LOS ERITROCITOS NUCLEADOS DE PECES EN LA INMUNIZACIÓN FRENTE A RHABDOVIRUS

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Tesis Doctoral

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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.

Este trabajo se ha desarrollado dentro del marco del proyecto titulado "*The Crosstalk Between Red and White Blood Cells: The case of fish*", referencia: GA639249, acrónimo: BloodCellsCrosstalk, en el Instituto de Investigación, Desarrollo e Innovación en Biotecnología Sanitaria de Elche (IDiBE), Universidad Miguel Hernández de Elche (UMH).

La Dra. **María del Mar Ortega-Villaizán Romo**, Profesora Ayudante Doctora del Departamento de Bioquímica y Biología Molecular de la Universidad Miguel Hernández (UMH) e Investigadora del Instituto de Investigación, Desarrollo e Innovación en Biotecnología Sanitaria (IDiBE); y el Dr. **Julio Coll Morales**, Profesor de Investigación del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

CERTIFICAN QUE:

El trabajo de investigación titulado **"LOS ERITROCITOS NUCLEADOS DE PECES EN LA INMUNIZACIÓN FRENTE A RHABDOVIRUS"**, presentado por **Sara Puente Marín** para optar al grado de Doctor, ha sido realizado bajo su dirección en el Instituto de Investigación, Desarrollo e Innovación en Biotecnología Sanitaria (IDiBE) de la Universidad Miguel Hernández de Elche. Considerando que esta tesis se halla concluida, **AUTORIZAN** su presentación para que pueda ser juzgada por el tribunal correspondiente.

Para que conste a los efectos oportunos, firman el presente certificado en Elche, a 14 de Junio de 2019.

Fdo.: Dra. María del Mar Ortega-Villaizán Romo

Fdo.: Dr. Julio Coll Morales

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 (IDiBE) de la Universidad Miguel Hernández de Elche.

DA SU CONFORMIDAD a la lectura de la tesis doctoral titulada "Los eritrocitos nucleados de peces en la inmunización frente a rhabdovirus", presentada por Sara Puente Marín.

Y para que así conste a los efectos oportunos, firma el presente certificado en Elche, a de Junio de 2019.

Fdo.: Dr. Ricardo Mallavia Marín

ABREVIATURAS

APCs	Células presentadoras de antígeno
CMC	Citotoxicidad mediada por células
DEGs	Genes expresados diferencialmente
DEPs	Proteínas expresadas diferencialmente
DNA	Ácido desoxirribonucleico
DNAc	Ácido desoxirribonucleico complementario
ELISA	Ensayo de inmunoabsorción ligado a enzima
FACS	Clasificación de células activadas por fluorescencia
frg16-GVHSV	Fragmento 16 de GVHSV
FSC	Dispersión frontal
GVHSV	Glicoproteína G de VHSV
HK-RBCs	Eritrocitos de riñón anterior
IB	Cuerpos de inclusión
$IB^{frg16G\text{-}VHSV}$	Cuerpos de inclusión del fragmento 16 de GVHSV
IB^{iRFP}	Cuerpos de inclusión de la proteína iRFP
$IB^{TNF\alpha}$	Cuerpos de inclusión de TNFα
IFN1	Interferón tipo 1
IHNV	Virus de la necrosis hematopoyética infecciosa
im	Intramuscular
IPNV	Virus de la Necrosis Pancreática Infecciosa
iRFP	Proteína fluorescente roja no inmunogénica
ISG	Genes estimulados por interferón
iv	Intravenoso
MC	Medio condicionado
MHCI	Complejo mayor de histocompatibilidad de clase I
MHCII	Complejo mayor de histocompatibilidad de clase II
mTFP1	Proteína fluorescente cian
Mx	Proteína de resistencia a myxovirus inducida por interferón
PAMPs	Patrones moleculares asociados a patógenos
PB-RBCs	Eritrocitos de sangre periférica
pmTFP1	Plásmido que codifica la proteína mTFP1
pmTFP1GVHSV	Plásmido que codifica la proteína GVHSV y mTFP1
PRV	Orthoreovirus acuático
qPCR	Reacción en cadena de la polimerasa cuantitativa
RBCs	Eritrocitos o células rojas de la sangre
RNA	Ácido ribonucleico
RTG-2	Línea celular de gónada de trucha arcoíris
RT-HKM	Macrófagos del riñón anterior de trucha arcoíris
RTS11	Línea celular de monocito/macrófago de trucha arcoíris
SAV	Alfavirus de los salmónidos
SSC	Dispersión lateral
TNFα	Factor de necrosis tumoral alfa
VHSV	Virus de la septicemia hemorrágica viral
WBCs	Leucocitos o células blancas de la sangre
ZFL	Línea celular de células del hígado de pez cebra

ÍNDICE

RESUMEN

ABSTRACT

Aquaculture is the farming of aquatic organisms in order to increase their yield and production. It is listed as one of the fastest growing food production sectors. The intense aquaculture developed in recent years favors the susceptibility to pathogens. Rhabdoviruses are responsible for great losses in aquaculture. Viruses of the *Rhabdoviridae* family and their hosts have been extensively studied in order to develop prophylactic measures against viral infection. Among these measures, vaccination has been the most effective and DNA vaccines, developed in recent years, the most promising type in aquaculture. However, the extrapolation of effective DNA vaccines against a broad range of pathogens has been unsatisfactory. Therefore, more research is necessary for the development of effective vaccines against farmed fish pathogens.

In this context, come into view the erythrocytes (RBCs) of fish. These nucleated cells have been barely investigated until recently. It was believed that their function was merely the transport of oxygen through the body. In the last decade it has been described that RBCs are able to internalize pathogens and release cytokines against stimuli, giving them other functions yet unknown. In this thesis, we intend to elucidate the role of these cells in the immunization of fish with a DNA vaccine against a rhabdovirus, the viral hemorrhagic septicemia virus (VHSV), and its potential use in the design of strategies for the development of new effective vaccines against rhabdoviruses. To achieve this goal, we analyzed the transcriptomic and proteomic profile of rainbow trout RBCs transfected with a DNA vaccine that encodes the glycoprotein G gene of VHSV (GVHSV), as well as of RBCs from individuals after immunization with GVHSV. The purification method of the RBCs by fluorescence-activated cell sorting (FACS) and the transcriptomic and proteomic analysis process for these cells were developed during this thesis. The results showed that rainbow trout RBCs overexpress genes and proteins related to the presentation of antigens, the interferon system, and signalling with other cells, among others. We also analyzed the crosstalk capacity of the RBCs transfected with GVHSV with other cell types. Finally, the study was extended to the evaluation of the immune response of the RBCs to recombinant subunit vaccines. All of this with the final objective of evaluating the potential use of RBCs as transporters or cell targets of DNA vaccines and/or immunostimulants in the prophylaxis of fish.

RESUMEN

La acuicultura es el cultivo de organismos acuáticos con el fin de aumentar su rendimiento y producción. Es catalogado como uno de los sectores de producción de alimentos de más rápido crecimiento. La intensa acuicultura desarrollada en los últimos años, favorece la susceptibilidad a patógenos. Los rhabdovirus se encuentran entre los virus que causan mayores pérdidas en acuicultura. Los virus de la familia *Rhabdoviridae* y sus hospedadores han sido ampliamente estudiados con el fin de desarrollar medidas profilácticas frente a las infecciones virales. Entre estas medidas, la vacunación ha resultado la más efectiva y las vacunas DNA, desarrolladas en los últimos años, las más prometedoras en acuicultura. Sin embargo la extrapolación de las vacunas DNA efectivas frente a algunos virus a otros virus patógenos ha resultado insatisfactoria. Por ello es necesaria más investigación para el desarrollo de vacunas eficaces frente a patógenos en acuicultura.

En este contexto, aparecen los eritrocitos (RBCs, del inglés "Red blood cells") de peces. Estas células nucleadas han sido poco investigadas ya que hasta hace poco se creía que su función era meramente el transporte de oxígeno en el organismo. En la última década se ha descrito que los RBCs son capaces de internalizar patógenos y liberar citoquinas frente a estímulos, otorgándoles otras funciones, hasta hace poco desconocidas. En esta tesis, pretendemos dilucidar el papel de estas células en la inmunización de peces con una vacuna DNA frente a un rhabdovirus, el virus de la septicemia hemorrágica viral (VHSV), y su posible utilización en el diseño de estrategias para el desarrollo de nuevas vacunas eficaces frente a rhabdovirus. Para ello, analizamos el perfil de transcriptómica y proteómica de RBCs de trucha arcoíris transfectados con una vacuna DNA que codifica el gen de la glicoproteína G de VHSV (GVHSV), así como de RBCs tras la inmunización de individuos con GVHSV. El método de purificación de los RBCs mediante clasificación de células activadas por fluorescencia (FACS) y el proceso de análisis de transcriptómica y proteómica para estas células fueron puestos a punto durante el desarrollo de esta tesis. Los resultados mostraron que los RBCs de trucha sobreexpresan genes y proteínas relacionados con la presentación de antígenos, el sistema de interferón y la comunicación con otras células, entre otros. También analizamos la capacidad de comunicación de los RBCs transfectados con GVHSV con otros tipos celulares. Finalmente, el estudio se extendió a la evaluación de la respuesta inmune de los RBCs a las vacunas basadas en proteínas recombinantes. Todo ello con el fin de evaluar el uso potencial de los RBCs como transportadores o dianas de vacunas DNA y/o inmunoestimulantes en la profilaxis de peces.

IDENTIFICACIÓN DEL PROBLEMA

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La acuicultura es el cultivo de organismos acuáticos, tanto de agua dulce como agua salada, con el fin de aumentar su rendimiento y producción. Es catalogado como uno de los sectores de producción de alimentos de más rápido crecimiento, si no el qué más. La acuicultura representa el 47% de la producción mundial de especies acuáticas y el 53% de la producción destinada al consumo humano según datos de 2018 de la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO). La FAO cataloga al sector de la pesca y la acuicultura como fundamental en la consecución del objetivo de un mundo sin hambre ni malnutrición [1].

La acuicultura intensiva, desarrollada en los últimos años, crea condiciones que favorecen la continua aparición de patógenos. El desplazamiento de especies de su hábitat natural, el hacinamiento, la poca diversidad de las especies cultivadas y el cambio en la dieta provoca un ambiente de mayor susceptibilidad a nuevos patógenos, más facilidad para la replicación de patógenos, la transmisión de enfermedades y una respuesta inmune comprometida por parte del hospedador. Esto hace de la acuicultura un sector muy vulnerable a la aparición de enfermedades [2-4].

Entre los patógenos que causan importantes pérdidas tanto en peces salvajes como cultivados, de agua dulce y salada, se encuentran los virus de la familia *Rhabdoviridae.* Debido a su impacto económico, estos virus y sus hospedadores han sido ampliamente estudiados [5]. Actualmente, la solución frente a una infección en acuicultura consiste en la eliminación de los peces infectados, evitar el contacto con áreas y/o peces no infectados, y en algunos casos la vacunación [6]. La vacunación con ácido desoxirribonucleico (DNA) es una de las tecnologías más recientes utilizadas en la profilaxis frente a virus de peces. La vacunación DNA frente a rhabdovirus ha demostrado su efectividad frente al virus de la septicemia hemorrágica viral (VHSV) [7], el virus de la necrosis hematopoyética infecciosa (IHNV) [8] y el alfavirus de salmónidos (SAV) [9]. La primera vacuna DNA aprobada para su uso y comercialización en acuicultura, en Canadá, fue la vacuna frente a IHNV, Apex-IHN ® (Novartis Aqua Health) [10]. Recientemente, otra vacuna DNA frente a SAV, Clynav® (Elanco) ha sido autorizada para su uso en la Unión Europea [11]. Sin embargo, aunque muchos aspectos relacionados con la comercialización de las vacunas DNA necesitan ser resueltos, la realidad es que todavía no se han desarrollado vacunas DNA eficaces frente a la mayoría de los virus de peces [6]. Las razones del porqué las vacunas DNA no son eficaces frente a algunos virus se desconoce. Por tanto, es necesaria más información acerca de la funcionalidad del sistema inmune de los peces, la inmunidad desarrollada por las vacunas DNA y por los virus y sus antígenos y comprender las interacciones patógeno-hospedador para desarrollar vacunas DNA más efectivas.

En los últimos años, los eritrocitos (RBCs, del inglés “red blood cells”) nucleados, característicos de vertebrados no mamíferos como los teleósteos, se han postulado como células con características inmunes. Sin embargo, todavía se desconoce su papel y participación en el sistema inmunitario de los peces. Dilucidar este aspecto nos permitiría un mejor entendimiento del funcionamiento del sistema inmune de los peces y desarrollar estrategias de profilaxis para acuicultura más efectivas.

OBJETIVOS

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El objetivo global de esta tesis consiste en dilucidar el papel de los RBCs nucleados de teleósteos en la inmunización frente a rhabdovirus. Para ello, se han utilizado dos modelos de trabajo, con el fin de investigar:

1. El papel de los RBCs de trucha arcoíris en la inmunización con una vacuna DNA que codifica la glicoproteína G de VHSV (GVHSV). Para ello, los objetivos específicos de este punto son:

i) analizar la expresión de la vacuna DNA en RBCs

ii) evaluar la respuesta de los RBCs a la vacuna DNA *in vitro* e *in vivo*

iii) evaluar la señalización/comunicación de los RBCs con otros tipos celulares

2. El papel de los RBCs de trucha arcoíris en la inmunización con cuerpos de inclusión de origen bacteriano hechos de proteína recombinante. Para ello, los objetivos específicos de este punto son:

i) analizar la endocitosis/fagocitosis de los cuerpos de inclusión por los RBCs

ii) evaluar la respuesta de los RBCs a la proteína recombinante

La motivación de esta tesis consiste en estudiar la función de los RBCs de teleósteos en la respuesta inmune del organismo a la inmunización frente a infecciones virales, con el objetivo final de contribuir con nuestros resultados a la búsqueda de tratamientos profilácticos más efectivos en acuicultura.

INTRODUCCIÓN

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1. LOS RBCs NUCLEADOS DE PECES

Los eritrocitos (RBCs, del inglés "Red blood cells") de peces suponen entre el 98-99% del total de las células de la sangre, según la especie [12]. Presentan forma elipsoidal, con una longitud entre 10-15 µm [12] y su vida media es de entre 80-500 días [13]. A diferencia de los mamíferos, los RBCs de peces poseen núcleo, mitocondria y otros orgánulos en el citoplasma [12] (Figura 1) y contienen hemoglobina tetramérica como otros vertebrados [14]. El principal órgano eritropoyético en peces teleósteos es el riñón anterior mientras que el bazo actúa como reservorio de RBCs [12].

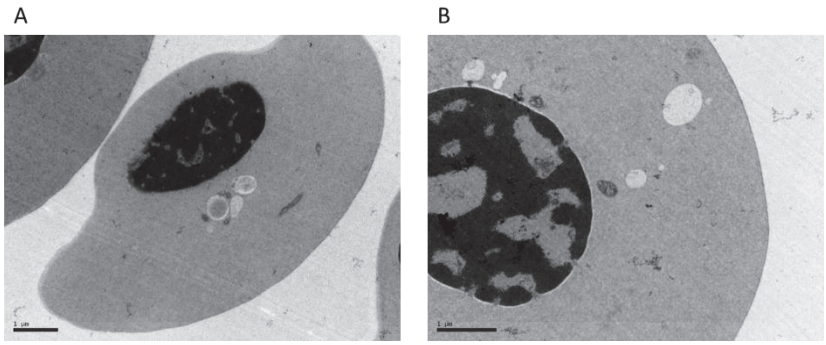

Figura 1: Micrografías de RBCs de trucha arcoíris por microscopía electrónica de transmisión. A) 12k× y B) 20k× de aumento.

La eritropoyesis en peces es similar a la de otros vertebrados y consta de las siguientes etapas: célula madre hematopoyética, célula madre mieloide, proeritroblasto, eritroblasto basófilo, eritroblasto policromático, eritroblasto ortocromático, eritrocito joven y finalmente el eritrocito maduro (Figura 2). El proeritroblasto se caracteriza por un núcleo de gran tamaño que ocupa la mayor parte de la célula. A medida que se produce la maduración la célula y el núcleo van adquiriendo su forma oval, el citoplasma va aumentando su tamaño y su acidez, y se condensa la cromatina en el núcleo [15]. Mientras que los primeros estadíos de la maduración tienen lugar en el órgano eritropoyético, la maduración final tiene lugar ya en el torrente sanguíneo, por lo que es probable encontrar precursores eritrocíticos en la sangre de peces [12].

Los RBCs de peces, al igual que los de otros vertebrados, tienen como función principal y más conocida el transporte e intercambio de oxígeno por el organismo. Además, ha sido descrita la participación de los RBCs de mamíferos en otras funciones secundarias como la inflamación, trombosis, coagulación y cicatrización de heridas [16, 17] así como en la modulación de la proliferación y supervivencia de células T [18-20].

En los últimos años los RBCs nucleados se han postulado como células multifuncionales, ya que a parte de las funciones ya conocidas, se ha observado su participación en la respuesta inmune del organismo frente a infecciones por hongos [21], bacterias [22, 23] y virus [24, 25].

Los RBCs en la respuesta inmune innata

La respuesta inmune innata es la primera línea de defensa tras la exposición a un patógeno. Los RBCs nucleados reconocen patrones moleculares asociados a patógenos (PAMPs) mediante los receptores de reconocimiento de patrones (PRRs), lo que les permite una respuesta frente a patógenos. Entre estos receptores, los receptores tipo Toll y la proteína de reconocimiento de peptidoglicanos han sido identificados en los RBCs nucleados [22, 26]. El reconocimiento de los PAMPs por parte de los PRRs desencadena la señalización que estimula genes característicos de la respuesta inmune innata, como la expresión del sistema de Interferón tipo 1 (IFN1) [27], producción de citoquinas [13, 22, 28, 29] y péptidos antimicrobianos [30] (Figura 3).

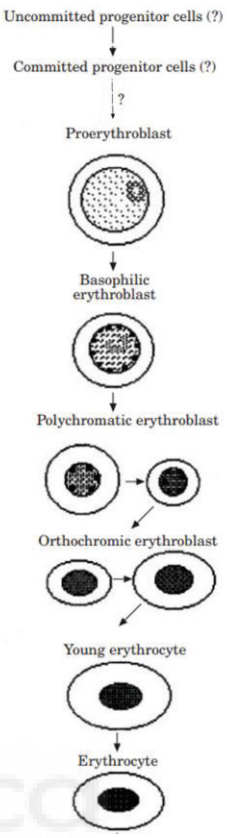

Figura 2: Morfogénesis de los eritrocitos de pez gato. Figura modificada de Fijan *et al.* [15].

El sistema de interferón juega un papel similar en vertebrados mamíferos y no-mamíferos [31]. La unión de IFN1 a sus receptores celulares desencadena la transcripción de los genes estimulados por interferón (ISGs), como por ejemplo la proteína de resistencia a myxovirus inducida por interferón (Mx), 2'-5' oligoadenilato sintetasa (OAS), la familia de proteínas con motivos tripartitos (TRIM), el gen estimulado por interferón 15 (ISG15), adenosina desaminasa que actúa sobre el ácido ribonucleico (RNA) (ADAR), proteínas inducidas por interferón con repeticiones de tetratricopéptidos (IFIT) y la viperina entre otros [32]. Los RBCs nucleados de trucha [22] y de pollo [26] sobreexpresan genes del sistema IFN1 en presencia de ácido polinosínico-policitidílico (poly(I:C)), un PAMP análogo del RNA de doble cadena que mimetiza el RNA viral (Figura 3). También, se ha descrito la replicación del virus de la anemia infecciosa de salmón (ISAV) [24] y el orthoreovirus acuático (PRV) [25] en RBCs de salmónidos y la expresión de IFN1 y sus ISGs por parte de los RBCs en respuesta a la infección. Recientemente, se ha descrito la respuesta inmune llevada a cabo por los RBCs de trucha arcoíris frente a la exposición a virus que no infectan RBCs como son el VHSV [33, 34] y el virus de la necrosis pancreática infecciosa (IPNV) [35]. Por todo ello, se ha propuesto a los RBCs nucleados de peces como células inmunes mediadoras de la respuesta antiviral [36].

Las citoquinas son proteínas involucradas en la regulación del sistema inmune innato y adaptativo. Tras el estímulo con diferentes PAMPs, los RBCs nucleados han demostrado activar la producción de genes y proteínas como la quimioquina ligando 4 (CCL4), un quimioatrayente de células inmunes [22], interleuquina 8 (IL8) [26, 33] e interleuquina 1 beta (IL1β) [33] (Figura 3). Además se ha visto que los RBCs nucleados son capaces de comunicarse con otros tipos celulares y estimular la expresión de citoquinas en estas células [33, 37].

