulate yellow fever virus replication.* Virol J, 2013. **10**: p. 205.

71. Solis, M., et al., *Gene expression profiling of the host response to HIV-1 B, C, or A/E infection in monocyte-derived dendritic cells.* Virology, 2006. **352**(1): p. 86-99.

727 72. Robertsen, B., *Expression of interferon and interferon-induced genes in salmonids in*
728 *response to virus infection, interferon-inducing compounds and vaccination.* Fish Shellfish
729 Immunol, 2008. **25**(4): p. 351-7.

730 73. Fensterl, V. and G.C. Sen, *Interferon-induced Ifit proteins: their role in viral pathogenesis.* J
731 Virol, 2015. **89**(5): p. 2462-8.

732 74. Verhelst, J., P. Hulpiau, and X. Saelens, *Mx proteins: antiviral gatekeepers that restrain the*
733 *uninvited.* Microbiol Mol Biol Rev, 2013. **77**(4): p. 551-66.

734 75. Pham, P.H., et al., *Differential effects of viral hemorrhagic septicaemia virus (VHSV)*
735 *genotypes IVa and IVb on gill epithelial and spleen macrophage cell lines from rainbow trout*
736 *(Oncorhynchus mykiss).* Fish Shellfish Immunol, 2013. **34**(2): p. 632-40.

737 76. Bauer, F., et al., *Structure determination of human and murine beta-defensins reveals*
738 *structural conservation in the absence of significant sequence similarity.* Protein Sci, 2001.
739 **10**(12): p. 2470-9.

PUBLICACIÓN 5

TÍTULO: Nucleated red blood cells: Immune cell mediators of the antiviral response

AUTORES: Ivan Nombela y María del Mar Ortega-Villaizan

REVISTA: *PLOS Pathogens*

DOI: 10.1371/journal.ppat.1006910

PLOS | PATHOGENS

PEARLS

Nucleated red blood cells: Immune cell mediators of the antiviral response

Ivan Nombela, Maria del Mar Ortega-Villaizan*

IBMC, Miguel Hernández University, Elche, Spain

* mortega-villaizan@umh.es

OPEN ACCESS

Citation: Nombela I, Ortega-Villaizan MdM (2018) Nucleated red blood cells: Immune cell mediators of the antiviral response. PLoS Pathog 14(4): e1006910. https://doi.org/10.1371/journal.ppat.1006910

Editor: Katherine R. Spindler, University of Michigan Medical School, UNITED STATES

Published: April 26, 2018

Funding: This work was supported by ERC Starting Grant GA639249 to MdMOV. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript

Competing interests: The authors have declared that no competing interests exist.

Introduction

The involvement of nucleated red blood cells (RBCs) as immune response cell mediators is a novel topic of research. RBCs are the most abundant cell type in the bloodstream and are best known for their roles in gas exchange and respiration. In mammals, mature RBCs are flexible, oval, biconcave disks that lack cell nuclei, organelles, and ribosomes (reviewed in Moras et al. 2017 [1]). In nonmammalian vertebrates, RBCs are oval, flattened, biconvex disks with a cytoskeleton composed of a marginal band of microtubules and a cell nucleus and organelles in their cytoplasm [2], which allow them to de novo synthesize proteins and molecules in response to stress and stimuli. In the recent past, a set of biological processes related to immunity–such as phagocytosis [3], antigen presentation [3], and interleukin-like production [4–7]–have been reported in nucleated RBCs from different species. However, elucidating the role of RBCs during viral infections is an emergent research topic of great interest. Here, we provide a brief overview of the novel role of nucleated RBCs against viral infections.

Viral pathogen-associated molecular patterns (PAMPs) induce pattern-recognition receptor (PRR) signaling in nucleated RBCs

Nucleated RBCs are implicated in the immune response to viral infections based on their response to viral PAMPs through various PRR signaling pathways. Among these receptors, the expression of Toll-like receptor 3 (TLR3) and TLR9–which are endosomal TLRs that recognize viral double-stranded RNA (dsRNA) and nonmethylated viral 5'-C-phosphate-G-3' (CpG)-containing DNA, respectively–and retinoic acid-inducible gene I (RIG-I)–a member of the RIG-I-like receptor (RLR) family that interacts intracellularly with viral dsRNA–have been reported in rainbow trout RBCs [5, 8] and Atlantic salmon [9], respectively. Chicken RBCs constitutively express *tlr3* and *tlr21*, which is a homolog of mammalian TLR9 [5, 10]. Stimulation of these receptors with their corresponding PAMPs leads to the activation of signaling networks that induce the transcription of a set of genes, resulting in a characteristic immune response.