Los péptidos antimicrobianos están presentes en todos los organismos vivos, y constituyen la primera línea de defensa frente a patógenos. La hemoglobina es la proteína más abundante en los RBCs y constituye una fuente de péptidos antimicrobianos que participan en el sistema inmune innato [38]. Su actividad antimicrobiana frente a microorganismos es uno de los

mecanismos antimicrobianos más antiguos [39, 40]. La hemoglobina libera radicales libres que destruyen la membrana y paredes de los patógenos [41]. Recientemente, se ha descrito que los RBCs de peces sobreexpresan la β-defensina 1 (BD1), un conocido péptido antimicrobiano quimiotáctico de células inmunes [33], y la NK-lisina, un ortólogo de la granulisina de humanos [34], en respuesta a la exposición al VHSV. Otro péptido antimicrobiano, la hepcidina, ha sido encontrado en RBCs de peces [33], aunque su papel todavía se desconoce. Fernandes *et al.* purificaron factores proteicos con actividad antibacteriana de RBCs de trucha arcoíris [42]. Por otro lado, se ha descrito que las histonas de RBCs de pollo ejercen actividad antimicrobiana frente a bacterias [43-45]. Así pues, los péptidos antimicrobianos de los RBCs contribuyen a la defensa del organismo frente a patógenos y respaldan la importancia de los RBCs en el sistema inmune innato (Figura 3).

El sistema de complemento es un sistema de proteínas plasmáticas que debe su nombre a su función de complementar y ayudar en el reconocimiento de patógenos, así como a la opsonización y lisis [46]. El sistema de complemento es un lazo de unión entre la inmunidad innata y la inmunidad adaptativa [47]. Los RBCs están en constante contacto con las proteínas plasmáticas del complemento y se ha descrito que los RBCs de trucha arcoíris son capaces de unirse a inmunocomplejos opsonizados, debido a la presencia de receptores de la membrana que actúan para eliminarlos de la circulación [48] (Figura 3).

Los RBCs en la repuesta inmune adaptativa

El sistema inmune adaptativo se caracteriza por un sistema de células especializadas que se encargan de una respuesta específica frente al patógeno. Está compuesto por dos elementos: la respuesta humoral, mediada por anticuerpos, y la respuesta celular, mediada por células T.

Todas las células nucleadas expresan moléculas del complejo mayor de histocompatibilidad (MHC) de clase I (MHCI) y por tanto, tendrían la capacidad de presentar antígenos en la membrana [49]. Los RBCs de vertebrados no mamíferos (peces [50], aves [51], reptiles [52] y anfibios [53]) como células nucleadas que son, expresan moléculas de MHCI en su membrana. Se ha descrito que la infección por PRV induce la sobreexpresión de moléculas de MHCI en RBCs de salmón atlántico [25], al igual que el tratamiento de RBCs de trucha arcoíris con poly(I:C) [13] y el estrés por calor [54]. También, se ha descrito que los RBCs de trucha arcoíris son capaces de endocitar patógenos y formar rosetas para la presentación y eliminación de patógenos por parte de los macrófagos [21]. Además, los RBCs de humanos han sido implicados en la diferenciación y supervivencia de células T [18-20, 55].

Por otro lado, el MHC de clase II (MHCII), es característico de las células presentadoras de antígeno (APCs), como son principalmente las células dendríticas, los macrófagos y los linfocitos B [56]. Sin embargo, se ha descrito la expresión de MHCII en RBCs de pollo [26] y de trucha arcoíris [57]. La presencia de MHCII en RBCs nucleados podría suponer la capacidad de éstas células de presentar antígenos y actuar como células APC atípicas (Figura 3). El concepto de células APC atípicas o no profesionales ha sido recientemente definido [56]. Entre estas células se incluyen los mastocitos, basófilos, eosinófilos, células linfoides innatas [56] y neutrófilos [58].

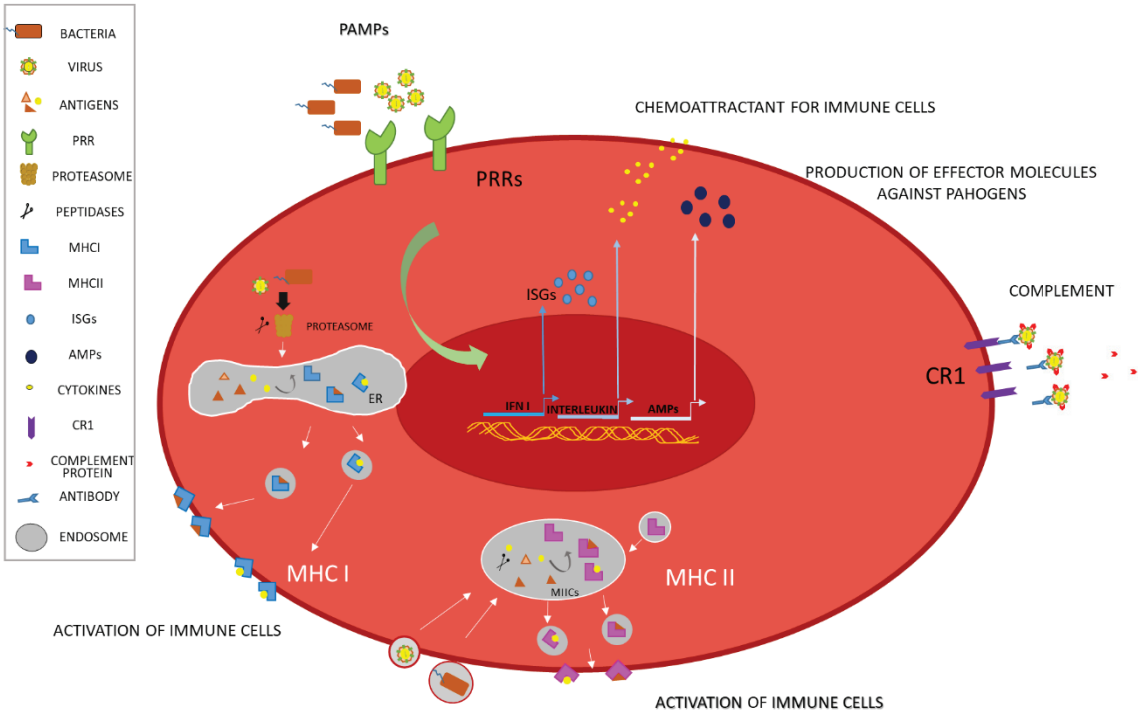

Figura 3: Resumen de la respuesta inmune de los RBCs nucleados según las funciones y moléculas descritas hasta la fecha [59].

2. CÉLULAS PRESENTADORAS DE ANTÍGENO COMO CÉLULAS DIANA DE VACUNAS

Las estrategias de vacunación pasan por una buena estimulación de las APCs, lo cual desencadena una respuesta inmune innata y adaptativa más eficaz. Recientemente se han llevado a cabo investigaciones sobre cómo dirigir las vacunas a moléculas de superficie de APCs para producir respuestas inmunes más específicas [60]. Las células dendríticas, las APCs por excelencia, capturan y procesan antígenos en la sangre y tejidos para presentarlos a los linfocitos T [61]. La transfección de células dendríticas con vacunas DNA basadas en antígenos virales y tumorales *ex vivo* y la posterior inmunización de los individuos con estas células ha mostrado resultados prometedores [62, 63]. Sin embargo, el reto es dirigir estas vacunas a APCs *in vivo*, una forma más directa y eficaz de inmunización. Por ejemplo, se ha descrito cómo la inmunización de ratones con una vacuna DNA tumoral encapsulada en liposomas y dirigida a células dendríticas induce una respuesta inmune antitumoral de memoria y de larga duración [64]. Por otro lado, Zaneti *et al.* mejoraron la inmunogenicidad de una vacuna DNA frente al virus del dengue dirigiendo la vacuna a células dendríticas *in vivo,* induciendo una respuesta humoral y celular [65]. Andersen *et al.* dirigieron una vacuna DNA, basada en la hemaglutinina del virus influenza, hacia la molécula MHCII y hacia receptores de quimioquinas presentes en APCs. Los resultados mostraron un incremento de los niveles de anticuerpos neutralizantes y de células T citotóxicas respecto a la vacuna no dirigida además de un aumento de la protección frente al desafío letal por el virus [66].

Las APCs se han identificado como las principales efectoras en la configuración de la respuesta inmune tras la vacunación con DNA [67]. Tras la inyección intramuscular (im) de la vacuna DNA, se ha encontrado la presencia de la vacuna DNA en APCs en el sitio de inyección. Las APC expresan el transgén, simulando una infección intracelular, y lo presentan vía MHCI a las células T. Además, las APCs también pueden endocitar el antígeno liberado por otras células transfectadas como podrían ser los miocitos, las principales células transfectadas tras la inyección im de una vacuna DNA [67]. Al igual que ocurre en mamíferos, entre las APCs de peces se han identificado células dendríticas [68], macrófagos [69] y células B [70]. Recientemente se ha identificado un subconjunto de células dendríticas especializadas, que llevan a cabo presentación cruzada de antígenos, en las agallas de teleósteos [71], un lugar donde también se observa estimulación del sistema inmune tras la vacunación [72]. Al igual que en mamíferos, la estrategia de dirigir la vacunación en peces a las APCs supone un reto. En cuanto a la profilaxis en acuicultura, uno de los grandes retos y objetivos consiste en la encapsulación de antígenos para la administración oral de vacunas, de manera que la vacunación en peces sea un proceso menos laborioso para el personal de la piscifactoría y menos estresante para los peces. Estas vacunas, administradas oralmente, tienen como célula diana los enterocitos del intestino de peces. Se ha visto que estas células de la membrana epitelial intestinal son capaces de captar eficientemente el antígeno, aunque el rápido recambio del epitelio intestinal supone la temprana pérdida del antígeno a menos que sea transferido a leucocitos y macrófagos intraepiteliales de manera eficiente [73]. Por ello, la búsqueda de nuevas dianas celulares y plataformas seguras de vacunas en acuicultura para lograr una protección eficaz y de larga duración es un tema de investigación de gran interés.

3. LOS RBCs COMO PLATAFORMAS DE VACUNAS/AGENTES TERAPÉUTICOS

La idea de utilizar los RBCs como plataforma de fármacos o antígenos ya ha sido previamente estudiada en RBCs no nucleados. Dado el elevado número de RBCs presentes en el organismo y su amplia difusión por todo el cuerpo, la posibilidad de utilizar los RBCs nucleados para el suministro de agentes terapéuticos y profilácticos es prometedora. Aunque es necesaria la optimización de aspectos como la farmacocinética, la estabilidad y la liberación de los agentes terapéuticos/vacunas así como el efecto en las diferentes poblaciones celulares, hay una ventaja que hace de los RBCs plataformas únicas en el transporte de fármacos o antígenos: el efecto sistémico en la circulación gracias a la vida media de los RBCs. Este efecto se lograría sin la necesidad de dirigir el fármaco o vacuna a una población concreta por lo que resulta ser una estrategia relativamente sencilla y una característica única de estas células [74]. Además el uso de los RBCs como plataformas de vacunas o inmunoestimulantes evita los problemas de seguridad relacionados con vectores virales o bacterianos [75-77].

Un paso más allá consistiría en dirigir el tratamiento a una población celular concreta para un tratamiento más específico. En los últimos años, se han llevado a cabo estudios de la biología de RBCs de humanos [78] y de primates [79] con el fin de identificar futuras diana moleculares en RBCs para una nueva generación de vacunas, especialmente para enfermedades tan importantes como la malaria. La manipulación *ex vivo* de los RBCs, las limitaciones de compatibilidad entre donadores/receptores y las infecciones de origen sanguíneo suponen inconvenientes para llevar a cabo esta estrategia (aunque las transfusiones de sangre y de productos sanguíneos están actualmente muy extendidas en la práctica médica, y se desarrollan con bastante seguridad). Como alternativa para evitar los inconvenientes mencionados anteriormente, cobran gran importancia las estrategias que pasan por dirigir fármacos hacia las moléculas de superficie de los RBCs para su adhesión o su internalización por parte de los RBCs *in vivo*. Esta supondría una técnica más directa que mejoraría su aplicabilidad en medicina. Resulta especialmente atractivo el diseño de complejos RBCs-fármacos para la administración intravascular, que controlen la respuesta inmune, aspectos patológicos de la homeostasis y eliminen patógenos y toxinas del torrente sanguíneo [74].

Por otro lado, se ha descrito la encapsulación de proteínas en RBCs de humanos con el fin de evaluar su potencial aplicabilidad como portadores de vacunas [80, 81]. Los RBCs han demostrado ser prometedores como plataforma de vacunas provocando respuestas humorales comparables o superiores a las obtenidas por la vía convencional de vacunación subcutánea en ratones [75]. También se ha testado a los RBCs como portadores de antígenos para inducir una tolerancia inmune específica que evite la producción por parte del organismo de anticuerpos específicos anti-fármacos tras la repetida administración de proteínas terapéuticas [82, 83]. Recientemente, Xiaoqi *et al.,* modificaron los RBCs de ratón anclando en la superficie celular moléculas características de APCs de manera que los RBCs actuaban como APCs artificiales, demostrando ser capaces de activar y cebar células T y promover la secreción de citoquinas inflamatorias [84].

4. LOS RHABDOVIRUS EN ACUICULTURA

Los rhabdovirus son una familia de virus que infectan tanto especies de animales como de plantas. En el caso de los peces, los rhabdovirus pueden causar graves enfermedades tanto en peces silvestres como cultivados [85].

Los rhabdovirus pertenecen al orden de los *Mononegavirales*, a la familia *Rhabdoviridae* [86]. El tamaño del virión presenta 100-430 nm de longitud y 45-100 nm de diámetro. Presentan un genoma RNA monocatenario negativo, de 11-16 kb de longitud que codifica para 5 proteínas estructurales. La nucleoproteína N (47-32 kDa) es el componente principal de la nucleocápsida y se encarga de regular la transcripción y replicación del genoma del virus. La fosfoproteína P (20-30 kDa) actúa como mediador durante el proceso de transcripción y replicación. La proteína de la matriz M (20-30 kDa) regula la transcripción del genoma RNA y se une a la nucleocápsida y a la glicoproteína G facilitando el proceso de salida de los viriones. La glicoproteína G (65-90 kDa) es una proteína transmembrana de superficie glicosilada de 500 aminoácidos [87]. Forma espículas en la superficie del virión en forma de homotrímeros y permite la unión y endocitosis en las células que infecta. Es la proteína más antigénica y es la única que induce protección frente a una infección con virus virulento [88]. Además provoca inmunidad mediada por células e induce la producción de anticuerpos neutralizantes [85]. Aparte, existen proteínas accesorias, no estructurales, como la proteína NV ("non-virion"), la cual es característica de algunos rhabdovirus que se engloban dentro del género *Novirhabdovirus*. Dentro de este género de virus infectivos para peces se encuentra el VHSV, uno de los rhabdovirus mejor estudiados en peces. El VHSV se encuentra entre las enfermedades de declaración obligatoria listadas por la Organización Mundial de Sanidad Animal (OIE) http://www.oie.int/es/sanidad-animal-en-el-mundo/oie-listed-diseases-2019/. Es el responsable de la septicemia hemorrágica viral de los peces, que provoca una alta mortalidad (puede llegar al 100% en alevines) [89]. Se clasifica en cuatro genotipos diferentes, distribuidos por las zonas templadas del hemisferio norte. Infecta tanto peces de agua dulce como salada, silvestres y cultivados. Hasta la fecha se ha aislado de aproximadamente 80 especies de teleósteos, siendo la trucha arcoíris (*Onchorynchus mykiss*) una de las especies más susceptibles y la septicemia hemorrágica viral una de las enfermedades más graves en acuicultura [89].

Con estos antecedentes, en esta tesis establecemos el VHSV y la trucha arcoíris como modelo de trabajo para dilucidar el papel de los RBCs nucleados de peces en la inmunización frente a rhabdovirus.

5. PROFILAXIS EN ACUICULTURA

A medida que la industria de la acuicultura crece, también crece la necesidad de buscar nuevas medidas profilácticas frente a patógenos de peces. La profilaxis en acuicultura abarca medidas como la higiene en las instalaciones y en la manipulación, la desinfección del material utilizado, asegurar la calidad del agua y de la alimentación, el uso de desinfectantes, antiparasitarios y antibióticos, la inmunoestimulación y la vacunación [90].

El uso masivo de antibióticos en acuicultura, muchos sin restricciones y no biodegradables, como tratamiento de infecciones bacterianas y también como medida de prevención, principalmente en países en vías de desarrollo, se ha convertido en un problema para el medioambiente y la salud humana. El uso indiscriminado de antibióticos provoca la aparición de cepas bacterianas resistentes en peces, la transferencia de resistencias a otras especies de animales incluidos los humanos, alteraciones en la flora bacteriana del agua y sedimentos y la aparición de antibióticos residuales en peces de consumo y sus productos derivados [91].

En acuicultura, no existen tratamientos frente a patógenos de origen viral aprobados. La única medida para luchar contra las enfermedades virales es la prevención.

La mayoría de las vacunas frente a virus utilizadas en acuicultura están basadas en virus inactivados/muertos [92, 93]. Por lo general estas vacunas han resultado ser poco inmunogénicas, aunque sí han demostrado ser eficaces, generalmente, si se administran mediante inyección, necesitando grandes dosis para lograr protección, por lo que su administración a gran escala resulta poco rentable [92]. Las vacunas basadas en virus atenuados han demostrado buenos resultados en cuanto a protección y rentabilidad sin embargo su uso es limitado por razones de seguridad [92]. Actualmente existen solo dos vacunas disponibles comercialmente basadas en virus atenuados [93].

Otro tipo de vacunas, también disponibles comercialmente, son las vacunas de subunidades, basadas en proteínas recombinantes de antígenos virales. Aunque son seguras, su efectividad depende de su forma de administración y de que asegure la estabilidad del antígeno [93]. Por ello, se sigue investigando sobre nuevas formas de administración de estas vacunas. Recientemente, ha aumentado el interés por el diseño y la producción de un nuevo formato de subunidades de vacunas, partículas que simulan virus (VLP), para su aplicación en acuicultura [93, 94]. Sin embargo, cuando se compara la efectividad de estas vacunas de subunidades basadas en la glicoproteína G de rhabdovirus con vacunas DNA estas últimas resultan mucho más eficaces [92].

5.1. LAS VACUNAS DNA

Desde la aparición del concepto de vacuna DNA, este tipo de vacuna se perfila como un agente profiláctico y terapéutico para un numeroso tipo de enfermedades y hospedadores. Actualmente, se investiga sobre vacunas DNA con aplicaciones frente a enfermedades infecciosas de tipo bacteriano, vírico o parasitario, frente a cáncer, alergias, enfermedades autoinmunes y como terapia génica [95, 96]. Hasta el momento, solo seis vacunas DNA han sido aprobadas, todas para uso veterinario (Tabla 1).

Animal	Frente a:	País de aprobación	Año de aprobación	Referencia
Cóndor californiano	Virus del Nilo occidental (WNV)	Estados Unidos	2003	[97]
Caballos			2005	[98]
Salmónidos	Virus de la necrosis hematopoyética infecciosa (IHNV)	Canadá	2005	[10]
Cerdas de cría	Terapia génica: hormona de crecimiento	Australia	2008	[99]
Perros	Anti-tumoral: Melanoma	Estados Unidos	2010	[97]
Salmón Atlántico	Alfavirus de salmónidos (SAV)	Europa	2016	[11]
Pollos	Influenza aviar (H5N1)	Estados Unidos	2017	[100]

Tabla 1: Tabla de vacunas DNA aprobadas para uso.

Las vacunas DNA consisten en un vector o plásmido en el que se inserta el gen de interés que codifica proteínas antigénicas o péptidos. El plásmido contiene un promotor y un terminador que flanquean el gen de interés para asegurar su correcta transcripción. Además, los plásmidos contienen un origen de replicación bacteriano y un gen de resistencia a antibiótico para su producción previa en bacterias (Figura 4).

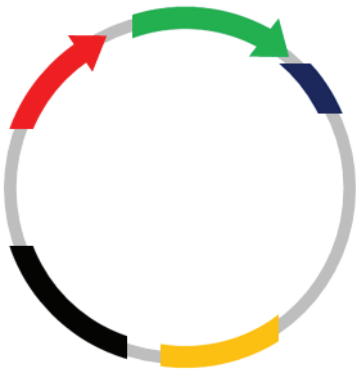

Figura 4: Representación esquemática de una vacuna DNA. Plásmido de doble cadena con el promotor de expresión eucariota (rojo), el gen de interés (verde), el terminador (azul), gen de resistencia a antibiótico (amarillo) y origen de replicación bacteriano (negro).

Las vacunas DNA presentan diversas ventajas respecto a las vacunas convencionales [6, 101]:

- capacidad de inducir inmunidad humoral y celular
- no hay riesgo de desarrollar la enfermedad al no usar patógenos atenuados
- confieren protección a largo plazo
- posibilidad de introducir más de un gen
- son eficaces a dosis más bajas
- son muy estables
- son relativamente sencillas de producir a gran escala y bajo coste

Y también desventajas [96]:

- pueden desarrollar autoinmunidad
- existe la posibilidad de su integración en el genoma de la célula
- suelen contener promotores de origen vírico que dificultan su comercialización
- pueden generar resistencias a antibióticos debido al gen de resistencia a antibiótico del plásmido
- ocasionan destrucción de tejido debido a respuestas citotóxicas
- se desconoce el impacto o persistencia en el medioambiente
- son más eficaces cuando se administran intramuscularmente, lo que aplicado a acuicultura resulta en un proceso muy laborioso y estresante para los peces

Vacunas DNA frente a rhabdovirus de peces

Las enfermedades virales en acuicultura provocadas por rhabdovirus causan grandes pérdidas económicas para el sector. Las vacunas DNA frente a rhabdovirus de peces han mostrado ser las más exitosas hasta la fecha [102]. La vacunación DNA basada en la glicoproteína G de los rhabdovirus [IHNV, VHSV, el virus de la viremia primaveral de la carpa (SVCV) y el virus del rabdovirus Hirame (HIRRV)] ha mostrado su eficacia frente a diferentes especies de peces [103]. Además, la vacunación DNA con la glicoproteína G de los rhabdovirus provoca una respuesta inmune temprana e inespecífica que protege frente a virus heterólogos [104-106]. Las vacunas DNA frente a VHSV e IHNV han resultado ser las más efectivas en la lucha frente a infecciones por rhabdovirus en acuicultura, mostrando una rápida y larga duración de la protección cuando se administran intramuscularmente [107, 108], que es la ruta de administración que mejores resultados ha dado hasta la fecha [67]. Sin embargo las vacunas DNA frente a otros tipos de patógenos en peces no han resultado tan efectivas [109].

Respuesta inmune innata en peces inducida por vacunas DNA

Tras la infección con virus se desarrolla una primera respuesta inmune inespecífica mediada por IFN1 e ISGs [110]. De la misma manera, esta primera repuesta mediada por el sistema de IFN1 también se desarrolla después de la inmunización con vacunas DNA frente a virus [111, 112]. La inducción del sistema de IFN1 y sus ISGs provoca un estado antiviral, activando células natural killer (NK), promoviendo la diferenciación de células T citotóxicas frente al virus y evitando la

propagación del virus a células vecinas [113, 114]. Entre los ISGs mejor caracterizados en peces se encuentra la proteína Mx. La proteína Mx es una GTPasa inducida por interferón con actividad antiviral frente a virus RNA que impide la replicación y ensamblaje de los viriones [115]. Se ha demostrado la participación de Mx en la respuesta inmune desencadenada por la infección por VHSV [110, 116, 117], así como después de la inmunización con una vacuna DNA frente a VHSV [111, 118]. Además, la expresión del gen *mx* se ha correlacionado con la protección inespecífica temprana generada por la vacuna DNA [108]. Esta respuesta inespecífica innata protege al pez durante un corto periodo de tiempo, y es de vital importancia para desencadenar la respuesta inmune específica, que dará lugar a una protección de larga duración [117]. La magnitud y especificidad de la respuesta de las células del sistema inmune innato conforman la subsecuente respuesta inmune adaptativa [119]. Por tanto, las estrategias de vacunación deben inducir una respuesta inmune innata efectiva que lleve a la generación de una respuesta inmune adaptativa que genere una protección duradera.

Respuesta inmune adaptativa en peces inducida por vacunas DNA

Después de una primera respuesta inmune inespecífica, se desarrolla una respuesta especifica frente al virus vacunado que puede durar hasta dos años después de la vacunación [120]. La vacunación DNA con la glicoproteína G pone de manifiesto la importancia de los anticuerpos neutralizantes frente al virus en esta protección a largo plazo [7, 103]. Sin embargo, McLauchlan et al. [108] detectaron anticuerpos neutralizantes hasta 6 meses después de la vacunación con GVHSV mientras que los peces resultaron protegidos durante 9 meses. Los autores sugirieron que o bien el título de anticuerpos neutralizantes estaba por debajo de los límites de detección u otros anticuerpos no neutralizantes estaban implicados en la protección. Se ha sugerido que la neutralización de rhabdovirus es dependiente del sistema de complemento, activando una ruta muy similar a la vía clásica de activación del complemento en mamíferos [121]. Aunque en teleósteos han sido identificados casi todos los componentes ortólogos del complemento presentes en mamíferos, todavía se desconoce su implicación en el sistema inmune y en la neutralización de virus [122].

En teleósteos se han identificado tres isotipos de inmunoglobulinas: IgM, producida en células B que responden a la estimulación antigénica en tejidos sistémicos [123], IgD, una inmunoglobulina con menos prevalencia que la IgM, pero altamente conservada entre especies que puede ser específica de tejidos [124] e IgT, producida por células B en la inmunidad relacionada con las mucosas [125]. A parte de su participación en la inmunidad humoral, las células B de teleósteos presentan actividad fagocítica y antimicrobiana, por lo que también presentan un papel importante en la inmunidad innata de teleósteos [126].

Existen otros mecanismos implicados en la protección generada por vacunas DNA. Cuesta y Tafalla [127] observaron que la vacunación DNA induce un estado de memoria en los peces que estimula la respuesta no específica en el posterior encuentro con el virus. Por otro lado, se ha visto una respuesta específica tras la vacunación con DNA sin detectarse anticuerpos neutralizantes [7, 103, 118]. Aquí entra en juego la citotoxicidad mediada por células (CMC), que puede ser específica o no específica. Se han identificado una gran cantidad de genes asociados a células T, los cuales se han visto sobreexpresados en respuesta a la infección por rhabdovirus

o a la vacunación con DNA [112, 128]. Existe una relación inversamente proporcional entre la actividad citotóxica y la carga viral. Las células citotóxicas son responsables de la reducción de la replicación viral [129]. Los linfocitos T citotóxicos reconocen y matan las células que presentan antígenos en su superficie a través de MHCI, mediante el receptor de células T (TCR) y la molécula CD8. Este sistema es similar al que encontramos en humanos [130]. Utke *et al*, observaron que leucocitos de sangre periférica de trucha arcoíris exhibían una respuesta CMC específica frente a células infectadas que presentaban un MHCI compatible [131]. De la misma manera, leucocitos de sangre periférica de truchas inmunizadas con una vacuna DNA frente a VHSV atacaron a células infectadas con VHSV pero no con IHNV, sugiriendo una respuesta de CMC específica de antígeno [114]. Esto pone de manifiesto la importancia de la respuesta CMC en peces.

Sin embargo, todavía se desconocen muchos de los mecanismos involucrados en la vacunación DNA, lo que complica la extrapolación de las vacunas DNA al resto de virus. Por ello, se sigue investigando en la búsqueda de nuevas vacunas DNA para mejorar su protección, ruta de administración, mejorar sus secuencias promotoras, su administración junto con coadyuvantes y el entendimiento de la inmunidad de peces para crear vacunas DNA más efectivas, seguras y comercializables.

5.2. VACUNAS BASADAS EN PROTEÍNAS RECOMBINANTES: CUERPOS DE INCLUSIÓN

Las vacunas basadas en patógenos inactivados o antígenos recombinantes, aun siendo más seguras que las vacunas basadas en patógenos atenuados, generalmente resultan menos inmunogénicas y/o no confieren protección por sí mismas [132]. Con el objetivo de subsanar este problema, se comenzó hace unas décadas a explorar la posibilidad de la coadministración de las vacunas con inmunoestimulantes o coadyuvantes, por ejemplo: emulsiones oleosas, nano/micropartículas, sales de aluminio, complejos inmunoestimulantes (ISCOMs), beta-glucanos, poly(I:C), saponinas, lipopeptidasas, flagelina, motivos CpG y citoquinas [132, 133]. Aunque las combinaciones y resultados de estos inmunoestimulantes son muy variables, se sigue considerando una estrategia prometedora. En el caso de las vacunas basadas en proteínas recombinantes, aparte de la necesidad de adyuvantes que potencien el sistema inmune, es imprescindible conseguir mantener la estabilidad y la funcionalidad de las proteínas *in vivo*. Por ello, se sigue investigando en busca de nuevas formulaciones que aseguren mantener las propiedades antigénicas o inmunoestimulantes de estas proteínas e incluso conseguir la administración oral de estas vacunas, el método más deseable en la inmunización de peces y uno de los mayores retos de la acuicultura [73]. En este contexto aparecen los cuerpos de inclusión bacterianos (IBs, del inglés “inclusion bodies”) como una alternativa prometedora.

Los IBs son agregados celulares (50-700 nm) de proteína recombinante, y presentan moléculas remanentes de la célula de producción [134]. Las características que hacen de los IBs excelentes candidatos para la administración de proteínas en peces son las siguientes:

- son seguros, no hay riesgo de integración de DNA en el hospedador, de invasión o reversión
- no son tóxicos [134]
- su origen bacteriano les confiere un fuerte poder inmunoestimulante [135].
- son estables, resisten condiciones fisicoquímicas extremas de pH y temperatura, y liofilización, manteniendo la funcionalidad [136, 137].
- su producción es escalable y rentable (no requiere de la purificación de la proteína recombinante) [136]
- pueden ser diseñados a la carta: para un amplio rango de moléculas frente a diferentes tratamientos [138]
- permiten la administración oral (aumenta la practicidad y disminuye el estrés de los peces) [136]
- permiten la liberación paulatina de las moléculas a dosis bajas [139]

IBs hechos de citoquinas y péptidos virales ya han sido testados en peces. IBs provenientes de bacterias transformadas con el plásmido que codificaba la proteína VP1, la proteína de la cápsida de la fiebre aftosa, y de la proteína iRFP, una proteína fluorescente infra-roja no-inmunogénica, demostraron proteger el pez cebra frente a la posterior infección con bacteria. Además los IBs estimularon la expresión de genes relacionados con la respuesta inmune en macrófagos de riñón anterior de trucha (RT-HKM) [140].

De la misma manera, IBs de citoquinas como el factor de necrosis tumoral alfa (TNFα) o el CCL4 demostraron su poder inmunoestimulante *in vivo*, administradas oralmente o por inyección, induciendo niveles de protección frente al desafío por bacteria [136].

Por último, tres IBs hechos de proteínas virales, la proteína de la cubierta del virus de la necrosis nerviosa viral (VNNV), la proteína viral 2 de IPNV y el fragmento 16 de la glicoproteína G de VHSV, fueron internalizados por células de hígado de pez cebra (ZFL) *in vitro* y por células del intestino de pez cebra *in vivo* y demostraron su capacidad de modular la respuesta inmune innata antiviral en células ZFL y RT-HKM [141].

MODELO DE TRABAJO

MODELO DE TRABAJO

Para el desarrollo de esta tesis y cumplir así el objetivo marcado de dilucidar el papel de los RBCs de peces en la inmunización frente a rhabdovirus, el modelo de trabajo utilizado consta de:

1. RBCs de trucha arcoíris (*Oncorhynchus mykiss)*. La trucha arcoíris pertenece a la familia de los salmónidos. Es originaria del pacífico norte aunque hoy en día ha sido introducida en numerosos países debido a su fácil adaptación. Esto hace que la trucha arcoíris esté catalogada como una de las especies exóticas invasoras más dañinas y también que sea una de las especies más cultivadas en acuicultura [142]. Es de gran importancia para el consumo humano y debido a su susceptibilidad al VHSV, ha sido una de las especies mejor estudiadas. Los RBCs de trucha (Figura 5) se utilizaron como modelo de estudio del efecto que tiene la inmunización frente rhabdovirus en estas células nucleadas.

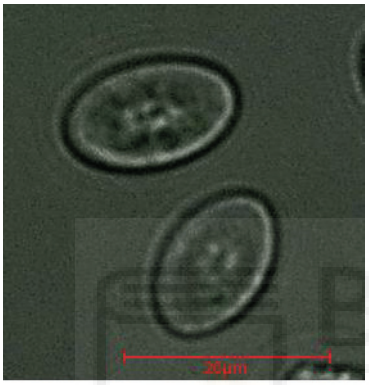

Figura 5: Eritrocitos de trucha arcoíris en campo claro, 40× aumento.

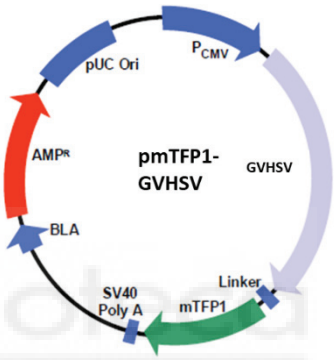

Figura 6: Construcción de la vacuna DNA, el plásmido pmTFP1GVHSV [106].

2. Vacuna DNA basada en la glicoproteína G de VHSV. Los plásmidos pmTFP1GVHSV y pmTFP1 se utilizaron para la transfección de los RBCs *in vitro* y la inyección de individuos *in vivo*. El plásmido pmTFP1GVHSV es un vector de expresión eucariota de 5471 pares de bases (pb) [106]. Consta de un promotor, el citomegalovirus humano (CMV), el gen de la glicoproteína G del VHSV (GVHSV), el gen de la proteína fluorescente mTFP1 (fusionada al extremo C' terminal del gen de interés), la señal de terminación del virus del simio 40 (SV40), un gen de resistencia a ampicilina y un origen de replicación pUC Ori para su replicación en *Echerichia coli* (Figura 6). El plásmido pmTFP1, sin el gen GVHSV, fue utilizado como control en todos los experimentos [106]. Estos plásmidos se utilizaron en las publicaciones 2 y 3.

3. Cuerpos de inclusión de proteínas recombinantes. Los IBs de origen bacteriano utilizados en esta tesis están hechos de las siguientes proteínas recombinantes: i) el fragmento 16 de GVHSV (frg16G-VHSV)($IB^{frg16G\text{-}VHSV}$) (Figura 7A), ii) el factor de la necrosis tumoral alfa (TNFα) ($IB^{TNF\alpha}$)(Figura 7B) y iii) la proteína no inmunogénica infraroja (iRFP) (IB^{iRFP}). Los IBs fueron

producidos en *Escherichia coli*. Las bacterias se transformaron con un plásmido codificando los genes de interés para después purificar los IBs de proteína recombinante. El IB^{iRFP} fue usado como control en todos los experimentos, según se indica en la publicación 4.

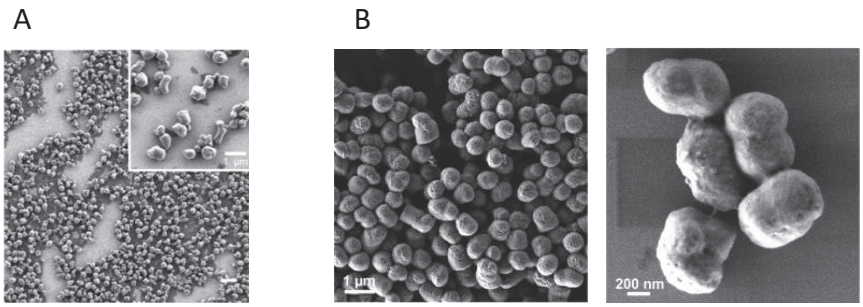

Figura 7: Imágenes de microscopía electrónica de barrido de emisión de campo (FESEM) de cuerpos de inclusión. A) Imagen de $IB^{frg16G\text{-}VHSV}$ [141] y B) Imágenes de $IB^{TNF\alpha}$ [136].

MATERIALES Y MÉTODOS

MATERIALES Y MÉTODOS

Para llevar a cabo los objetivos propuestos, la metodología utilizada se expone a continuación en tres apartados, que engloban los experimentos *in silico, in vitro* e *in vivo*, utilizados a lo largo de la tesis.

1. EXPERIMENTOS *IN SILICO*

En primer lugar, para poder abordar la evaluación de la respuesta de los RBCs nucleados a la vacuna DNA de GVHSV, tanto *in vitro* como *in vivo*, se analizaron la transcriptómica y proteómica de RBCs de trucha arcoirís según se explica a continuación y en la publicación 1.

1.1. Secuenciación del transcriptoma de RBCs

Para analizar el transcriptoma de los RBCs de trucha arcoíris, los RBCs se lisaron y se sintetizó el DNA complementario (DNAc) utilizando el kit SMART-Seq v4 Ultra Low Input RNA (Clontech, Takara Bio). La construcción de la librería de DNAc se llevó a cabo utilizando el kit Illumina Nextera XT Library Preparation (Illumina Inc., San Diego, CA, USA). La secuenciación del DNAc se realizó con la plataforma Illumina Hiseq 2500, usando lecturas de secuenciación de 100 pb por los dos extremos. A continuación, se realizó un ensamblaje *de novo* de las lecturas generadas utilizando el programa CLC Genomics Worbench (versión 9.5.4) [143] con el fin de crear una base de datos específica de RBCs de trucha arcoíris. Los cóntigos generados se mapearon frente a una base de datos local de Teleósteos (https://www.ncbi.nlm.nih.gov, última actualización: 20072017) para la anotación de genes utilizando el programa Blast2GO PRO versión 4.1.9 [144] y se seleccionaron las anotaciones con mayor identidad de secuencia (Top Hit). Esta base de datos de secuencias/genes de RBCs de trucha arcoíris se enriqueció con secuencias/genes de las bases de datos de trucha arcoíris (NCBI, última actualización: 09082017), salmón atlántico (NCBI, última actualización: 09082917), y pez cebra (NCBI, última actualización: 31072017). Esta base de datos, llamada "RBCs transcripts database" en la publicación 1, es la base de datos de referencia que se utilizó para el mapeo y anotación de las muestras de RBCs de los ensayos *in vitro* e *in vivo* realizados en esta tesis. El nivel de expresión de genes se cuantificó utilizando el número de lecturas mapeadas a cada gen. En la Figura 8 se representa un resumen del proceso, el cual se explica en detalle en la publicación 1.

1.2. Secuenciación del Proteoma de RBCs

Para analizar el proteoma de los RBCs de trucha arcoíris, las células se lisaron y la fracción citosólica se separó de la fracción de membrana antes de su digestión. La fracción de membrana se homogeneizó por ultrasonidos y 40 µg de las proteínas solubilizadas se precipitaron mediante metanol/cloroformo y se resuspendieron en buffer UTT (una solución multicaotrópica). A la fracción citosólica, previamente a la digestión, se le deplecionó la hemoglobina utilizando el Kit Hemovoid [145], la elución fue concentrada y 40 µg de proteína se diluyeron en buffer UTT. Finalmente se procedió a la digestión proteica con tripsina de cada fracción celular (citosólica y

membrana) por separado. El fraccionamiento peptídico de la fracción citosólica se llevó a cabo en cromatografía líquida de alta presión (HPLC) con pH de fase reversa. La fracción de membrana se analizó en una única fracción peptídica. La fracción citosólica se dividió en fracciones peptídicas, según está indicado en cada experimento. Después se procedió a la cuantificación de cada fracción mediante cromatografía líquida y espectrometría de masas LC-MS/MS. Los datos se procesaron mediante el software PeakView 2.2 [146] y se enfrentaron a la base de datos de Teleósteos (última actualización: 20170412, 2.542.118 secuencias) mediante un software de identificación de proteínas (Mascot Server v2.5.1). En la Figura 8 se representa un resumen del proceso, el cual se explica en detalle en la publicación 1.

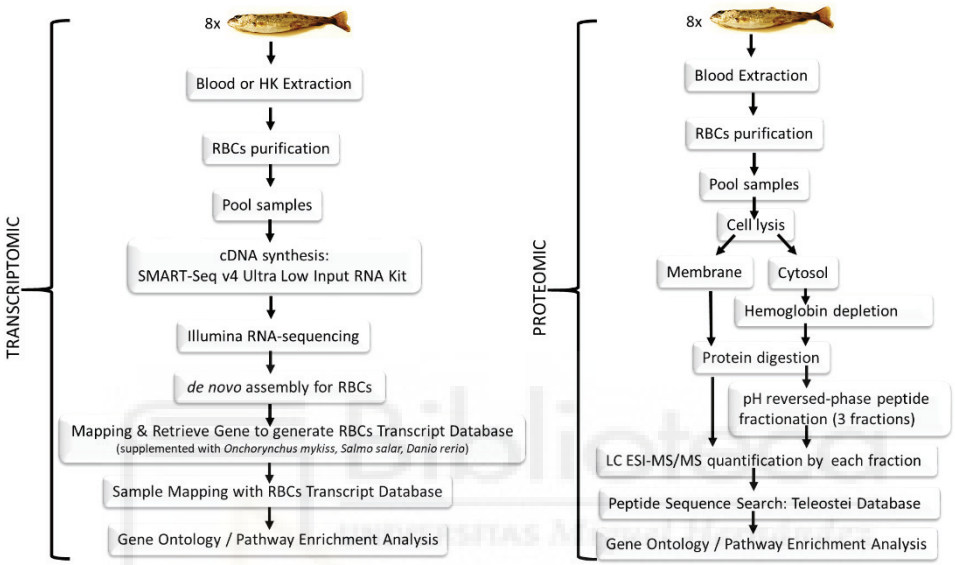

Figura 8: Esquema de trabajo seguido para analizar el transcriptoma y proteoma de los RBCs de trucha arcoíris (Figura 1, publicación 1).