The activation of these receptors by viral pathogens induces expression of the interferon system [11, 12]. Stimulation of rainbow trout RBCs with polyinosinic:polycytidylic acid (poly I:C, a molecule structurally similar to dsRNA) induces the de novo synthesis of mRNAs from immune genes such as chemokine (C-C motif) ligand 4 (*ccl4*), interferon-α (*ifn-α*), and myxovirus resistance gene *(mx)* [5]; and in chickens, RBCs respond to poly I:C by upregulating type I IFN (*ifn1*) and interleukin-8 (*il-8*) genes [10]. Moreover, the infectious pancreatic necrosis virus (IPNV)–a dsRNA virus–has been reported to stimulate the expression of *tlr3*, *ifn1* and *mx* genes [13]. The piscine orthoreovirus (PRV) also increases the expression of *rig-I*, *mx*, and *ifn-α* genes in Atlantic salmon RBCs [6].

The roles that other members of the RLR family, such as melanoma differentiation-associated protein 5 (MDA5) or probable ATP-dependent RNA helicase DExH-box helicase 58 (LGP2), assume in RBCs are still unknown. In addition, we still do not know if RBCs express other PRRs that recognize viral genomic RNA, such as TLR7 or TLR8. While IFN1 is thought to play a similar role in mammalian and nonmammalian species and induce similar sets of genes [14], the extent of nucleated RBCs' involvement in the global organism IFN1 response and how RBCs' involvement influences defense against viral infections remain to be defined.

Nucleated RBCs may be capable of inducing an adaptive immune response

Nucleated RBCs are linked to the adaptive immune response. Major histocompatibility complex I (MHCI) plays a key role in the antigen presentation of intracellular pathogens, which initiates adaptive immunity mechanisms. MHCI is expressed on the surface of RBCs from rainbow trout [15], Atlantic salmon [6], African clawed frogs [16], and chickens [17]. However, to date, it has only been reported that PRV infection induces genes involved in antigen presentation via MHCI in salmon RBCs [6] and that poly I:C upregulates gene ontology (GO) categories related to antigen processing, antigen presentation, and MHCI receptor activity in rainbow trout RBCs [18].

Molecules bearing the immunoreceptor tyrosine-based activation motif (ITAM), which is contained in certain transmembrane proteins of the immune system and is important for signal transduction in immune cells, are known markers of hematopoietic and immune cells [19]. ITAM-bearing molecules are expressed on rainbow trout RBCs [20]. Further, Epstein–Barr virus G-protein-coupled receptor 2 (EBI2) plays a critical role in the regulation of T cell–dependent antibody responses and provides a mechanism to balance short- versus long-term antibody responses [21]. EBI2 is highly expressed in rainbow trout young RBCs [22]. The presence of these molecules in nucleated RBCs may indicate a role of these cells in the adaptive immune response. However, the function of these molecules on RBCs and their effect on the antiviral adaptive immune response remain to be studied.

Nucleated RBCs trigger diverse immune responses against viral aggression

Three viruses from different families that infect or replicate inside nucleated RBCs have been identified: (i) infectious salmonid anemia virus (ISAV) from the Orthomyxoviridae family with single-stranded RNA (ssRNA) [7], (ii) PRV from the Reoviridae family with dsRNA [6, 23], and (iii) erythrocytic viral infections, reviewed in Paperna and Alves de Matos [24]. Fig 1 schematically summarizes the response of nucleated RBCs to these viruses. Unfortunately, information on the immune response of RBCs to erythrocytic viral infections is not available.