1.3. Análisis de enriquecimiento de rutas funcionales

La ontología génica y el enriquecimiento de las rutas funcionales implicadas en la respuesta de los RBCs a la vacuna DNA se analizó mediante el programa Cytoscape [147] y las aplicaciones ClueGO [148] y CluePedia [149], que establecen una red de rutas funcionales enriquecidas. Las bases de datos utilizadas fueron ontología génica de procesos del sistema inmune, ontología génica de procesos biológicos, Reactome rutas y Reactome reacciones. El análisis de las interacciones proteína-proteína se evaluó utilizando el programa STRING v11 [150]. Los símbolos de genes ortólogos de *Homo sapiens* se obtuvieron por identidad de secuencia usando el programa Blast2GO version 4.1.9 [144] y la base de datos de *Homo sapiens*.

2. EXPERIMENTOS *IN VITRO*

Los experimentos *in vitro* que se llevaron a cabo para evaluar la respuesta de los RBCs a la vacuna DNA y a los IBs de proteína recombinante fueron los siguientes:

2.1. Evaluación de la respuesta de los RBCs a la vacuna DNA *in vitro*

Los RBCs se extrajeron de la sangre de la vena caudal de individuos y se purificaron mediante dos centrifugaciones consecutivas en gradiente de densidad mediante Ficoll [33]. A continuación los RBCs se transfectaron mediante electroporación con 4 µg por cada 10^6 células con el plásmido pmTFP1GVHSV o con el plásmido control pmTFP1. Los materiales y métodos de este apartado se explican en detalle en la publicación 2.

2.1.1. Aislamiento de célula única de RBCs que expresan la proteína GVHSV

Para seleccionar los RBCs transfectados que expresan la proteína GVHSV fluorescente se elaboró un protocolo de clasificación de células activadas por fluorescencia (FACS) de célula única (FACS célula única) utilizando el equipo BD FACSJazz (BD Biosciences, Madrid, Spain).

Los RBCs purificados y transfectados se analizaron según los parámetros de citometría de flujo que miden la dispersión frontal (forward scatter, FSC), la dispersión lateral (side scatter, SSC) y la fluorescencia (FITC). La parte más fluorescente del histograma (población P2), depende de la población con más FSC y SSC del gráfico de puntos (población P1). El filtro aplicado a FSC y SSC nos excluye la posible contaminación de debris celular y células más pequeñas fruto del estrés. La población P2 fue aislada en modo 1.0 drop single. Las poblaciones seleccionadas se indican en la Figura 9. Este modo de selección nos permite una población donde prima la pureza a costa de un bajo rendimiento. La recuperación de células es baja, pero nos asegura que estas son 100% puras. No hay contaminación con otras células que no sean RBCs (aunque la población había sido previamente purificada mediante ficoll) ni contaminación por RBCs que no expresan GVHSV. Los RBCs aislados fueron visualizados por microscopía de fluorescencia utilizando el equipo IN Cell Analyzer 6000 (GE Healthcare, Little Chalfont, UK) para confirmar la pureza. A continuación, los RBCs se almacenaron a -80ºC en buffer de lisis e inhibidor de RNAsas hasta la construcción de la librería de DNAc.

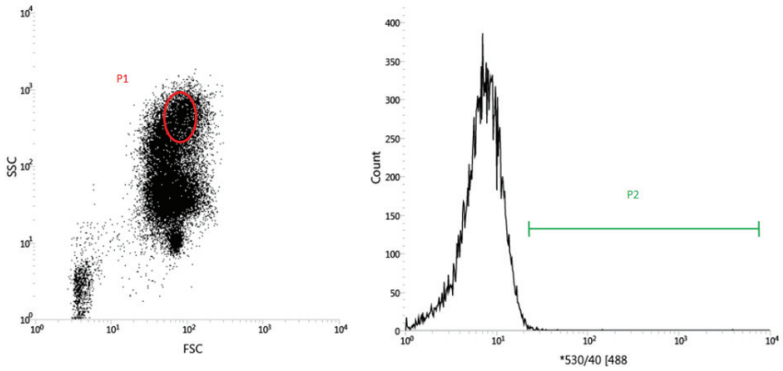

Figura 9: Aislamiento mediante FACS célula única de RBCs que expresan la proteína GVHSV. Gráfico de puntos e histograma de las poblaciones seleccionadas para aislar RBCs que expresan la proteína GVHSVmTFP1 o mTFP1. Para ello, se seleccionó la población P2 dependiente de P1 usando el clasificador de células BD FACSJazz (Figura suplementaria 1, publicación 2).

2.1.2. Análisis del transcriptoma de RBCs que expresan la proteína GVHSV

Para evaluar el transcriptoma de los RBCs que expresan la proteína GVHSV utilizamos la población de RBCs aislados mediante FACS célula única (en la publicación 2 nos referimos a esta población como "FACS Single-Cell Sorted GVHSV-expressing RBCs"). RBCs de 24 individuos se transfectaron con pmTFP1GVHSV o pmTFP1. Seis días post-transfección, se aislaron entre 6 y 10 RBCs por individuo que expresaban la proteína GVHSV (o mTFP1 como control) y se procedió con la secuenciación del transcriptoma según se explica en el apartado 1.1 de materiales y métodos. Los RBCs se agruparon en 6 grupos, de 8 individuos cada uno, resultando en 3 grupos de RBCs que expresan GVHSVmTFP1 y 3 grupos de RBCs que expresan mTFP1 (Figura 10).

2.1.3. Análisis del proteoma de RBCs transfectados con pmTFP1GVHSV

Para evaluar el proteoma de los RBCs transfectados con pmTFP1GVHSV se utilizó toda la población de RBCs compuesta de RBCs que expresaban o no la proteína GVHSV (en la publicación 2 nos referimos a esta población como "GVHSV transfected RBCs"). Para ello, 10^7 RBCs de 16 individuos se transfectaron con pmTFP1GVHSV o pmTFP1. Seis días post-transfección, las muestras se recogieron y se procedió con la secuenciación del proteoma según se explica en el apartado 1.2 de materiales y métodos. Los RBCs se agruparon en 4 grupos, de 8 individuos cada uno, resultando en 2 grupos de RBCs transfectados con pmTFP1GVHSV y 2 grupos de RBCs transfectados con pmTFP1 (Figura 10).

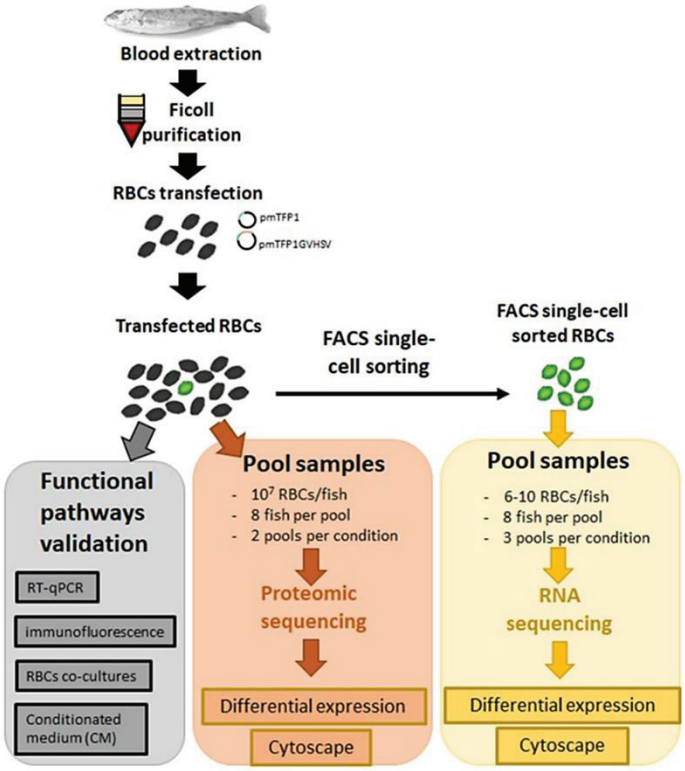

Figura 10: Esquema de trabajo seguido para evaluar la respuesta de los RBCs a la vacuna DNA *in vitro.* (Figura 1, publicación 2).

2.1.4. Análisis de la expresión de genes en RBCs transfectados con pmTFP1GVHSV

Los genes y proteínas de las rutas sobrerrepresentadas y sobreexpresadas en el perfil de transcriptómica y proteómica de los RBCs se analizaron mediante la reacción en cadena de la polimerasa (PCR) cuantitativa (qPCR), inmunofluorescencia y citometría de flujo, según se resume en la Figura 10 y se indica en el material y métodos de la publicación 2.

2.1.5. Comunicación de RBCs transfectados con pmTFP1GVHSV con otros tipos celulares

Analizamos la comunicación de RBCs transfectados con pmTFP1GVHSV (o pmTFP1 como control) con una línea celular de monocitos / macrófagos de trucha arcoíris (RTS11) y una línea celular de gónada de trucha arcoíris (RTG-2) (Figura 10). El cocultivo de RBCs transfectados con RTS11 se realizó en pocillos con insertos "Transwell" donde se cultivaron las dos líneas celulares y se analizó la expresión de marcadores de diferenciación en RTS11. Por otro lado, RTG-2 se pretrató con medio condicionado (MC) de RBCs transfectados con pmTFP1GVHSV para después analizar la expresión de genes relacionados con IFN1. También se evaluó la protección desencadenada por el MC de RBCs transfectados con pmTFP1GVHSV en RTG2 frente a una infección posterior por VHSV. En la publicación 2 se describen los materiales y métodos relacionados con estos ensayos.

Por otro lado, también realizamos cocultivo de RBCs transfectados con pmTFP1GVHSV con leucocitos de sangre periférica (WBCs, del inglés "White blood cells"). Para ello, los RBCs y los

WBCs se purificaron de sangre periférica mediante dos centrifugaciones consecutivas en gradiente de densidad mediante Ficoll. Los RBCs se transfectaron con pmTFP1GVHSV y se cocultivaron con los WBCs autólogos a un ratio 1:1. Tras 7 días de cocultivo se evaluó la proliferación celular mediante contaje del número de células y análisis de marcadores de células T y B por medio de qPCR. En la publicación 3 se describen los materiales y métodos relacionados con este ensayo.

2.2. Evaluación de la respuesta de los RBCs a los cuerpos de inclusión de proteína recombinante *in vitro*

Para evaluar la respuesta de los RBCs a los IBs de las proteínas de interés, los RBCs se trataron con $IB^{TNF\alpha}$, $IB^{frg16G\text{-}VHSV}$, o IB^{iRFP} (como control). La entrada de los IBs, marcados con fluorescencia, en los RBCs se evaluó mediante citometría de flujo y microscopía confocal, a distintos concentraciones de IBs y a distintos tiempos post-tratamiento. La expresión de genes relacionados con la respuesta inmune en los RBCs tratados con los IBs se analizó mediante qPCR a 24 h tras el tratamiento. En la publicación 4 se describen los materiales y métodos relacionados con estos ensayos. Los IBs fueron donados por la Dra. Nerea Roher (Universitat Autònoma de Barcelona) y parte de los ensayos se realizaron en su laboratorio.

3. EXPERIMENTOS *IN VIVO*

Los experimentos *in vivo* que se llevaron a cabo para evaluar la respuesta de los RBCs a la vacuna DNA y a los IBs de proteína recombinante fueron los siguientes:

3.1. Evaluación de la respuesta de los RBCs a la vacuna DNA *in vivo*

Para evaluar el papel que tienen los RBCs en la inmunización frente a VHSV con una vacuna DNA, se llevó a cabo una inmunización im de individuos con 10 µg del plásmido pmTFP1GVHSV o con el plásmido control pmTFP1.

3.1.1. Aislamiento de RBCs de riñón anterior y sangre periférica

El procedimiento de aislamiento mediante FACS célula única para aislar los RBCs del riñón anterior y de la sangre fue puesto a punto en esta tesis y se explica en los materiales y métodos de la publicación 3. Brevemente, el riñón anterior de individuos inyectados con pmTFP1GVHSV o pmTFP1 fue extraído y filtrado por un tamiz de 40 µm y la sangre periférica fue extraída de la vena caudal. A continuación, las células fueron incubadas con 500 nM de SYTO RNASelect (Molecular Probes), un agente que tiñe el RNA de las células. La tinción del RNA nos permitió separar los RBCs del resto de células de la sangre o del riñón anterior. En la publicación 3 nos referimos a la población de RBCs aislados de sangre periférica como "PB-RBCs" (del inglés "Peripheral blood RBCs") y a los RBCs aislados de riñón anterior como "HK-RBCs" (del inglés "Head Kidney RBCs"). Los PB-RBCs fueron aislados mediante FSC, SSC y FITC. La máscara empleada fue 2.0 drop enrich. Los HK-RBCs fueron aislados mediante SSC y FITC (Figura 11). La máscara empleada fue 1.0 drop single.

Los PB-RBCs y los HK-RBCs asilados fueron visualizados por microscopía en el equipo IN Cell Analyzer 6000 para confirmar la pureza. A continuación se almacenaron a -80ºC en buffer de lisis e inhibidor de RNAsas hasta la construcción de la librería de DNAc.

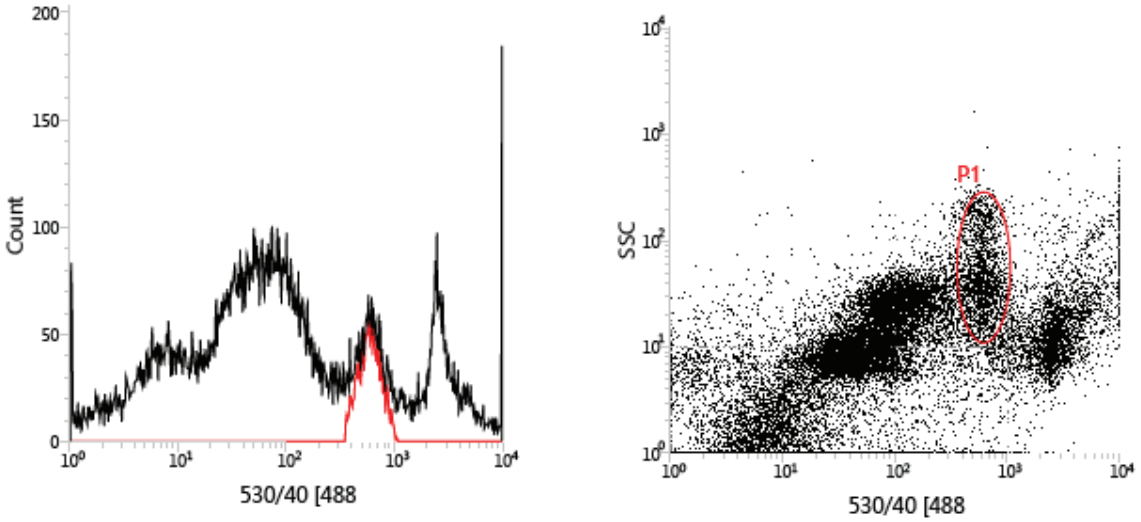

Figura 11: Asilamiento mediante FACS célula única de HK-RBCs de individuos inyectados con pmTFP1GVHSV o pmTFP1. Gráfico de puntos e histograma donde se muestra la población seleccionada (P1) para el aislamiento de RBCs utilizando el equipo BD FACSJazz™ cell sorter (Figura suplementaria 1, Publicación 3).

3.1.2. Análisis del transcriptoma de PB-RBCs y HK-RBCs tras la inmunización con GVHSV

Para evaluar el transcriptoma de los PB-RBCs y HK-RBCs de individuos inyectados con pmTFP1GVHSV (o pmTFP1 como control), se extrajo el riñón anterior y la sangre periférica a 14 días post-inyección. A continuación, se aislaron 10^2 HK-RBCs de riñón anterior y 10^6 PB-RBCs de sangre periférica, como se indica en el apartado anterior, de 32 individuos (16 inyectados con pmTFP1GVHSV y 16 con pmTFP1). Los HK-RBCs o PB-RBCs se agruparon en 4 grupos, de 8 individuos cada uno, resultando en 2 grupos inyectados con pmTFP1GVHSV y 2 grupos inyectados con pmTFP1 y se procedió con la secuenciación del transcriptoma según se explica en el apartado 1.1 de materiales y métodos. La Figura 12 muestra un esquema del flujo de trabajo, que se explica en detalle en los materiales y métodos de la publicación 3.

3.1.3. Análisis del proteoma de PB-RBCs tras la inmunización con GVHSV

Para evaluar el proteoma de los PB-RBCs de individuos inyectados con pmTFP1GVHSV, los PB-RBCs se aislaron mediante dos centrifugaciones consecutivas en gradiente de densidad mediante Ficoll a 14 días post-inyección. Se recogieron 10^7 PB-RBCs de 32 individuos (16 inyectados con pmTFP1GVHSV y 16 con pmTFP1). Los PB-RBCs se agruparon en 4 grupos de 8 individuos cada uno, resultando en 2 grupos inyectados con pmTFP1GVHSV y 2 grupos inyectados con pmTFP1 y se procedió con la secuenciación del proteoma según se explica en el apartado 1.2 de materiales y métodos. La Figura 12 muestra un esquema del flujo de trabajo, que se explica en detalle en los materiales y métodos de la publicación 3.

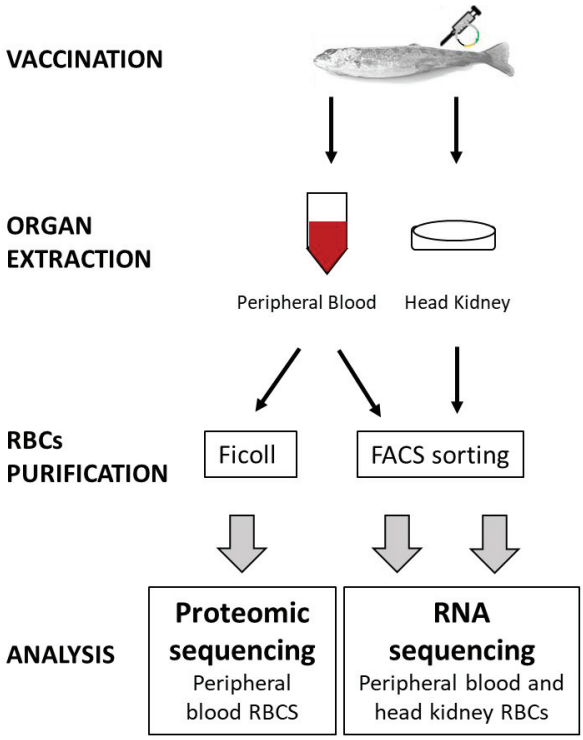

Figura 12: Esquema del flujo de trabajo seguido para evaluar el transcriptoma y el proteoma de los RBCs a la vacuna DNA *in vivo* (Figura 1, publicación 3).

3.1.4. Análisis de la expresión de genes en RBCs tras la inmunización con GVHSV

Para evaluar la expresión de los genes/proteínas sobreexpresados en los resultados de transcriptómica y proteómica, PB-RBCs de individuos inyectados con pmTFP1GVHSV fueron aislados mediante dos centrifugaciones consecutivas en gradiente de densidad mediante Ficoll a 14 días post-inyección. A continuación se evaluó la expresión de genes mediante qPCR.

3.1.5. Transfusión de RBCs autólogos transfectados con pmTFP1GVHSV

Para evaluar la capacidad de los RBCs de inducir una respuesta humoral, 15×10^6 RBCs autológos, purificados mediante dos centrifugaciones consecutivas en gradiente de densidad mediante Ficoll y transfectados *in vitro* con pmTFP1GVHSV (4 µg pmTFP1GVHSV/10^6 RBCs) se reinfundieron en la vena caudal de individuos adultos (20-25 cm). Treinta días después se extrajo sangre periférica y se analizaron los niveles de anticuerpos anti-VHSV en suero mediante el ensayo de inmunoabsorción ligado a enzima (ELISA). El protocolo de este ensayo se explica en detalle en los materiales y métodos de la publicación 3.

3.2. Evaluación de la respuesta de los RBCs a los cuerpos de inclusión de proteína recombinante *in vivo*

Los cuerpo de inclusión $IB^{TNF\alpha}$ y $IB^{frg16G\text{-}VHSV}$ (5.5 mg/kg) se inyectaron en la vena caudal de individuos adultos (15-20 g). A continuación, 24 y 48 h post-inyección, se extrajo sangre periférica y los RBCs fueron purificados mediante dos centrifugaciones de gradiente de densidad mediante Ficoll. La expresión de genes relacionados con la respuesta inmune se evaluó mediante qPCR. Por otro lado, la expresión proteica se analizó mediante citometría de flujo e inmunofluorescencia. IB^{iRFP} se utilizó como control en los experimentos. Los protocolos de estos ensayos se explican en detalle en los materiales y métodos de la publicación 4.

RESULTADOS Y DISCUSIÓN

RESULTADOS Y DISCUSIÓN

1. LA RESPUESTA INMUNE ANTIVIRAL DE LOS RBCs TRANSFECTADOS CON UNA VACUNA DNA QUE CODIFICA LA PROTEÍNA GVHSV

Este trabajo tuvo como objetivo evaluar la capacidad de los RBCs de trucha arcoíris de expresar una proteína codificada por un plásmido (una vacuna DNA), la respuesta de los RBCs a dicha vacuna y su señalización hacia otros tipos celulares *in vitro* con el fin de explorar la utilización de RBCs para mejorar la respuesta inmune de peces a las vacunas DNA. Los resultados de este apartado corresponden con los resultados mostrados en la publicación 2 (P2).