A study of nucleated RBCs from ISAV-infected Atlantic salmon first demonstrated the ability of RBCs to induce an immunological response against a viral pathogen. This response was characterized by the induction of *ifn-α* in hemagglutinated RBCs [7]. Recently, it has been shown that PRV also can induce the expression of *ifn-α*–in addition to *mx*, protein kinase RNA-activated (*pkr*) [6], *viperin*, and interferon-stimulated gene 15 (*isg15*) [25] antiviral genes–in PRV-challenged Atlantic salmon RBCs.

Recently, Nombela and colleagues demonstrated that nucleated RBCs can generate immune responses to viruses despite not being infected. Rainbow trout RBCs are nonpermissive to viral hemorrhagic septicemia virus (VHSV) [26] and infectious pancreatic necrosis virus (IPNV) infections [13], likely due to the inability of VHSV and IPNV to replicate in ex vivo purified rainbow trout RBCs. This phenomenon is known as nonproductive or abortive infection in nonpermissive cells and occurs when a virus enters a host cell and some or all viral components are synthesized but nonproductive or defective viruses are ultimately released because

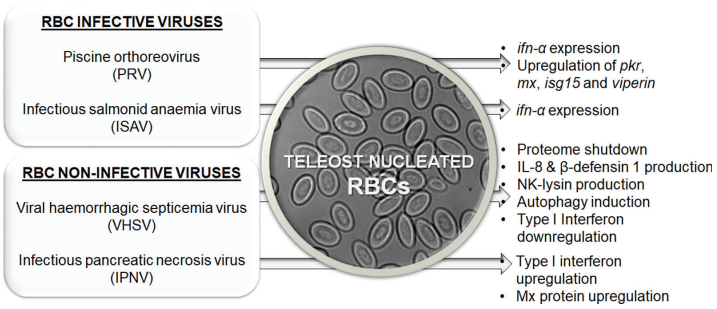


Fig 1. Schematic representation of teleost nucleated RBC immune responses against different infective (target: RBCs) or noninfective (target: other cell types) viral pathogens. *ifn-α*, interferon-α; IL-8, interleukin-8; *isg15*, interferon-stimulated gene 15; *mx*, myxovirus resistance gene; *pkr*, protein kinase RNA-activated; RBC, red blood cell.

https://doi.org/10.1371/journal.ppat.1006910.g001

the host cell is nonpermissive or inhibits the replication of the virus. Previously, abortive infection in a macrophage cell line was linked with the constitutive expression of the antiviral Mx protein by macrophages [27]. Similarly, high levels of constitutive Mx transcripts and protein have been identified in rainbow trout RBCs (Fig 2), suggesting a possible mechanism for aborted or halted infections in RBCs [13, 26]. Nevertheless, rainbow trout RBCs can develop diverse immune responses to VHSV halted replication, a process characterized by global proteome downregulation–mainly of proteins from the proteasome and RNA stability processes–increased expression of IL-8 and β-defensin 1, decreased expression of genes related to the IFN1 pathway, and an antioxidant response [13]. In the case of IPNV aborted infection in rainbow trout RBCs, there was an increase in the expression of *ifn1*, *mx*, interferon regulatory factor 7 (*irf7*), and *pkr* genes, followed by upregulation of Mx protein expression [13] (summarized in Fig 1).

Considering their ability to produce immune proteins related to interferon, pro-inflammatory cytokines, antimicrobial peptides, proteasome [26], and autophagy [28] pathways, nucleated RBCs likely are able to trigger an immune response similar to that of their leukocyte counterparts by activating diverse immune mechanisms to complement the protection against infection conferred to the host organism.