1.1. Los RBCs son capaces de expresar el antígeno GVHSV

En este trabajo se muestra por primera vez la expresión de un antígeno codificado por un plásmido en RBCs. La expresión de la proteína GVHSV se observó mayoritariamente en la zona perinuclear. Se ha descrito que la proteína G del virus de la rabia (RABV), un rhabdovirus, puede expresarse en la zona perinuclear de células de neuroblastoma de ratón [151]. Sin embargo la expresión de proteína mTFP1 se observó distribuida uniformemente en el núcleo y citoplasma de RBCs (Figura 2A,B, P2). También se observó la expresión de GVHSV en células derivadas de RBCs (shape-shifted RBCs, shRBCs [37]). La transfección de los RBCs se monitorizó a distintas concentraciones de plásmido y tiempos post-transfección (Figura 2C, P2). Por otro lado, se comparó la expresión del gen y de la proteína GVHSV en RBCs con la línea celular RTS11. RTS11 mostró una mayor expresión del gen GVHSV, decreciendo con el tiempo al contrario que sucedió en los RBCs (Figura 2D, P2). La expresión de proteína GVHSV en RBCs fue menor (Figura 2E,G, P2) que en RTS11 (Figura 2F,H, P2).

1.2. El transcriptoma de RBCs que expresan la proteína GVHSV inducen la expresión de genes relacionados con el complemento y moléculas señalizadoras

La evaluación del transcriptoma de RBCs que expresaban la proteína GVHSV, en comparación con RBCs que expresaban la proteína mTFP1, identificó 3249 genes expresados diferencialmente (DEGs). De ellos, 1786 se encontraban sobreexpresados y 1463 infraexpresados. Destacamos la sobreexpresión de genes implicados en la señalización de interferón alfa/beta en mecanismos antivirales, en la señalización mediante interleuquinas y en la cascada de complemento. El análisis de enriquecimiento de rutas funcionales reveló la sobrerrepresentación de las rutas de expresión génica activada por RORA, señalización por los receptores acoplados a proteínas G (GPCR) y la ruta de activación del complemento (Figura 3, P2). Los GPCR son una familia de receptores de proteína que detectan señales extracelulares y activan una cascada de señalización celular interna [152]. Están implicados en numerosos procesos y por ello son muy utilizados como dianas de fármacos. Los GPCR participan en el proceso de inflamación, regulan la función de los macrófagos y son receptores de quimioquinas entre otros [153, 154]. Arrestina beta 2 (ARRB2) ha sido identificada como un regulador de GPCR de las rutas NFκB regulando la expresión de interleuquina 6 (IL6), IL8 e interleuquina1 β (IL1β) [155]. *arrb2* se observó sobreexpresada en la transcriptómica de RBCs que expresaban la proteína GVHSV y su expresión

fue confirmada por qPCR de RBCs transfectados con GVHSV (Figura 5, P2). Igualmente, las proteínas IL8, TNFα y IL1β resultaron sobreexpresadas en RBCs transfectados con GVHSV (Figura 6, P2). Por otro lado, la ruta de expresión génica activada por RORA, la cual está implicada en respuestas inflamatorias regulando la diferenciación de células Th17 [156] e implicada la producción de citoquinas [157], se encontró sobreexpresada en RBCs que expresaban GVHSV. Finalmente, también encontramos sobrerrepresentada el proceso de activación del complemento. En este sentido, es conocido que el sistema del complemento actúa como conexión entre la inmunidad innata y la adaptativa, y es un mediador de la respuesta inflamatoria [46]. Se han descrito receptores del complemento en RBCs de trucha arcoíris que participan en la opsonización de inmunocomplejos [48].

1.3. El proteoma de RBCs transfectados con GVHSV induce procesos antivirales, antioxidantes y de presentación de antígenos

La evaluación del proteoma de RBCs transfectados con GVHSV, en comparación con RBCs transfectados con el plásmido control, identificó 199 proteínas expresadas diferencialmente (DEPs), 75 sobreexpresadas y 124 infraexpresadas (FDR<0.001 y aplicando un filtro de [−1.5 < Log2fold change (FC)> 1.5]). El análisis de enriquecimiento de rutas funcionales utilizando la base de datos de Reactome rutas, reveló la sobreexpresión del mecanismo antiviral ISG15, la detoxificación de especies reactivas de oxígeno, el "splicing" de RNAm e interacciones del hospedador de factores de virus (Figura 4A, P2). ISG15, es un ISG que pertenece a la familia de las ubiquitinas y lleva a cabo el proceso de ISGilación. Entre las proteínas que se conjugan con ISG15 se encuentran proteínas relacionadas con mecanismos antivirales inducidos por interferón como la proteína quinasa R (PKR), proteínas IFIT y Mx [158]. Se ha descrito que la infección por VHSV y vacunación o transfección de células con plásmidos que codifican la GVHSV inducen la expresión de los genes *isg15* [159, 160] y *mx* [160-162].

Otra ruta sobreexpresada en RBCs transfectados con GVHSV fue la detoxificación de especies reactivas de oxígeno, como ya ha sido descrito anteriormente para RBCs expuestos a VHSV [33] y a daño por metales [163]. En este trabajo vemos como una vacuna DNA que codifica GVHSV también es capaz de producir una respuesta celular antioxidante inducida por especies reactivas de oxígeno. Proteínas antioxidantes como la peroxiredoxina 4 (PRDX4), superóxido dismutasa 1 (SOD1) y las tioredoxinas (TRX y TXNL1) resultaron sobreexpresadas entre los DEPs.

Por otro lado, el enriquecimiento de rutas funcionales llevado a cabo utilizando la base de datos de Reactome reacciones reveló la sobrerrepresentación de procesos como la escisión proteosomal de antígenos exógenos, importación y liberación del snRNP nuclear y la formación del complejo de AT-AC C. Además la base de datos de ontología génica de procesos del sistema inmune reveló la sobreexpresión de la ruta de presentación y procesamiento de péptidos exógenos vía MHC de clase I, dependiente de TAP (Figura 4C,E, P2). Esto indicaría que los RBCs son capaces de presentar antígenos codificados por la vacuna DNA. La presentación de péptidos exógenos vía MHCI y la capacidad de inducir la activación de células T se discutirá en el siguiente apartado de resultados y discusión, aunque más investigación es necesaria para determinar el papel de los RBCs en la presentación de antígenos.

1.4. Análisis de la expresión de genes de rutas sobrerrepresentadas en transcriptómica y proteómica

Los genes/proteínas relacionados con las rutas y procesos sobrerrepresentados en los análisis de transcriptómica y proteómica fueron analizados mediante qPCR. Los genes *sod1*, peroxiredoxina 6 (*prdx6*), factor potenciador de células NK (*nkef*) y *trx* resultaron igualmente sobreexpresados confirmando la ruta de detoxificación de especies reactivas de oxígeno. La sobreexpresión de los genes *arrb2* y receptor α relacionado con rar (*rora*), pertenecientes a los procesos de señalización por GPCR y la expresión génica activada por RORA, respectivamente, también se confirmó mediante qPCR (Figura 5, P2). También se observó una elevada sobreexpresión de los genes *mx*, proteína 5 inducida por interferón con repeticiones de tetratricopéptidos (*ifit5*) y *pkr,* moléculas efectoras de la ruta de mecanismo antiviral mediado por IFN1 (Figura 5, P2). Sin embargo, se observó una infraregulación significativa de la expresión génica de *ifn1* (Figura 5, P2), lo cual podría indicar que la expresión de los ISGs sería independiente de la expresión de *ifn1*. Este hecho ya ha sido previamente descrito en células tras la infección con virus [164-166]. Así mismo, también se ha descrito la infraregulación del gen *ifn1* en RBCs expuestos al virus a VHSV [33]. Finalmente, en relación a la ruta de presentación de antígenos, se observó la sobreexpresión de los genes *mhcI*, *mhcII*, dinamina 2 (*dnm2*) y el componente del complejo de revestimiento COPII y de poro nuclear homólogo de SEC13 (*sec13*) mediante qPCR (Figura 5, P2). Por otro lado, a nivel de proteína, observamos la sobreexpresión de Mx, IL1β, IL8, TNFα y óxido nítrico sintetasa inducible (INOS) en RBCs transfectados con GVHSV mediante citometría de flujo, aunque los resultados no fueron estadísticamente significativos (Figura 6, P2).

1.5. RBCs transfectados con la GVHSV protege a RTG-2 frente a la infección por VHSV y estimula marcadores de proliferación en RTS11

Para evaluar la capacidad de los RBCs de propagar la repuesta desencadenada por GVHSV hacia otras células, analizamos la capacidad de los RBCs transfectados con GVHSV de conferir protección antiviral frente a VHSV en la línea celular RTG-2, así como su capacidad de inducir diferenciación en la línea celular RTS11.

Los resultados mostraron que el MC de RBCs transfectados con GVHSV, tanto a 3 como a 6 días post-transfección, provocó una disminución del número de unidades formadoras de focos por mL (UFF/mL) en las células RTG-2, en comparación con el MC de RBCs transfectados con el plásmido control tras la infección con VHSV (Figura 7A,B, P2). Además, se analizó la expresión de los genes *ifn1* y *mx* en RTG-2 pre-tratadas con MC para evaluar si esta protección conferida a RTG-2 frente a VHSV estaba relacionada con el sistema IFN1. La expresión de *mx* resultó significativamente sobreexpresada en RTG-2 pre-tratada con MC de RBCs transfectados con GVHSV 3 y 6 días post-transfección, y la expresión de *ifn1* resultó significativamente sobreexpresada con el MC de 6 días post-transfección (Figura 7C, P2). A pesar de que la expresión de *ifn1* resultó infraexpresada en RBCs transfectados con GVHSV (Figura 5, P2), el MC de RBCs fue capaz de proteger RTG-2 frente a la infección por VHSV así como estimular la expresión de *ifn1* y *mx* en RTG-2.

Por otro lado, RBCs transfectados con GVHSV fueron cocultivados con la línea celular RTS11 durante 3 días. A continuación se analizó la expresión de genes de diferenciación de macrófagos en la línea celular RTS11. Los resultados mostraron la sobreexpresión de interleuquina 10 (*il10*), marcador de macrófagos M2 [167], y la infraregulación de interleuquina 12 subunidad beta (*il12β*) e *inos*, marcadores de macrófagos M1 [167, 168], aunque los resultados no fueron estadísticamente significativos (Figura 8, P2). Los macrófagos M1 secretan niveles altos de IL12 y bajos de IL10. Cuando se activan los macrófagos M2, la expresión de estas citoquinas cambia, y producen niveles altos de IL10 y bajos de IL12 [168]. Las citoquinas o moléculas secretadas por los RBCs transfectados con GVHSV fueron capaces de inducir marcadores de macrófagos M2 en RTS11. Aunque se desconocen las moléculas implicadas, se sabe que los macrófagos M2 son alternativamente activados por citoquinas como IL4, IL10 o IL13 y participan en procesos antiinflamatorios, curación de heridas y reparación de tejidos [168, 169].

2. LOS RBCs COMO MEDIADORES DE LA RESPUESTA INMUNE INDUCIDA POR VACUNAS DNA

El objetivo de este trabajo fue evaluar el papel de los RBCs nucleados en la inmunización con vacunas DNA. Para ello, analizamos la implicación de los RBCs en la respuesta inmune desarrollada tras la inmunización im de individuos con GVHSV. Se llevó a cabo un análisis del transcriptoma de RBCs de riñón anterior (HK-RBCs) y de sangre periférica (PB-RBCs), y el análisis del proteoma de PB-RBCs tras la inmunización. Además exploramos la capacidad de los RBCs de actuar como portadores de la vacuna DNA e inducir una respuesta humoral *in vivo* así como de modular la proliferación de leucocitos *in vitro*. Los resultados de este apartado corresponden con los resultados mostrados en la publicación 3 (P3).

2.1. El transcriptoma de HK-RBCs de individuos inmunizados con GVHSV revela rutas de presentación de antígenos y comunicación con leucocitos

El análisis transcripcional de HK-RBCs de individuos inmunizados con GVHSV identificó 479 DEGs, de los cuales 287 resultaron sobreexpresados y 192 infraexpresados respecto al control (HK-RBCs de individuos inyectados con el plásmido control). El análisis de enriquecimiento de rutas funcionales utilizando de la base de datos de ontología génica de procesos biológicos reveló la sobrerrepresentación de rutas del proceso de síntesis de sustancias orgánicas, localización de proteínas, transporte mediado por vesículas y respuesta celular a estrés (Figura 2A, P3).

El riñón anterior es el principal órgano hematopoyético en peces. Allí ocurre gran parte de la respuesta inmune innata y adaptativa tras la vacunación con DNA. La fagocitosis, el procesamiento de antígenos y la maduración y diferenciación de células B tienen lugar principalmente en el riñón anterior de peces [170]. Entre los DEGs destacamos la sobreexpresión de la quimioquina ligando 25 (*ccl25*) y de su receptor el receptor de quimioquina tipo 9 (*ccr9*). Este sistema ha sido implicado en el reclutamiento de células T tras la vacunación oral [171] y tras la infección con parásitos [172] en peces. También encontramos la quimioquina ligando 13 (*ccl13*), una citoquina involucrada en el transporte mediado por vesículas y cuya expresión se evaluó mediante qPCR aunque no resultó significativa (Figura 5, P3). Junto con *ccl13*, el receptor de quimioquinas C-X-C tipo 4 (*cxcr4*) resultó sobreexpresado en HK-RBCs, al contrario que sucede en RBCs de sangre periférica de organismos infectados con PRV, donde se ha descrito la infraregulación de estos dos genes [25]. Por otro lado, el análisis de enriquecimiento de rutas funcionales utilizando de la base de datos de ontología génica de procesos del sistema inmune reveló la sobrerrepresentación de procesos relacionados con el procesamiento y presentación de péptidos exógenos vía MHC clase I, dependiente de TAP, procesos relacionados con la regulación de células T, diferenciación de leucocitos mieloides y la señalización de receptores Fc (Figura 3A,D, P3), procesos que indicarían la comunicación entre RBCs y WBCs en el riñón anterior de peces. Se ha visto que RBCs de mamíferos pueden inducir la proliferación de células T y participar en la respuesta inmune mediante su comunicación con otras células del sistema inmune [18-20, 55]. La comunicación de los RBCs de trucha arcoíris con otros tipos celulares evidenció la capacidad de señalización de los RBCs mediante el sistema de IFN1. RBCs expuestos a VHSV [33] o transfectados con GVHSV indujeron la respuesta de IFN en las líneas celulares TSS y RTG-2, respectivamente. Como ya se comentó anteriormente en esta tesis, RBCs transfectados

con GVHSV indujeron la expresión de marcadores de diferenciación en RTS11. Finalmente, entre los DEGs destacamos la quinasa de células hematopoyéticas (*hck*), un miembro de la familia tirosina-quinasa Scr implicado en la regulación del sistema inmune innato. Participa en el reclutamiento y activación de neutrófilos [173], en la regulación de la migración de células mieloides [174] y se ha sugerido su implicación en la activación de monocitos/macrófagos [175]. Además su expresión en PB-RBCs de individuos inmunizados con GVHSV fue confirmada por qPCR (Figura 5, P3). Se ha descrito la expresión de *hck* en RBCs de salmón atlántico desafiados con PRV, aunque en ese caso *hck* resultó infraregulado [25]. Se ha sugerido que una de las estrategias para que la vacunación resulte eficaz es que haya una eficiente comunicación hacia linfocitos [176]. La inmunización dirigida a APCs ha demostrado mejorar la respuesta humoral y de células T [65]. Los procesos y genes sobreexpresados en los RBCs de individuos inmunizados con GVHSV indican por tanto que los RBCs tienen las herramientas necesarias para participar en la comunicación con los leucocitos.

Finalmente, el análisis de la interacción proteína-proteína de los genes sobrerrepresentados en los HK-RBCs de individuos inmunizados con GVHSV reveló una alta interacción entre los genes identificados confirmando las rutas sobrerrepresentadas mediante el análisis de enriquecimiento de rutas funcionales (Figura 4A, P3).

2.2. El transcriptoma de PB-RBCs de individuos inmunizados con GVHSV revela rutas de presentación de antígenos, autofagia y señalización celular

El análisis transcripcional de PB-RBCs de individuos inmunizados con GVHSV, reveló 1018 DEGs, de los cuales 892 resultaron sobreexpresados y 126 infraexpresados respecto al control (PB-RBCs de individuos inyectados con el plásmido control). El análisis de enriquecimiento de rutas funcionales utilizando la base de datos de ontología génica de procesos biológicos reveló la sobrerrepresentación de rutas relacionadas con los procesos metabólicos, localización de proteínas, respuesta celular a estrés, desarrollo de órganos hematopoyéticos o linfoides, señalización apoptótica y autofagia, entre otras (Figura 2B, P3).

La autofagia es un proceso natural de la célula y muy conservado entre especies, de vital importancia para la supervivencia de la célula en condiciones de estrés [177], como por ejemplo la infección de la célula por virus [178]. La autofagia en peces ha sido descrita como un proceso que inhibe [179] o facilita [180, 181] la replicación del virus. Recientemente se ha descrito que los RBCs nucleados utilizan la autofagia como un mecanismo de defensa antiviral [34, 57] y que la transfección con un plásmido que codifica la proteína GVHSV también desencadena el proceso de autofagia en líneas celulares de peces [106]. En este trabajo observamos que los RBCs de individuos inmunizados con GVHSV también desencadenan procesos de autofagia. Entre los genes de autofagia destacan la proteína 1 de dominio WD de interacción con fosfoinosítido (*wipi1*), serina/treonina quinasa ulk1 (*ulk1*) y la proteína asociada al receptor de ácido gamma-aminobutírico (*gabarap*), y cuya sobreexpresión fue confirmada mediante qPCR (Figura 5, P3). De la misma manera que la autofagia, el proceso de apoptosis forma parte del mantenimiento de la homeostasis celular y participa en la regulación del sistema inmune [182]. Entre los genes relacionados con la regulación de la apoptosis encontramos sobreexpresados BCL2-like 1 (*bcl2l1*), atanogen 3 asociado a BCL2 3 (*bag3*), atanogen 5 asociado a BCL2 (*bag5*), proteína 3 de

interacción con BCL2 (*bnip3*). El análisis del proteoma de RBCs del besugo de roca (rock bream) infectados con el iridovirus del besugo de roca (rock bream iridovirus, RBIV) mostró la sobreexpresión de la ruta de apoptosis [183]. También se ha descrito apoptosis en RBCs bajo estrés oxidativo [184]. Se ha observado que RBCs expuestos a VHSV desencadenaban una respuesta antioxidante [33], de la misma manera que RBCs transfectados con GVHSV, como se comentó anteriormente en esta tesis. El análisis del transcriptoma de PB-RBCs de individuos inmunizados con GVHSV mostró la sobreexpresión de genes involucrados en la respuesta antioxidante como *sod1*, and superóxido dismutasa 2 (*sod2*). Por ello, los RBCs podrían inducir una respuesta antioxidante para contrarrestar el estrés oxidativo provocado por el virus o la vacuna DNA.

Los genes relacionados con la respuesta de IFN1, el factor regulador del interferón 8 (*irf8*), *mx*, *ifit5* y *pkr* también resultaron sobreexpresados por qPCR (Figura 5, P3) en los PB-RBCs de individuos inmunizados con GVHSV, aunque no de manera significativa. Ya se ha descrito que RBCs expuestos a VHSV *in vitro* sobreexpresaban genes relacionados con esta ruta [33] y previamente en esta tesis ya se ha visto que RBCs transfectados con GVHSV *in vitro* también sobreexpresaban genes de la ruta de IFN1.

Por otro lado, el análisis del enriquecimiento de rutas funcionales utilizando la base de datos de ontología génica de procesos del sistema inmune reveló la sobrerrepresentación del procesamiento y presentación de péptidos antigénicos vía MHC clase I (Figura 3B,E P3). Entre los genes sobreexpresados destacamos beta-2-microglobulina (*b2m*), calnexina (*canx*), proteína de unión a TAP -like (*tapbpl*), y genes relacionados con el proteosoma como subunidad alfa 3 del proteosoma (*psma3*) y la subunidad alfa 7 del proteosoma (*psma7*). Más aún, la expresión de genes relacionados con la presentación de antígenos como *mhcI*, *mhcII,* cluster de diferenciación 83 (*cd83*) y *dnm2* resultó sobreexpresada por qPCR, aunque estos resultados no fueron estadísticamente significativos (Figura 5, P3).

Finalmente, el análisis de la interacción proteína-proteína de los genes sobrerrepresentados en los PB-RBCs de individuos inmunizados con GVHSV reveló una alta interacción entre los genes identificados confirmando las rutas sobrerrepresentadas mediante el análisis de enriquecimiento de rutas funcionales (Figura 4B, P3).