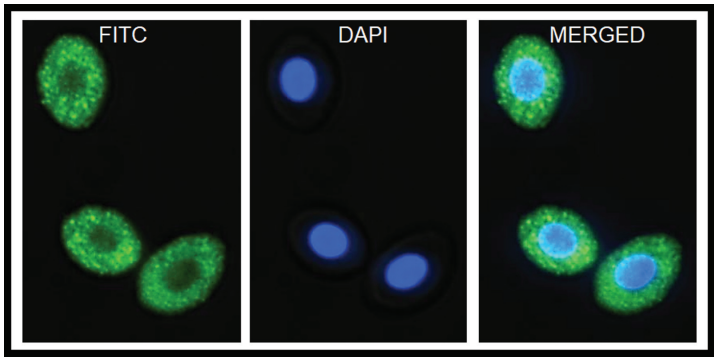


Fig 2. Constitutive expression of Mx antiviral protein in rainbow trout nucleated RBCs. Immunofluorescence images of Mx protein expression in nucleated RBCs. FITC: Mx protein expression; DAPI: nuclei. Images were obtained using an INCell Analyzer 6000 Cell imaging system (GE Healthcare, Little Chalfont, United Kingdom). DAPI, 4',6-diamidino-2-phenylindole; FITC, Fluorescein-5-isothiocyanate; Mx, myxovirus resistance gene; RBC, red blood cells.

https://doi.org/10.1371/journal.ppat.1006910.g002

Nucleated RBCs can mount immune responses against nonviral pathogens

The RBCs of mammalian and nonmammalian vertebrates are hosts for approximately 40 genera, including protists, prokaryotes, and viruses [18, 29]. Few blood infections of fish, amphibians, reptiles, and birds have proven pathogenicity, in contrast to the many known intraerythrocytic mammalian pathogens [29]. To date, few studies have reported diverse immune responses of nucleated RBCs to this broad spectrum of pathogens.

As previously described, the immune response against viruses is generally associated with the expression of IFN1 and ISGs. In response to bacterial lipopolysaccharide (LPS), rainbow trout RBCs upregulate the expression of tumor necrosis factor receptor-like (*tnfr-like*), oxidative-stress response 1 (*oxsr1*), *irf1*, and *mhcI* genes. Several reports have shown that hemoglobin, the most abundant protein of RBCs, has antibacterial activity and can elicit antimicrobial activity through reactive oxygen species production when under pathogen attack [30]. In rainbow trout, acid-soluble extracts from RBCs showed antibacterial activity against a variety of bacteria, including *Planococcus citreus* and *Escherichia coli* [31]. In the presence of the fungus *Candida albicans*, rainbow trout [3], and chickens [4], RBCs performed innate immunity functions, using phagocytosis to bind and engulf *C. albicans* and present to macrophages. Ultimately, little is known regarding the immune response triggered by nucleated RBCs against the broad range of pathogens that infect them.

Nucleated RBCs are future targets for vaccines

Human non-nucleated RBCs have long been investigated for the transportation of drugs or antigens through the blood [32, 33]. Proteomic studies of human [34] and nonhuman primate species [35] aim to further characterize the biology of human RBCs and identify future targets for newer-generation vaccines, especially against malaria. Because of the ability of nucleated RBCs to generate and modulate immune responses, development of a new generation of vaccines targeting membrane receptors or intracellular molecules of nucleated RBCs capable of triggering and stimulating the antiviral immune response is a promising and exciting field. Such vaccines may contribute greatly to organism survival, given the large volume of RBCs and their fast distribution through the organism. However, additional proteomic studies of nucleated RBCs are needed to identify potential therapeutic targets.

Acknowledgments

Special thanks are due to Prof. Henny Gevers for helping with English editing. We would also like to thank the two anonymous reviewers for their valuable comments and suggestions.

References

1. Moras M., Lefevre S.D., and Ostuni M.A., *From Erythroblasts to Mature Red Blood Cells: Organelle Clearance in Mammals*. Front Physiol, 2017. 8: p. 1076. https://doi.org/10.3389/fphys.2017.01076 PMID: 29311991
2. Glomski C.A., Tamburlin J., and Chainani M., *The phylogenetic odyssey of the erythrocyte. III. Fish, the lower vertebrate experience*. Histol Histopathol, 1992. 7(3): p. 501–28. PMID: 1504472
3. Passantino L, et al., *Fish immunology. I. Binding and engulfment of Candida albicans by erythrocytes of rainbow trout (Salmo gairdneri Richardson)*. Immunopharmacol Immunotoxicol, 2002. 24(4): p. 665–78. https://doi.org/10.1081/IPH-120016050 PMID: 12510797
4. Passantino L, et al., *Antigenically activated avian erythrocytes release cytokine-like factors: a conserved phylogenetic function discovered in fish*. Immunopharmacol Immunotoxicol, 2007. 29(1): p. 141–52. https://doi.org/10.1080/08923970701284664 PMID: 17464774
5. Morera D, et al., *RNA-Seq reveals an integrated immune response in nucleated erythrocytes*. PLoS ONE, 2011. 6(10): p. e26998. https://doi.org/10.1371/journal.pone.0026998 PMID: 22046430

6. Dahle M.K, et al., *Transcriptome analyses of Atlantic salmon (Salmo salar L.) erythrocytes infected with piscine orthoreovirus (PRV)*. Fish Shellfish Immunol, 2015. 45(2): p. 780–90. https://doi.org/10.1016/j.fsi.2015.05.049 PMID: 26057463
7. Workenhe S.T, et al., *Infectious salmon anaemia virus replication and induction of alpha interferon in Atlantic salmon erythrocytes*. Virol J, 2008. 5: p. 36. https://doi.org/10.1186/1743-422X-5-36 PMID: 18307775
8. Rodriguez M.F, et al., *Characterization of Toll-like receptor 3 gene in rainbow trout (Oncorhynchus mykiss)*. Immunogenetics, 2005. 57(7): p. 510–9. https://doi.org/10.1007/s00251-005-0013-1 PMID: 16086174
9. Wessel O, et al., *Piscine orthoreovirus (PRV) replicates in Atlantic salmon (Salmo salar L.) erythrocytes ex vivo*. Vet Res, 2015. 46: p. 26. https://doi.org/10.1186/s13567-015-0154-7 PMID: 25888832
10. St Paul M, et al., *Chicken erythrocytes respond to Toll-like receptor ligands by up-regulating cytokine transcripts*. Res Vet Sci, 2013. 95(1): p. 87–91. https://doi.org/10.1016/j.rvsc.2013.01.024 PMID: 23433682
11. Robertsen B, *The interferon system of teleost fish*. Fish Shellfish Immunol, 2006. 20(2): p. 172–91. https://doi.org/10.1016/j.fsi.2005.01.010 PMID: 15939626
12. Zou J, Bird S, and Secombes C, *Antiviral sensing in teleost fish*. Curr Pharm Des, 2010. 16(38): p. 4185–93. PMID: 21184657
13. Nombela I, et al., *Infectious pancreatic necrosis virus triggers antiviral immune response in rainbow trout red blood cells, despite not being infective*. F1000Research, 2017. 6: p. 1968. https://doi.org/10.12688/f1000research.12994.2 PMID: 29333244
14. Schultz U, Kaspers B, and Staeheli P, *The interferon system of non-mammalian vertebrates*. Dev Comp Immunol, 2004. 28(5): p. 499–508. https://doi.org/10.1016/j.dci.2003.09.009 PMID: 15062646
15. Sarder M.R, et al., *The MHC class I linkage group is a major determinant in the in vivo rejection of allogeneic erythrocytes in rainbow trout (Oncorhynchus mykiss)*. Immunogenetics, 2003. 55(5): p. 315–24. https://doi.org/10.1007/s00251-003-0587-4 PMID: 12879308
16. Nedelkovska H, et al., *Effective RNAi-mediated beta2-microglobulin loss of function by transgenesis in Xenopus laevis*. Biol Open, 2013. 2(3): p. 335–42. https://doi.org/10.1242/bio.20133483 PMID: 23519478
17. Delany M.E, et al., *Cellular expression of MHC glycoproteins on erythrocytes from normal and aneuploid chickens*. Dev Comp Immunol, 1987. 11(3): p. 613–25. PMID: 3678561
18. Morera D and MacKenzie S.A, *Is there a direct role for erythrocytes in the immune response*? Vet Res, 2011. 42(1): p. 89.
19. Humphrey M.B, Lanier L.L, and Nakamura M.C, *Role of ITAM-containing adapter proteins and their receptors in the immune system and bone*. Immunol Rev, 2005. 208: p. 50–65. https://doi.org/10.1111/j.0105-2896.2005.00325.x PMID: 16313340
20. Ohashi K, et al., *A molecule in teleost fish, related with human MHC-encoded G6F, has a cytoplasmic tail with ITAM and marks the surface of thrombocytes and in some fishes also of erythrocytes*. Immunogenetics, 2010. 62(8): p. 543–59. https://doi.org/10.1007/s00251-010-0460-1 PMID: 20614118
21. Gatto D, et al., *Guidance of B cells by the orphan G protein-coupled receptor EBI2 shapes humoral immune responses*. Immunity, 2009. 31(2): p. 259–69. https://doi.org/10.1016/j.immuni.2009.06.016 PMID: 19615922
22. Gotting M and Nikinmaa M.J, *Transcriptomic Analysis of Young and Old Erythrocytes of Fish*. Front Physiol, 2017. 8: p. 1046. https://doi.org/10.3389/fphys.2017.01046 PMID: 29311976
23. Finstad O.W, et al., *Piscine orthoreovirus (PRV) infects Atlantic salmon erythrocytes*. Vet Res, 2014. 45: p. 35. https://doi.org/10.1186/1297-9716-45-35 PMID: 24694042
24. Paperna I and Alves de Matos A.P, *Erythrocytic viral infections of lizards and frogs: new hosts, geographical locations and description of the infection process*. Annales de Parasitologie Humaine et Comparée, 2016. 68(1): p. 11–23.
25. Haatveit H.M, et al., *Viral Protein Kinetics of Piscine Orthoreovirus Infection in Atlantic Salmon Blood Cells*. Viruses, 2017. 9(3): p. 49.
26. Nombela I, et al., *Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus*. F1000Research, 2017. 6: p. 1958. https://doi.org/10.12688/f1000research.12985.2 PMID: 29527292
27. Pham P.H, et al., *Differential effects of viral hemorrhagic septicaemia virus (VHSV) genotypes IVa and IVb on gill epithelial and spleen macrophage cell lines from rainbow trout (Oncorhynchus mykiss)*. Fish Shellfish Immunol, 2013. 34(2): p. 632–40. https://doi.org/10.1016/j.fsi.2012.12.004 PMID: 23257204