2.3. El proteoma de PB-RBCs de individuos inmunizados con GVHSV revelan rutas de presentación de antígenos y regulación de hemopoyesis

El proteoma de PB-RBCs de individuos inmunizados con GVHSV identificó 848 DEPs, de las cuales 573 resultaron sobreexpresadas y 275 infraexpresadas comparadas con el control (PB-RBCs de individuos inyectados con el plásmido control). El análisis de enriquecimiento de rutas funcionales utilizando la base de datos de ontología génica de procesos biológicos reveló la sobrerrepresentación de procesos metabólicos relacionados con nitrógeno, transporte intracelular y regulación de respuesta a estrés, entre otros (Figura 2C, P3).

Por otro lado, el análisis de enriquecimiento de rutas funcionales utilizando la base de datos de ontología génica de procesos del sistema inmune reveló la sobrerrepresentación de la ruta de procesamiento y presentación de péptidos exógenos y la regulación de la hemopoyesis (Figura

3C,F, P3). Entre las DEPs, destacamos las proteínas del complejo mayor de histocompatibilidad clase I, B (HLA-B) y la proteína de unión a TAP (TAPBP). La sobrerrepresentación de la ruta de presentación de antígenos vía MHC clase I se identificó en el proteoma y también en el transcriptoma de los RBCs de sangre periférica y riñón anterior, como se comentó en los apartados anteriores. El complejo MHCI se caracteriza por la presentación de péptidos endógenos y juega un papel importante en la defensa frente a virus [49]. RBCs de individuos infectados con los virus PRV y RBIV mostraron la sobreexpresión de genes y proteínas relacionadas con la presentación vía MHCI.

Finalmente, el análisis de la interacción proteína-proteína de las proteínas sobrerrepresentados en los PB-RBCs de individuos inmunizados con GVHSV reveló una alta interacción entre los genes identificados confirmando las rutas sobrerrepresentadas mediante el análisis de enriquecimiento de rutas (Figura 4C, P3).

En general, nuestros resultados indicaron que la principal ruta sobrerrepresentada en el transcriptoma y proteoma de HK-RBCs y PB-RBCs tras la inmunización de individuos con GVHSV, fue la ruta de presentación de péptidos exógenos vía MHCI. La presentación de péptidos exógenos vía MHCI se denomina presentación cruzada ("crosspresentation") [185-188]. Los péptidos exógenos se presentan en la superficie de las células, junto con el MHCI. La presentación cruzada ha sido especialmente descrita para APCs [189, 190]. Como ya se comentó anteriormente en esta tesis, el análisis del proteoma de RBCs transfectados con GVHSV *in vitro* también mostró la sobreexpresión de la ruta de presentación de péptidos exógenos vía MHCI, dependiente de TAP y el transcriptoma reveló la sobrerrepresentación de la ruta de escisión proteosomal de antígenos exógenos. Por otro lado, aunque ya se ha descrito la expresión de MHCII en RBCs nucleados, como ya se comentó en la introducción de esta tesis, la evaluación e implicación de esta molécula y su ruta en RBCs se encuentra bajo estudio.

2.4. Los RBCs transfectados con GVHSV inducen proliferación y marcadores de diferenciación en leucocitos

Dado que los RBCs se encuentran distribuidos por todo el organismo, una comunicación eficaz entre RBCs y otras células del sistema inmune sería muy ventajosa a la hora de utilizar los RBCs como células diana para vacunas o inmunoestimulantes. El cocultivo de RBCs transfectados con GVHSV y WBCs autólogos resultó en un aumento del número de células con respecto a los RBCs transfectados con el plásmido control y cultivados con WBCs autólogos, aunque estos resultados no fueron estadísticamente significativos (Figura 6A, P3). Por otro lado, se analizó la expresión de genes marcadores de células T, como el receptor de células T (*tcr*) y el cluster de diferenciación 8 (*cd8*) y de células B, como el factor de transcripción pax5 (*pax5*) y la inmunoglobulina M de membrana (*igm*) en los cocultivos. La expresión de estos genes resultó sobreexpresada en el cocultivo de RBCs transfectados con GVHSV y WBCs autólogos en comparación con el control (Figura 6B, P3), sugiriendo que la expresión de GVHSV en RBCs es capaz de estimular las poblaciones de células T y B.

2.5. RBCs transfectados con GVHSV inducen una respuesta humoral

Para analizar si los RBCs nucleados portadores de una vacuna DNA eran capaces de generar una respuesta humoral *in vivo,* RBCs transfectados *in vitro* con GVHSV se reinfundieron en la vena caudal de individuos adultos y 30 días post-inyección se detectaron anticuerpos específicos anti-VHSV en el suero de los individuos inyectados. La reinfusión de RBCs transfectados con GVHSV resultó en el mismo nivel de anticuerpos que la inyección im con el plásmido. Sin embargo, la administración intravenosa (iv) del plásmido pmTFP1GVHSV resultó en niveles negativos de detección igual que el control negativo de peces no infectados ni inmunizados (Figura 7, P3). Se ha descrito que los RBCs no nucleados tienen capacidad de inducir una respuesta humoral como portadores de una vacuna [75]. Por tanto, nuestros resultados demuestran que RBCs nucleados portadores de la vacuna son capaces de generar una respuesta humoral en el organismo tan efectiva como la administración de la vacuna vía im.

Todo esto, sumado a la capacidad de los RBCs de producir citoquinas bajo estímulo, inducir proliferación de células T y B y su involucración en el procesamiento y presentación de antígenos, nos lleva a sugerir que los RBCs nucleados podrían comportarse como APCs atípicas. Recientemente, células como mastocitos, basófilos, eosinófilos, células linfoides innatas y neutrófilos [56, 58] han sido propuestas como APCs atípicas. Estas células principalmente difieren de las APCs profesionales en que su expresión de MHCII no es constitutiva y no se conoce su capacidad o son incapaces de primar células T CD4+ [56]. Recientemente, se ha propuesto a los neutrófilos como APCs atípicas por su capacidad de inducir MHCII y moléculas asociadas bajo un estímulo, presentar un antígeno a células T CD4+, establecer comunicación con leucocitos, sintetizar citoquinas frente a un estímulo y comportarse como lazo de unión entre la respuesta innata y adaptativa entre otras [58]. Durante esta tesis, y basándonos en estudios recientes de RBCs nucleados, observamos que los RBCs de teleósteos comparten algunas de las funciones descritas para neutrófilos. Los RBCs inducen moléculas relacionadas con MHCII bajo estímulos como la transfección o inmunización con vacunas DNA, como se ha mostrado en las publicaciones 2 y 3 de esta tesis. Como se había descrito previamente y en esta tesis, los RBCs son capaces de sobreexpresar citoquinas tanto *in vitro* como *in vivo* y además son capaces de inducir una respuesta humoral cuando actúan como portadores de una vacuna DNA. Además, la elevada cantidad de RBCs presente en el organismo supliría la limitada capacidad de presentación que podrían tener estas APCs atípicas en comparación con las APCs profesionales [58]. Siendo que las APCs son excelentes candidatas para las estrategias de vacunación [60, 62-66], nuestros resultados nos llevan a sugerir a los RBCs como células diana candidatas para el diseño de nuevas estrategias de vacunación o inmunoestimulación.

3. LOS RBCs MODULAN LA EXPRESIÓN GÉNICA EN RESPUESTA A CUERPOS DE INCLUSIÓN DE TNFα Y DE UN FRAGMENTO DE LA PROTEÍNA GVHSV

En este trabajo exploramos la capacidad de los RBCs de trucha arcoíris de endocitar IBs así como de generar una respuesta inmune *in vitro* e *in vivo* frente a $IB^{TNFα}$ y $IB^{frg16G\text{-}VHSV}$. Los resultados de este apartado corresponden con los resultados mostrados en la publicación 4 (P4).

3.1. Internalización/adhesión de $IB^{TNFα}$ y $IB^{frg16G\text{-}VHSV}$ en RBCs

El tratamiento de RBCs de trucha arcoíris con los IBs mostró que conforme aumentaba la concentración de IBs aumentaba el porcentaje de internalización/adhesión en RBCs. El porcentaje internalización/adhesión fue del 5% en los RBCs incubados con 50 µg/ml de $IB^{frg16G\text{-}VHSV}$ y del 7% en los RBCs incubados con $IB^{TNFα}$ (Figura 1A, P4). Por otro lado, a concentraciones más altas de IBs la internalización/adhesión llegó hasta el 17% en RBCs incubados con $IB^{TNFα}$. Estos resultados aunque significativos son inferiores a los descritos para ZFL y RT-HKM, donde se observó el 40% y 80% de internalización de $IB^{TNFα}$, respectivamente [136], y casi el 100% de internalización de $IB^{frg16G\text{-}VHSV}$ en células ZFL [141]. No se observaron diferencias de internalización/adhesión entre los distintos tiempos de incubación, lo que indicó que el máximo de internalización/adhesión ocurre a las 6 h post-incubación (Figura 1B, P4). Este resultado contrasta con lo descrito anteriormente para ZFL y RT-HKM, donde la máxima internalización fue alcanzada a las 24 h [140, 141]. Imágenes 3D de microscopía confocal nos permitieron confirmar la internalización de IBs en el citosol de RBCs (Figura 2A,B, P4). Hasta el momento, se desconoce el mecanismo responsable de esta internalización, que podría ocurrir mediante micropinocitosis, como se ha propuesto para células de mamífero [139].
Finalmente, se evaluó la distribución de $IB^{TNFα}$ en individuos inyectados iv a 3 h post-inyección, y se detectaron, por microscopía de fluorescencia, RBCs de sangre periférica y riñón anterior portadores de $IB^{TNFα}$, siendo la presencia de RBCs con $IB^{TNFα}$ más evidente en riñón anterior (Figura 2C, P4).

3.2. Respuesta inmune de RBCs inducida por $IB^{TNFα}$ y $IB^{frg16G\text{-}VHSV}$ *in vitro*

La expresión transcripcional de genes relacionados con la respuesta inmune fue evaluada, mediante qPCR, en RBCs incubados durante 24 h con $IB^{frg16G\text{-}VHSV}$, $IB^{TNFα}$ o IB^{iRFP} como control. El tratamiento de RBCs con $IB^{TNFα}$ provocó la infraregulación de la mayoría de los genes analizados, siendo significativamente infraregulada la expresión de los genes relacionados con la presentación de antígenos (*cd83* y *mhcI*) y el gen antioxidante glutatión S-transferasa P1 (*gstp1*). El tratamiento de RBCs con $IB^{frg16G\text{-}VHSV}$ mostró la sobreexpresión de genes relacionados con actividad antioxidante, siendo significativamente sobreexpresado el gen *trx* (Tabla 2, P4). Como ya se describió anteriormente en esta tesis, los RBCs incrementan la expresión de genes relacionados con la actividad antioxidante cuando se transfretan con GVHSV. De la misma manera, se ha descrito el incremento de la expresión de genes de enzimas antioxidantes tras la exposición de RBCs al virus VHSV [33].

La expresión génica en respuesta a cada IB se evaluó en su conjunto mediante un análisis multivariante de la matriz de datos de expresión génica. El análisis de componentes principales de la expresión génica mostró dos poblaciones muy diferenciadas entre los RBCs tratados con $IB^{frg16G\text{-}VHSV}$ o $IB^{TNF\alpha}$ en comparación con el control (Figura 3A, P4). El mapa de clusterización/agrupación también evidenció esta diferenciación entre tratamientos (Figura 3B, P4).

3.3. Respuesta inmune de RBCs de individuos tratados con $IB^{TNF\alpha}$ y $IB^{frg16G\text{-}VHSV}$

La expresión transcripcional de genes relacionados con la respuesta inmune fue evaluada, mediante qPCR, en RBCs de individuos inyectados iv con $IB^{frg16G\text{-}VHSV}$, $IB^{TNF\alpha}$, o IB^{iRFP} a 24 y 48 h post-inyección.

En general, como ya se observó *in vitro*, observamos una infraregulación de la expresión de genes en RBCs de individuos inyectados con $IB^{TNF\alpha}$ en comparación con RBCs de individuos inyectados con IB^{iRFP}. Esta tendencia se confirma por la infraregulación de la expresión de los genes receptor tipo toll 9 (*tlr9*), *tnfα*, *il1β*, *il12β* e interleuquina 2 (*il2*) tanto a 24 como a 48 h post-inyección (Figura 4, P4). TNFα es una citoquina con un amplio rango de acción involucrada en la regulación de células inmunes y del proceso de inflamación. Es producida por monocitos y macrófagos y otras células como linfocitos, células NK, leucocitos polimorfonucleares y eosinófilos en respuesta a un estímulo [191]. Recientemente se ha descrito que los RBCs también son capaces de modular la expresión de TNFα en respuesta a virus [35]. En este trabajo, se observa como $IB^{TNF\alpha}$ infrareguló la respuesta inflamatoria en RBCs, siendo estadísticamente significativa la infraregulación de *cd83* a 24 h post-inyección, como también se observó *in vitro.* Por otro lado, destacamos la sobreexpresión significativa de *il6* a 24 h post-inyección (Figura 4A, P4). A 48 h post-inyección se acentúa esta tendencia de infraregulación siendo estadísticamente significativa para los genes *tlr9*, *ifn1*, *il1β*, *il2*, *mhcII* y *nkef* (Figura 4B, P4). La expresión de las proteínas Mx e IL8 en RBCs de individuos inyectados con $IB^{TNF\alpha}$ resultó infraexpresada respecto a los demás tratamientos, y significativamente infraexpresada fue la expresión de Mx en comparación con IB^{iRFP} (Figura 7, P4) confirmando la tendencia infrareguladora de este tratamiento también a nivel de proteína. Por último, se analizó la expresión de estas proteínas (Mx e IL8) en la sangre total, observando la misma tendencia para Mx. No se observaron diferencias en la expresión de IL8 entre los diferentes tratamientos (Figura 8, P4). Se ha descrito que la familia TNF puede tener efectos tanto beneficiosos como perjudiciales. Una exposición prolongada a TNFα recombinante puede ocasionar efectos parecidos a un "shock séptico" [191]. Además, la inhibición por parte de TNFα de la citoquina IL12β inducida por IFN gamma es capaz de regular funciones antiinflamatorias y de reparación mediante señales de retroalimentación positivas y negativas, sin que se manifieste el proceso de inflamación [192]. Todo esto, podría explicar la infraregulación observada en RBCs en respuesta a $IB^{TNF\alpha}$. Dado el gran número de RBCs presentes en el organismo, la infraregulación de la expresión de genes relacionados con la respuesta inflamatoria podría suponer una estrategia para modular la inflamación provocada tras la exposición sistémica a TNFα recombinante. Por otro lado, también se observó infraregulación de genes relacionados con la presentación de antígenos, *cd83* y *mhcII*, a 24 y 48 h respectivamente para RBCs de individuos inyectados con $IB^{TNF\alpha}$. También la expresión génica de *cd83* y *mhcI* resultó infraexpresada en RBCs tratados con $IB^{TNF\alpha}$ *in vitro*, 24 h post-tratamiento.

Previamente, se ha descrito que TNFα modula la expresión de MHC clase II inducida por IFN gamma según el tipo celular y estadio de diferenciación. Por otro lado, puntualizar que la expresión de *cd83* y *mhcII*, genes específicamente relacionados con células APCs profesionales, ha sido recientemente descrita para RBCs de trucha [57], como se ha visto anteriormente en esta tesis, y también ha sido descrita la expresión de *mhcII* en pollo [26].

El tratamiento con $IB^{frg16G\text{-}VHSV}$ mostró una tendencia hacia la sobreexpresión génica en RBCs a 24 y 48 h post-inyección, en comparación con IB^{iRFP}. Los resultados mostraron la sobreexpresión de *il2*, *il6* y *nkef* a 24 h post-inyección (Figura 4A, P4). Los resultados también mostraron una sobreexpresión, aunque ligera, de genes relacionados con la actividad antioxidante, tanto a 24 como a 48 h post-inyección, como se observó en los ensayos *in vitro,* probablemente como un esfuerzo por parte del RBC de compensar la primera respuesta inflamatoria frente al estímulo. La expresión de genes relacionados con IFN1, receptor tipo Toll 3 (*tlr3*) y *mx* y de presentación de antígenos, *cd83* y *mhcII*, fue significativamente sobreexpresada a 48 h post-inyección (Figura 4B, P4). Se ha demostrado que la GVHSV induce la expresión de *ifn1* y *mx* [105, 161, 193] y péptidos de la GVHSV también inducen respuestas relacionadas con IFN1 [105, 161, 162] en otros tipos celulares. La expresión de la proteína Mx e IL8 se analizó a 48 h post-inyección. La expresión de Mx en RBCs de individuos inyectados con $IB^{frg16G\text{-}VHSV}$ resultó sobreexpresada en comparación con los demás tratamientos, aunque no significativamente (Figura 7A,C, P4) lo que coincide con la expresión del gen *mx* a 48 h post-inyección. También hubo un incremento de la expresión de IL8 respecto a los demás tratamientos (Figura 7B,D, P4). A nivel de proteína se confirma esta tendencia a la sobreexpresión de genes en RBCs de individuos inyectados con $IB^{frg16G\text{-}VHSV}$, que se observó en la expresión génica. Por otro lado, como se ha visto anteriormente en esta tesis, la transfección de RBCs con GVHSV aumentó significativamente la expresión de *mhcII* (Figura 5, P2). Igualmente, la inmunización de individuos con GVHSV también aumentó ligeramente la expresión de *mhcII* y *cd83* (Figura 5, P3). Estos resultados en su conjunto indican que la exposición de RBCs a un antígeno viral (sea cual sea su forma de administración) desencadena una respuesta relacionada con la presentación de antígenos, poniendo de manifiesto que los RBCs podrían comportarse como APCs no-profesionales o atípicas.
Finalmente, el análisis de componentes principales de la matriz de datos de expresión génica mostró la clusterización/agrupación diferenciada de RBCs tratados con $IB^{frg16G\text{-}VHSV}$, $IB^{TNF\alpha}$ o IB^{iRFP} (Figura 5A,6A, P4). El mapa de clusterización/agrupación también evidenció esta diferenciación entre tratamientos (Figura 5B,6B, P4).

CONCLUSIONES

CONCLUSIONES

1. Los RBCs de trucha arcoíris son capaces de expresar un antígeno codificado por una vacuna DNA.

2. Los RBCs de trucha arcoíris son capaces de modular la expresión de genes y proteínas relacionados con la respuesta inmune cuando expresan la proteína antigénica GVHSV.

3. Los RBCs de trucha arcoíris ejercen comunicación paracrina con otras células cuando se transfectan con GVHSV.

4. La inmunización de trucha arcoíris con una vacuna DNA que codifica la proteína antigénica GVHSV induce procesos de presentación de péptidos exógenos en RBCs de riñón anterior y sangre periférica, a nivel transcripcional y traduccional.

5. La transfusión de trucha arcoíris con RBCs transfectados con GVHSV desencadena una respuesta humoral en el organismo.

6. Los RBCs de trucha arcoíris son capaces de endocitar cuerpos de inclusión (IBs) de origen bacteriano y de modular la expresión de genes del sistema inmune en respuesta a la proteína recombinante TNFα y un fragmento de la proteína GVHSV.

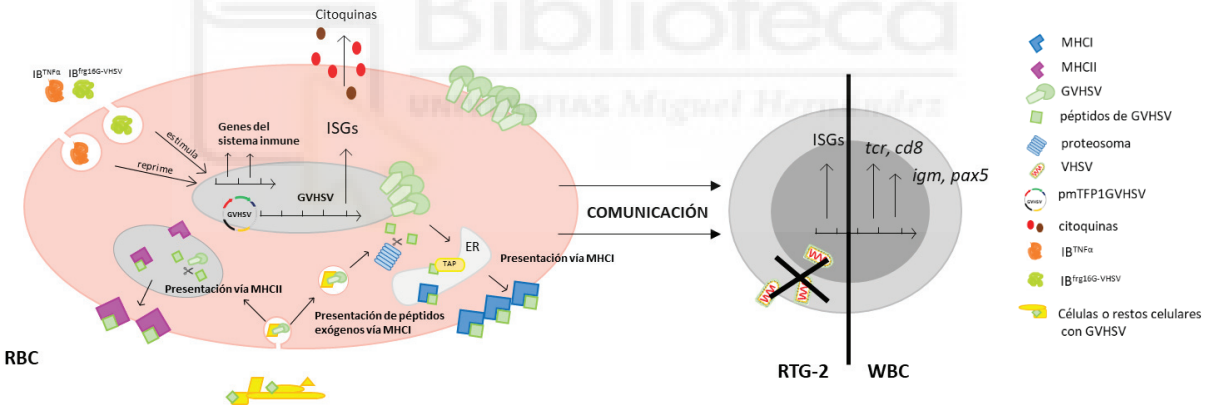

Figura 13: Resumen de los procesos descritos o sugeridos en esta tesis para los RBCs de trucha arcoíris en la inmunización frente a rhabdovirus.