28. Pereiro P, et al., *Nucleated Teleost Erythrocytes Play an Nk-Lysin- and Autophagy-Dependent Role in Antiviral Immunity*. Frontiers in Immunology, 2017. 8: p. 1458. https://doi.org/10.3389/fimmu.2017.01458 PMID: 29163526

29. Davies A.J and Johnston M.R, *The biology of some intraerythrocytic parasites of fishes, amphibia and reptiles*. Adv Parasitol, 2000. 45: p. 1–107. PMID: 10751939

30. Jiang N, et al., *Respiratory protein-generated reactive oxygen species as an antimicrobial strategy*. Nat Immunol, 2007. 8(10): p. 1114–22. https://doi.org/10.1038/ni1501 PMID: 17721536

31. Fernandes J.M and Smith V.J, *Partial purification of antibacterial proteinaceous factors from erythrocytes of Oncorhynchus mykiss*. Fish Shellfish Immunol, 2004. 16(1): p. 1–9. PMID: 14675829

32. Cremel M, et al., *Red blood cells as innovative antigen carrier to induce specific immune tolerance*. Int J Pharm, 2013. 443(1–2): p. 39–49. https://doi.org/10.1016/j.ijpharm.2012.12.044 PMID: 23305866

33. Muzykantov V.R, *Drug delivery by red blood cells: vascular carriers designed by mother nature*. Expert Opin Drug Deliv, 2010. 7(4): p. 403–27. https://doi.org/10.1517/17425241003610633 PMID: 20192900

34. D'Alessandro A and Zolla L, *Proteomic analysis of red blood cells and the potential for the clinic: what have we learned so far*? Expert Rev Proteomics, 2017. 14(3): p. 243–252. https://doi.org/10.1080/14789450.2017.1291347 PMID: 28162022

35. Moreno-Perez D.A, et al., *The Aotus nancymaae erythrocyte proteome and its importance for biomedical research*. J Proteomics, 2017. 152: p. 131–137. https://doi.org/10.1016/j.jprot.2016.10.018 PMID: 27989940