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PUBLICACIÓN 1

TÍTULO: In silico functional networks identified in fish nucleated red blood cells by means of transcriptomic and proteomic profiling

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Communication

In Silico Functional Networks Identified in Fish Nucleated Red Blood Cells by Means of Transcriptomic and Proteomic Profiling

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Abstract: Nucleated red blood cells (RBCs) of fish have, in the last decade, been implicated in several immune-related functions, such as antiviral response, phagocytosis or cytokine-mediated signaling. RNA-sequencing (RNA-seq) and label-free shotgun proteomic analyses were carried out for in silico functional pathway profiling of rainbow trout RBCs. For RNA-seq, a de novo assembly was conducted, in order to create a transcriptome database for RBCs. For proteome profiling, we developed a proteomic method that combined: (a) fractionation into cytosolic and membrane fractions, (b) hemoglobin removal of the cytosolic fraction, (c) protein digestion, and (d) a novel step with pH reversed-phase peptide fractionation and final Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC ESI-MS/MS) analysis of each fraction. Combined transcriptome- and proteome- sequencing data identified, in silico, novel and striking immune functional networks for rainbow trout nucleated RBCs, which are mainly linked to innate and adaptive immunity. Functional pathways related to regulation of hematopoietic cell differentiation, antigen presentation via major histocompatibility complex class II (MHCII), leukocyte differentiation and regulation of leukocyte activation were identified. These preliminary findings further implicate nucleated RBCs in immune function, such as antigen presentation and leukocyte activation.

Keywords: rainbow trout; red blood cells; RNA-seq; de novo assembly; transcriptome; peptide fractionation; LC ESI-MSMS; proteome; functional network; immune response

1. Introduction

Red blood cells (RBCs) are the most copious cell type in blood circulation and are well-known for their roles in respiration. Also, other roles such as modulation of angiogenesis, coagulation, vascular tone and inflammation have been described for mammalian RBCs (reviewed in Akbari A. 2011) [1]. In mammals, mature RBCs lack cell nuclei, organelles, and ribosomes [2]. In contrast, in non-mammalian vertebrates, RBCs have cell nuclei and organelles in their cytoplasm [3]. The role of nucleated RBCs as immune response intermediaries is a novel field of research [4]. RBCs, rich in hemoglobin, were thought to drive processes of gas exchange to tissues. However, in the recent past, a set of biological processes related to immunity, such as phagocytosis and presentation [5], interferons production [6–9] and cytokines production [7,8,10], have been

reported in nucleated RBCs from different non-mammal vertebrate species. During the last decade, transcriptomic and proteomic sequencing have allowed us to identify many more genes and proteins in RBCs. Transcriptome sequencing of nucleated RBCs has identified the genes responsible for the expression of a wide spectrum of biological processes, including immune response [6,11]. On the other hand, proteomics sequencing of non-nucleated RBCs has significantly evolved [12–15], allowing us to significantly increase the number of identified proteins from a few hundred to almost 2700 proteins [12,13]. However, to our knowledge, no study on nucleated RBCs proteome sequencing has been published (although extensive research exists on cell proteome of the different cell types during human erythroid differentiation [16]).

In this manuscript we show a combined transcriptomic and proteomic evaluation of rainbow trout nucleated RBCs (see Figure 1 for a representative schema of the procedure). In order to achieve this, we performed RNA-sequencing (RNA-seq) and label-free shotgun proteomic analyses of RBCs pooled from eight fishes. For transcriptome profiling, a de novo assembly of rainbow trout RBCs was conducted to create a transcriptome database for RBCs gene mapping. For proteome profiling, we developed a novel proteomic analysis method that combined: (a) fractionation into cytosolic and membrane fractions, (b) hemoglobin removal of the cytosolic fraction, (c) protein digestion, and (d) a novel step with pH reversed-phase peptide fractionation and final Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC ESI-MS/MS) analysis of each of the fractions.

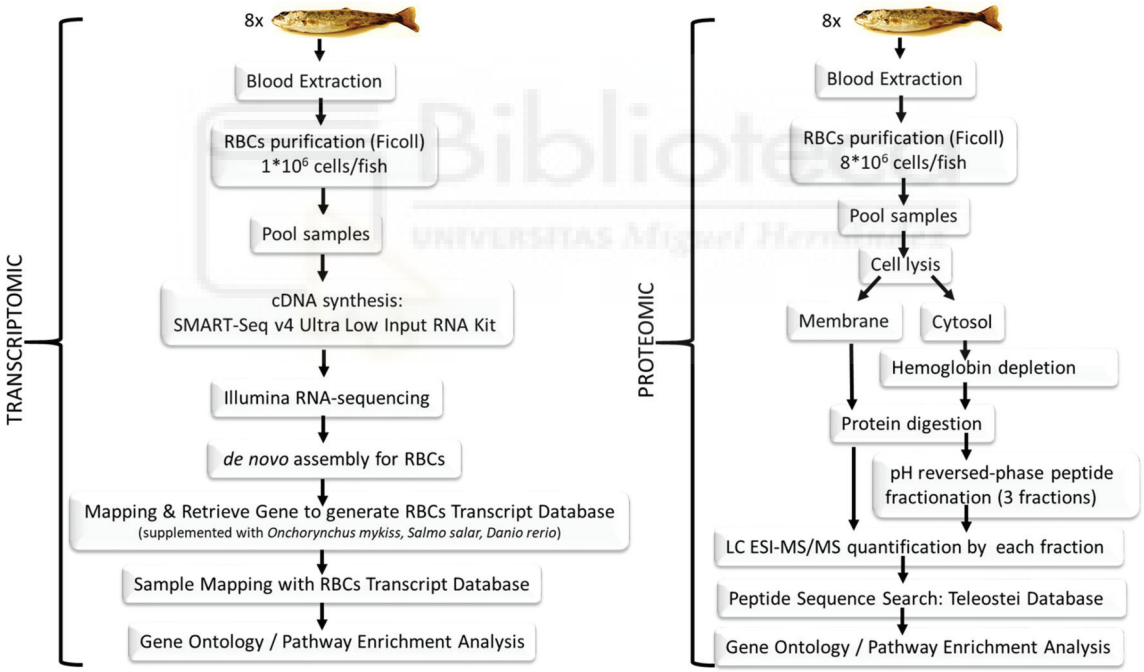


Figure 1. Schema representing the different steps in the experiment described here, from sample collection to data analysis.

In silico functional profiling revealed the presence of novel and striking networks for rainbow trout nucleated RBCs, mainly related to innate and adaptive immunity. Functional pathways related to regulation of hematopoietic cell differentiation, antigen presentation via major histocompatibility complex class II (MHCII), leukocyte differentiation, and regulation of leukocyte activation were found in rainbow trout RBCs transcriptome. This study provides new knowledge on the immune functions of nucleated RBCs.

2. Materials and Methods

2.1. Fish

Rainbow trout (*Oncorhynchus mykiss*) of approximately 6 cm, obtained from a commercial fish farm (PISZOLLA S.L., CIMBALLA FISH FARM, Zaragoza, Spain), were maintained at University Miguel Hernandez (UMH) facilities at 14 °C, with a re-circulating dechlorinated-water system, at a stocking density of 1 fish/3 L, and fed daily with a commercial diet (Skretting, Burgos, Spain). Fish were acclimatized to laboratory conditions over 2 weeks. All activities involving animal handling and animal care were done in accordance with EU Directive EC86/609.

2.2. Blood Sampling and Red Blood Cells Purification

Rainbow trout RBCs were obtained from the peripheral blood of fish which died through overexposure to tricaine (tricaine methanesulfonate, Sigma-Aldrich, Madrid, Spain; 0.2 g/L), as previously described [8]. Briefly, peripheral blood was sampled from the caudal vein. Then, RBCs were purified by two consecutive density gradient centrifugations (7206 *g*, Ficoll 1.007; Sigma-Aldrich). Purity of RBCs of 99.9% was estimated by optical microscopy evaluation (Figure S1).

2.3. Transcriptome Sequencing

2.3.1. Complementary DNA Library Preparation and Illumina Sequencing

RBCs isolated from eight fishes (10^6 cells per fish) were lysed with 9.5 µL of 10× Lysis buffer (Clontech, Takara Bio, Mountain View, CA, USA) and 0.5 µL of ribonuclease (RNase) Inhibitor (Invitrogen, Thermo-Fischer Scientific Inc., Waltham, MA, USA), and preserved at −80 °C, until complementary DNA (cDNA) library construction.

Lysed RBCs from the eight fishes were pooled, and cDNA was directly produced from lysed cells using a SMART-Seq v4 Ultra Low Input RNA Kit (Clontech, Takara Bio). cDNA integrity was tested using a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). The library construction was carried out using an Illumina Nextera XT Library Preparation Kit (Illumina Inc., San Diego, CA, USA). Generated cDNA fragments were sequenced with the lllumina Hiseq 2500 platform, using 100 bp paired-end sequencing reads. Sequence reads are available at SRA-NCBI, SRA-NCBI Accession SRP133501. RNA-Seq library preparation and sequencing were carried out by STABVida Lda (Caparica, Portugal).

2.3.2. De Novo Assembly Bioinformatics Procedure

In order to create a transcript database specific to, or enriched for, rainbow trout RBCs, a de novo assembly of RBCs expressed short reads was carried out. CLC Genomics Workbench (version 9.5.4) [17] was used for expressed short reads de novo assembly. Raw data were filtered by removing short, duplicated and low quality reads. For each original read, the regions of the sequence to be removed were determined independently for each type of trimming operation: Quality trimming (based on quality ratings), and Ambiguity trimming. The trimming parameters applied were: ambiguous limit = 2 nucleotides, quality limit = 0.01 (error probability), minimum number of nucleotides = 30. After quality trimming, raw sequence data were de novo assembled. One list of sequences corresponding to generated contigs, and one mapping file were generated. After initial contig creation, reads were mapped back to contigs for assembly correction, using the following parameters: word size = 54, bubble size = 50, length fraction = 0.8 and similarity fraction = 0.8. To remove redundancy from assemblies, generated contigs were analyzed with CD HIT EST (Version 4.6) [18,19], using the following parameters: -c 0.85 -n 8.

2.3.3. BLASTing of Assembled Contigs, Gene Sequence Retrieval, Red Blood Cells Transcript Database Construction and Functional Annotation

De novo assembled contigs were BLASTed (using Nucleotide Basic Local Alignment Search Tool, BLASTn, https://blast.ncbi.nlm.nih.gov/Blast.cgi), with a cut off E-value of 1.00×10^{-3}) against a local Teleostei messenger RNA (mRNA) Reference Sequence (RefSeq) database downloaded from NCBI (https://www.ncbi.nlm.nih.gov, last update: 20072017), using Blast2GO PRO version 4.1.9 [20]. Then, full sequences of the top blast hit obtained in the previous step were retrieved from NCBI, based on accession ids. Duplicated and similar sequences with 95% similarity were removed. These steps were performed using Blast2GO PRO version 4.1.9. Resulting mRNA RefSeq curated database{Ortega-Villaizan, 2018 #28} (referred to hereafter as RBCs transcript database [21]), was enriched with rainbow trout (NCBI, last update: 09082017), Atlantic salmon-*Salmo salar*-(NCBI, last update: 09082917), and zebrafish-*Danio rerio*-(NCBI, last update: 31072017) mRNA RefSeq annotations in NCBI. This curated and enriched database was used as a reference for following sample mapping and annotation analyses. The RBCs transcript database was finally annotated against local Teleostei protein RefSeq database downloaded from NCBI (last update: 17072017), using Basic Local Alignment Search Tool (BLASTx, https://blast.ncbi.nlm.nih.gov/Blast.cgi) with a cut off E value of 1.00×10^{-3}.

2.3.4. Red Blood Cells Transcriptome Mapping and Gene Expression Profiling

Sample raw sequence data was mapped against the RBCs transcript database using CLC Genomics Workbench (version 10.1.1) [22]. Before mapping, analysis started with the trimming of raw sequences to generate high quality data only. For each original read, the regions of the sequence to be removed were determined independently for each type of trimming operation: Quality trimming (based on quality ratings), Ambiguity trimming, and Length trimming. The following trimming parameters were applied: ambiguous limit = 2 nucleotides, quality limit = 0.01 (error probability), minimum number of nucleotides = 15. High quality sequencing reads (approximately 40 million reads) were mapped against RBCs transcript database, using the following parameters: length fraction = 0.6, similarity fraction = 0.5. Gene prediction and annotation was conducted on the RBCs transcripts database. The expression level of genes was obtained by counting the number of reads mapped to a gene.

2.4. Proteome Sequencing

2.4.1. Protein Digestion

RBCs isolated from eight fishes (8×10^6 cells per fish) were pooled and pelleted by centrifugation (5 min, 700× *g*). Supernatant was removed, and cell pellet (~70–100 µL) was mixed with 250 µL of deionized water, and then frozen at −80 °C for 3 h. After thawing, it was centrifuged at 17,000× *g* for 20 min at 4 °C, to separate cytosolic supernatant and pelleted membrane fractions. To purify the membrane fraction, the pellet was washed twice with 500 µL of deionized water, and then centrifuged at 20,000× *g* for 10 min at 4 °C. After washing, the new membrane pellet was dissolved with 200 µL of chaotropic lysis buffer containing 8.4 M urea (USB Corporation, Cleveland, OH, USA), 2.4 M thiourea (Sigma-Aldrich), 5% CHAPS (Sigma-Aldrich), 5 mM TCEP (Sigma-Aldrich) and a protease inhibitor cocktail (Sigma-Aldrich), for 15 min, on ice. Homogenization of the membrane pellet was achieved by ultrasonication for 5 min on ultrasonic bath Branson 2510 (Marshall Scientific, Hampton, NH, USA). The sonicated membrane was centrifuged again at 20,000× *g* for 10 min at 4 °C, and the supernatant containing solubilized membrane fraction proteins was used for further analysis. Then, 40 µg of protein was precipitated using the methanol/chloroform method [23], and re-suspended in 20 µL of multichaotropic sample solution composed of 7 M Urea, 2 M thiourea and 10 mM triethylammonium bicarbonate (TEAB) (Sigma-Aldrich), called hereafter UTT buffer.

The cytosolic fraction, approximately 300 µL, was depleted of hemoglobin using HemoVoid™ kit (Biotech Support Group, Monmouth Junction, NJ, USA), in accordance with the manufacturer's instructions [24]. After hemoglobin removal, the eluted fraction was transferred to a Pall Omega Nanosep® (Pall Corporation, Port Washington, NY, USA) centrifugal filter device (molecular weight cut-off (MWCO) 10 kDa), and concentrated by centrifugation at 14,000× *g* for 15 min at 4 °C. Finally, the hemoglobin depleted-cytosolic fraction were dialyzed with 300 µL of UTT buffer, and concentrated into a volume of ~50–80 µL (20 min, 14,000× *g*, 4 °C). Then, 40 µg of protein were diluted with 20 µL of multichaotropic sample solution UTT buffer.

Both protein fractions were reduced with 2 µL of 50 mM TCEP, pH 8.0, at 37 °C for 60 min, before 1 µL of 200 mM cysteine-blocking reagent MMTS (SCIEX, Foster City, CA, USA) was added for 10 min at room temperature. Then, the cytosolic and membrane fractions were diluted to 140 µL with 25 mM TEAB, to reduce the urea concentration. Finally, digestions were initiated by adding 6 and 2 µg respectively of Pierce MS-grade trypsin (Thermo-Fisher Scientific Inc., Waltham, MA, USA) to each fraction, in a ratio of 1:20 (*w*/*w*), and then incubated at 37 °C overnight on a shaker. The fraction digestions were evaporated to dryness in a vacuum concentrator.

2.4.2. pH Reversed-Phase Peptide Fractionation

Offline high pH reversed-phase peptide fractionation of peptides from cytosolic fraction was performed on a SmartLine (Knauer, Berlin, Germany) high pressure liquid chromatography (HPLC) system using an XBridge C18 column (100 × 2.1 mm, 5 µm particle) (Waters Corporation, Milford, MA, USA). Mobile phases A and B were used for chromatography. The composition of mobile phase A was 10 mM ammonium hydroxide (pH 9.4) (Sigma-Aldrich), whereas composition of mobile phase B was 80% methanol (Scharlab S.L, Barcelona, Spain) and 10 mM ammonium hydroxide (pH 9.3). Dried-up peptide pellet was dissolved in 100 µL of mobile phase A, injected into a sample loop, and then fractionated using a linear gradient of 0–100% mobile phase B at 150 µL/min for 90 min. Thirty fractions were collected and then pooled, with an alternating numerical sequence, into three fractions (i.e., fractions 1 + 4 + 7 + 10 + 13 + 16 + 19 + 22 + 25 + 28) and dried.

2.4.3. Liquid Chromatography and Mass Spectrometry Analysis

Peptide fractions were cleaned/desalted using Stage-Tips with Empore 3M C18 disks (Sigma-Aldrich). One microgram of each peptide fraction was used for a 1D-nano LC ESI-MS/MS analysis, using a nano-liquid chromatography system (Eksigent Technologies nano LC Ultra 1D plus; SCIEX, Foster City, CA, USA), coupled to a 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 (Waters Corporation). The trap column was a C18 Acclaim PepMap™ 100 (Thermo-Fisher Scientific Inc.), 100 µm × 2 cm, 5 µm particle diameter, 100 Å pore size, switched on-line with the analytical column. A 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. Cytosolic peptide fractions were separated using a 150 min gradient ranging from 2% to 90% mobile phase B (mobile phase A: 2% acetonitrile (Scharlab S.L), 0.1% formic acid (Sigma-Aldrich); mobile phase B: 100% acetonitrile, 0.1% formic acid). Two hundred & fifty minutes' gradient was used for the membrane fraction, using the same gradient conditions. Injection volume was 5 µL.

Data were acquired using an ionspray voltage floating 2300 V, curtain gas 35, interface heater temperature 150, ion source gas 125 and declustering potential 150 V. For Information-Dependent Acquisition (IDA) parameters, 0.25 s mass spectrometry (MS) survey scan in the mass range of 350–1250 Da were followed by 35 MS/MS scans of 100 ms 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 a charge state of 2–5 and an abundance threshold >90 counts (cps). Former target ions were excluded for 15 s.

2.4.4. Proteomics Data Analysis and Sequence Search

Mass spectrometry data obtained were processed using PeakView 2.2 Software (SCIEX [25]) and exported as mgf files, which were then searched, using Mascot Server v2.5.1 (Matrix Science, London, UK), against a protein database including Teleostei protein sequences from Uniprot/Swissprot Knowledgebase (last update: 20170412, 2.542.118 sequences), together with commonly occurring contaminants. Search parameters were set as follows: enzyme, trypsin; allowed missed cleavages, 2; methylthiolation (C) as fixed modification; and acetyl (Protein N-term), Oxidation (M), Gln → pyro-Glu (N-term Q) and Glu → pyro-Glu (N-term E) as variable modifications. Peptide mass tolerance was set to ±25 ppm for precursors and 0.05 Da for fragment masses. 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% False Discovery Rate (FDR) at PSM (peptide-to-spectrum matches) level were considered to have been correctly identified.

2.5. Pathway Enrichment Analysis

In order to evaluate functionally grouped Gene Ontology (GO) and pathway annotation networks of expressed genes and proteins, pathway enrichment analysis was performed using the ClueGO [26] and CluePedia [27] Cytoscape [28] plugins. The GO Immune System Process functional pathway database was used. p value ≤ 0.05 and Kappa score of 0.4 served as threshold values. Protein-protein interaction (PPI) networks were analyzed using STRING v10.5 (http://string.embl.de/) [29], with a medium confidence score threshold of 0.4. The *Homo sapiens* model organism was used for ClueGO and STRING analyses. Gene symbols were obtained through sequence homology of RBCs transcript database genes with *Homo sapiens* orthologues, using Blast2GO version 4.1.9.

2.6. RNA Extraction and Reverse Transcription Real Time Polymerase Chain Reaction Analysis

RNA extraction and reverse transcription real time polymerase chain reaction (RT-qPCR) was performed as previously described [8], using specific primers and probe for *mhcII* gene (Forward:TGCCATGCTGATGTGCAG; Reverse: GTCCCTCAGCCAGGTCACT; Probe: CGCCTATGACTTCTACCCCAAACAAAT) [30]. Gene expression was analyzed by the $2^{-\Delta Ct}$ method [31] and *ef1α* gene (Forward: ACCCTCCTCTTGGTCGTTTC; Reverse: TGATGACACCAACAGCAACA; Probe: GCTGTGCGTGACATGAGGCA) [32] was used as endogenous control.

Besides, RNA extracted from RTS-11 cell line [33] (donated by Dr. Niels Bols) and RTG-2 cell line (ATCC® CCL-55™) were respectively used as antigen presenting cell (APC) and non-APC cell types, for *mhcII* gene expression comparison.

2.7. RBCs Single-Cell Sorting

RBCs from one fish were single-cell sorted, in order to obtain a sample of pure RBCs (20–30 cells), using BD FACSJazz™ cell sorter (BD Biosciences, Madrid, Spain). Sorted RBCs were visualized using an IN Cell Analyzer 6000 (GE Healthcare, Little Chalfont, UK) cell imaging system. The sample was lysed with 9.5 µL of 10× Lysis buffer and 0.5 µL of RNase Inhibitor, and preserved at −80 °C until cDNA library construction.

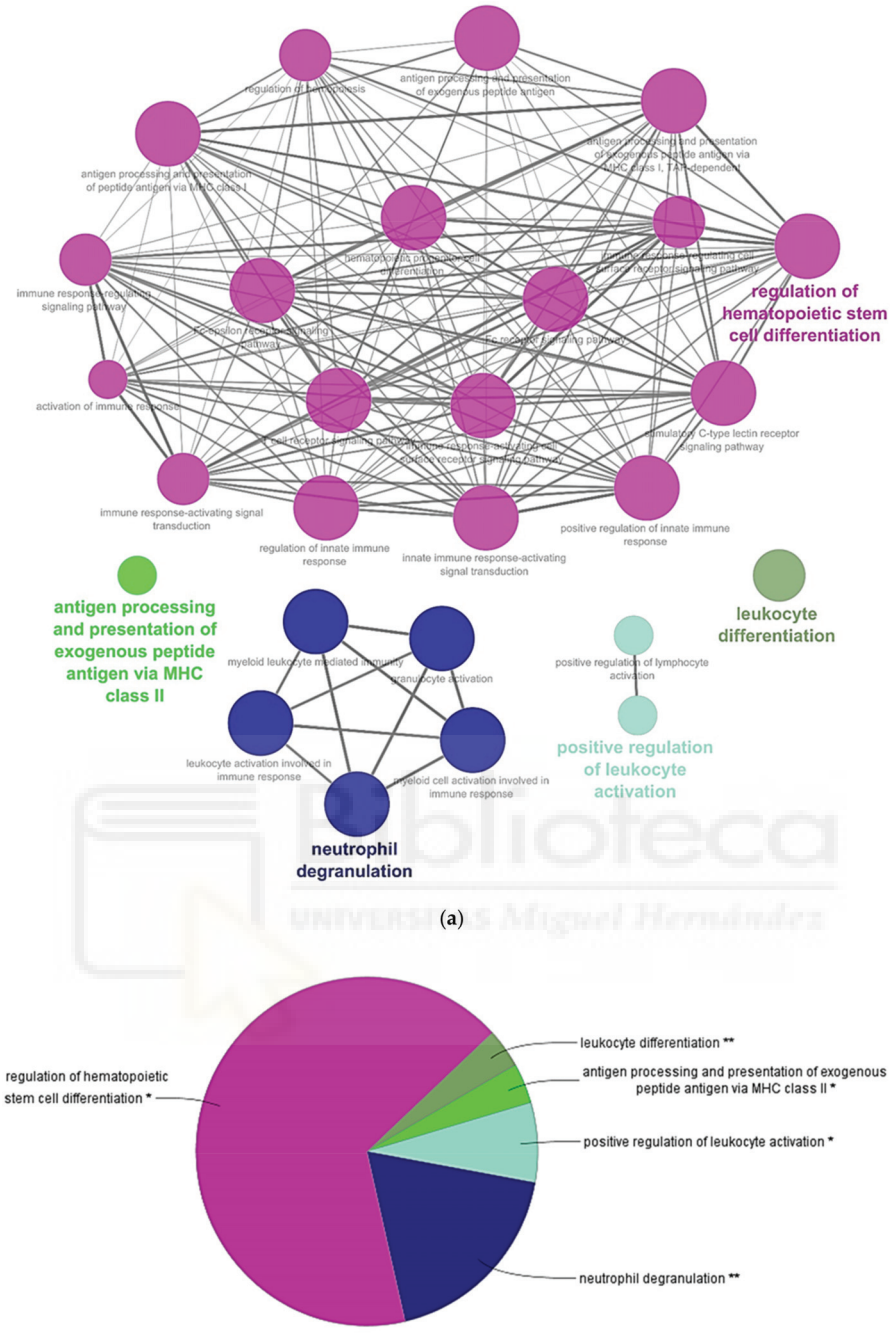
3. Results and Discussion

RBCs transcriptome profiling identified 14,008 genes, from which 13,937 genes were considered expressed since they were detected above a threshold of 10 reads mapped. Table 1 shows statistics of de novo assembly, RNA-seq raw data and mapping. Conversely, proteome profiling identified 1.770 proteins, where 724 proteins had more than 2 PSMs. Among those genes with more than 10 gene reads and proteins with more than 2 PSMs and 670 genes and proteins (Table S1), were common to both transcriptome and proteome profiling respectively (Figure S2). A Cytoscape

pathway enrichment analysis, with Immune System Process GO-terms, was performed in order to evaluate functionally grouped GO-terms and pathway annotation networks which are mainly represented in rainbow trout nucleated RBCs immune response. Results showed five strongly represented networks of interest: (i) regulation of hematopoietic stem cell differentiation, (ii) neutrophil degranulation, (iii) positive regulation of leukocyte activation, (iv) antigen processing and presentation of exogenous peptide antigen via MHCII, and (v) leukocyte differentiation (Figure 2a,b, Table S2). Subsequently, an interactome network was built for each GO-term set of proteins, to identify protein-protein interactions, and predict functional associations. We found that proteins grouped in antigen processing and presentation of exogenous peptide antigen via MHCII network highly interacted with each other (Figure 3), with a FDR p value 1.37×10^{-27} and PPI enrichment p value $< 1.0 \times 10^{-16}$. Fourteen proteins identified in this GO-term were: ACTR1B (ARP1 actin related protein 1 homolog B), AP1B1 (adaptor related protein complex 1 beta 1 subunit), AP2A1 (adaptor related protein complex 2 alpha 1 subunit), AP2A2 (adaptor related protein complex 2 alpha 2 subunit), ARF1 (ADP ribosylation factor 1), CANX (calnexin), CAPZA1 (capping actin protein of muscle Z-line alpha subunit 1), CLTA (clathrin light chain A), CLTC (clathrin heavy chain), CTSD (cathepsin D), DNM2 (dynamin 2), DYNC1H1 (dynein cytoplasmic 1 heavy chain 1), DYNLL2 (dynein light chain LC8-type 2), RAB7A (member RAS oncogene family). Moreover, the expression of these genes was corroborated in a single-cell sorted RBCs RNA-seq. Gene reads are indicated in Table S3. Besides, MHCII gene reads were detectable in single-cell sorted RBCs RNA-seq (Table S3).

Table 1. De novo assembly, RNA-sequencing (RNA-seq) raw data and mapping statistics.

De Novo Assembly	
Total reads	404,825,036
Number of aligned reads	286,555,140
Total contigs	1,056,546
Contigs after CD HIT EST c 0.85	862,667
RBCs Transcript Database	
Genes after assembly BLAST, gene retrieval, removal of duplicates and 95% similar sequences	106,361
Genes after adding *Oncorhynchus mykiss*, *Salmo salar*, and *Danio rerio* NCBI sequences	137,444
Raw Data and Mapping	
Total reads	93,177,954
Reads after trimming	92,391,474
Mapped reads	52,118,053
Un-mapped reads	40,273,421



(**a**)

(**b**)

Figure 2. Cytoscape pathway network of significantly over-represented Immune System Process Gene Ontology (GO)-terms in RBCs transcriptome and proteome profiling common genes and proteins. (**a**) Pathway network. Each node represents a GO-term from Immune System Process. Node size shows GO-term significance (*p* value): smaller *p* value, larger node size. Edge (lines) between nodes indicate the presence of common genes: thicker line implies a larger overlap. GO-terms are classified into several function groups (different node color). The label of the most significant GO-term for each group is highlighted. (**b**) A pie chart of Immune System Process function groups. Asterisks denote GO-term significance. Functional groups are labelled as follows: Dark pink = regulation of hematopoietic stem cell differentiation, dark blue = neutrophil degranulation, light blue = positive regulation of leukocyte activation, light green = antigen processing and presentation of exogenous peptide antigen via MHCII, and dark green = leukocyte differentiation. A list of all over-represented terms and statistics is provided in Table S2.

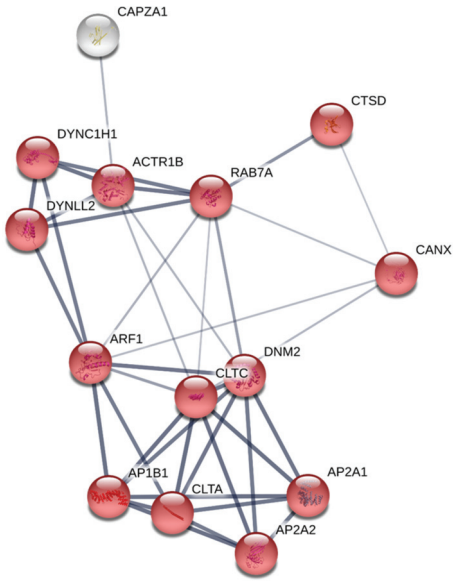


Figure 3. Constructed protein-protein interactions of a set of proteins of antigen processing and presentation of exogenous peptide antigen via MHCII GO-term using STRING software. Nodes represent proteins, while edges denote the interactions between two proteins. Red nodes highlight proteins functionally annotated with STRING software in GO-term antigen processing and presentation of exogenous peptide antigen via MHCII. White nodes represent proteins not functionally annotated in the highlighted GO-term. Network edge line thickness indicates the strength of data support.

These proteins, among others, may provide nucleated RBCs with the essential machinery to participate in the production of antigenic peptides, and their loading onto MHCII molecules within the compartments of endosomal–lysosomal system [34]. Unlike MHCI molecule—which is widely expressed on the cell surface of nearly all nucleated cells, including nucleated RBCs [35]—MHCII molecules are generally restricted to some endothelial cells and a subset of antigen-presenting cells (APCs), such as macrophages, dendritic cells, and B cells [36]. To our knowledge, there is only one record describing low levels of transcripts expression for MHC II in chicken nucleated RBCs [37]. We have also observed transcripts expression in rainbow trout nucleated RBCs (Figure S3). Moreover, it has been described how rainbow trout nucleated RBCs were shown to engulf *Candida albicans*, and presented it to macrophages [5]. Taken altogether, this evidence strongly suggests that nucleated RBCs may participate in antigen presentation, via MHCII, as professional APCs.

These findings have broad implications in the knowledge of nucleated RBCs immune functions, since they open a novel topic of investigation where nucleated RBCs may act as professional APCs, and may be participants of the immunological synapse of T- and NK-cells. The function of MHCII pathway molecules in nucleated RBCs, and their role under viral infection scenarios, remains to be studied, and constitutes a part of our ongoing research.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/9/4/202/s1, Figure S1: Brightfield microscopy image of RBCs after two consecutive density gradient centrifugations, Figure S2: Venn diagram of proteomic and transcriptomic results, Figure S3: *mhcII* gene expression, by means of RT-qPCR, in rainbow trout nucleated RBCs, RTS-11 and RTG-2 cell types, relative to *ef1α* endogenous control, Table S1: Common genes and proteins related to Immune System Process GO-terms overrepresented in RBCs, Table S2: List of all over-represented Immune System Process GO-terms in RBCs transcriptome and proteome profiling common genes and proteins, Table S3: Sorted RBCs RNA-seq reads mapped to the set of genes of antigen processing and presentation of exogenous peptide antigen via MHC class II GO-term.

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Author Contributions: M.d.M.O.-V. conceived, designed the research and analyzed the data. S.P.-M., I.N. and V.C. performed the experiments and analyzed the data. S.C. and M.C.M. performed the proteomic experiments. J.C. contributed to the writing of the manuscript. M.d.M.O.-V. wrote the manuscript with contribution from other authors. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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PUBLICACIÓN 2

TÍTULO: Rainbow trout erythrocytes ex vivo transfection with a DNA vaccine encoding VHSV Glycoprotein G induces an antiviral immune response

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Rainbow Trout Erythrocytes *ex vivo* Transfection With a DNA Vaccine Encoding VHSV Glycoprotein G Induces an Antiviral Immune Response

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Fish red blood cells (RBCs), are integral in several biologic processes relevant to immunity, such as pathogen recognition, pathogen binding and clearance, and production of effector molecules and cytokines. So far, one of the best strategies to control and prevent viral diseases in aquaculture is DNA immunization. DNA vaccines (based on the rhabdoviral glycoprotein G [gpG] gene) have been shown to be effective against fish rhabdoviruses. However, more knowledge about the immune response triggered by DNA immunization is necessary to develop novel and more effective strategies. In this study, we investigated the role of fish RBCs in immune responses induced by DNA vaccines. We show for the first time that rainbow trout RBCs express gpG of viral hemorrhagic septicaemia virus (VHSV) (GVHSV) when transfected with the DNA vaccine *ex vivo* and modulate the expression of immune genes and proteins. Functional network analysis of transcriptome profiling of RBCs expressing GVHSV revealed changes in gene expression related to G-protein coupled receptor (GPCR)-downstream signaling, complement activation, and RAR related orphan receptor α (RORA). Proteomic profile functional network analysis of GVHSV-transfected RBCs revealed proteins involved in the detoxification of reactive oxygen species, interferon-stimulated gene 15 (ISG15) antiviral mechanisms, antigen presentation of exogenous peptides, and the proteasome. Conditioned medium of GVHSV-transfected RBCs conferred antiviral protection and induced *ifn1* and *mx* gene expression in RTG-2 cells infected with VHSV. In summary, rainbow trout nucleated RBCs could be actively participating in the regulation of the fish immune response to GVHSV DNA vaccine, and thus may represent a possible carrier cells for the development of new vaccine approaches.

Keywords: rainbow trout, erythrocytes, red blood cells, VHSV glycoprotein G, DNA vaccine, transcriptome, proteome, immune response

INTRODUCTION

The fish immune system is characterized by an active innate immune system that is of primary importance in combating infections (1). However, differences between the fish immune system and that of mammals complicate the extrapolation of knowledge, thus limiting our ability to control infectious diseases in fish. Piscine infectious diseases, especially viral infections, cause significant losses globally, which implies a negative impact on aquaculture industry. For that reason, efforts have been made to understand the fish antiviral immune response over the last few decades. A particular area of interest is the role that nucleated red blood cells (RBCs) play as immune cell mediators (2–4).

In contrast to mammalian RBCs, fish RBCs are nucleated and contain organelles in their cytoplasm (5). In addition, RBCs contain transcriptome machinery that plays an active role in the immune response against viral infections (2). The involvement of nucleated RBCs in the immune response has been demonstrated both *in vivo* and *in vitro*. Moreover, RBCs can act as phagocytes and antigen-presenting cells and release cytokine-like factors such as interferon gamma that could affect macrophage function (6). Nucleated RBCs also are able to develop specific responses to different pathogen-associated molecular patterns (PAMPs) (4) and can modulate leukocyte activity by producing soluble factors (4, 7). Recently, it has been demonstrated that fish RBCs are also involved in the immune response to viral infections (7–10).

Currently, the prevention of viral diseases is only possible through vaccination or immune stimulation. In aquaculture, one of the best strategies for controlling and preventing viral diseases is DNA immunization (11). This method triggers long-term protection against viral infections (11). However, more knowledge about the immune response triggered by DNA immunization is necessary in order to develop new and more effective viral prevention strategies. It is necessary to understand both the viral molecules involved in triggering the host immune responses (immunogenicity and antigenicity) and the viral-induced immune host responses in efforts to improve current and develop new vaccination strategies. In addition, new applications targeting immune cells are being developed to improve the immune response of DNA vaccines (12).

At the present time, only DNA vaccines based on the rhabdoviral glycoprotein G (gpG) gene have been found to be effective for all fish rhabdoviruses tested (13, 14). However, the immune mechanisms responsible for their efficacy remain largely unknown. Therefore, this topic is of special importance to supplement the knowledge of how DNA vaccines confer immune protection, especially given the recent revelations of the importance of RBCs in the piscine immune response.

In this study, we show for the first time that rainbow trout RBCs can express the gpG of VHSV (GVHSV) and modulate the expression of genes related to interferon, such as interferon-inducible myxovirus resistance gene (*mx*), interferon-inducible RNA-dependent protein kinase (*pkr*), and interferon-induced protein with tetratricopeptide repeats 5 (*ifit5*). Transcriptomic and proteomic profiles network analyses revealed genes and proteins involved in G-protein coupled receptor (GPCR)-downstream signaling, complement activation, RORA-activates gene expression, ISG15 antiviral mechanisms, and detoxification of reactive oxygen species. Further, conditioned medium (CM) of GVHSV-transfected RBCs conferred protection to RTG-2 cell line against VHSV infection. Our results lead us to suggest that RBCs are immune cell mediators that play an active role in GVHSV DNA vaccine immune stimulation. Therefore, RBCs could be considered promising target or carrier cells in the development of new vaccine approaches.

MATERIALS AND METHODS

Animals

Rainbow trout (*Oncorhynchus mykiss*) of ∼7–10 cm were obtained from a VHSV-free commercial farm (PISZOLLA S.L., CIMBALLA FISH FARM, Zaragoza, Spain), and maintained at the University Miguel Hernandez (UMH) facilities at 14°C. Prior to experiments, fish were acclimatized to laboratory conditions for 2 weeks.

Cell Cultures

RBCs were obtained from the peripheral blood of fish sacrificed by overexposure to tricaine (tricaine methanesulfonate, Sigma-Aldrich, Madrid, Spain) (0.2 g/L). Peripheral blood was collected from the caudal vein using insulin syringes (NIPRO, Bridgewater, NJ, USA). RBCs were purified by two density gradient centrifugations (1,600 rpm, Ficoll 1.007; Lymphoprep, Reactiva, Sigma-Aldrich) as previously described (8). Purified RBCs were placed in RPMI-1640 medium (Dutch modification) (Gibco, Thermo Fisher Scientific Inc., Carlsbad, CA) supplemented with 10% gamma irradiated 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), 100 U/mL penicillin (Sigma-Aldrich), and 100 μg/mL streptomycin (Sigma-Aldrich). The cells were cultured at 14°C.

The rainbow trout cell line RTG-2 (Rainbow Trout Gonad-2) was purchased from the American Type Culture Collection (ATCC 50643) and 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. RTS11, a rainbow trout monocyte/macrophage-like cell line (donated by Dr. Niels Bols) (15) isolated from a spleen hematopoietic culture was maintained at 21°C in Leibovitz's medium (L-15) (Sigma-Aldrich) supplemented with 20% FBS, 1 mM pyruvate, 2 mM L-glutamine, 50 μg/mL gentamicin, and 2 μg/mL fungizone.

Antibodies

Primary antibodies used in the manuscript included rabbit polyclonal antibody against Mx protein produced at the laboratory of Dr. Amparo Estepa (16, 17), and mouse polyclonal antibodies against IL1β (interleukin 1 beta) (18, 19), IL8 (interleukin 8) (20), and TNFα (tumor necrosis factor alpha) (21) produced at the laboratory of Dr. Luis Mercado. A mouse monoclonal 2C9 antibody produced at laboratory of Dr. Julio Coll against the N protein of VHSV was used for VHSV labeling (22). For GVHSV labeling, we used a mixed of anti-GVHSV monoclonal antibodies (MAbs) (C10, 3F1A2, and I16) (23)

produced at Dr. Julio Coll's laboratory. Secondary antibodies used in these studies included anti-rabbit IgG CF™ 647 and anti-mouse IgG CF™ 647 (Sigma-Aldrich, Madrid, Spain) produced in goat.

Plasmids

Plasmid pmTFP1 (Allele Biotechnology, ABP-FP-TCNCS), encoding the teal fluorescent protein 1 (mTFP1) (24), used as control plasmid, and pmTFP1GVHSV, encoding mTFP1 fused to the C-terminus of the membrane gpG of VHSV (GVHSV) (GenBank accession A10182.1), described previously (25), were used for transfection assays.

Cell Transfection Assays

RBC transfection assays were performed by electroporation using the Neon™ Transfection System (Life Technologies, Thermo Fisher Scientific, Inc.) one day after Ficoll purification. For each electroporation reaction, we used 4 μg of plasmid construct (pmTFP1 or pmTFP1GVHSV plasmid) per 1×10^6 cells resuspended in Buffer T (Neon™ Transfection System Kit, Life Technologies). RBCs were electroporated at 1600 V, 30 ms, and 1 pulse and incubated at 14°C for one to six days in RPMI 10% FBS.

The RTS11 cell line was transfected by electroporation with 4 μg of plasmid construct (pmTFP1 or pmTFP1GVHSV) per $1{\cdot}10^6$ cells using the Neon™ Transfection System and resuspended in Buffer R (Neon™ Transfection System Kit). RTS11 was electroporated at 1,600 V, 30 ms, and 1 pulse and incubated at 21°C for one to six days in L-15 20% FBS.

Transcriptome Analysis of FACS Single-Cell Sorted GVHSV-Expressing RBCs

Ficoll-purified RBCs from 24 fish were transfected as described above with pmTFP1 or pmTFP1GVHSV (**Figure 1**). At six days post-transfection, TFP1- or GVHSV-expressing RBCs (6–10 cells per fish) were sorted by FACS single-cell sorting using the BD FACSJazz™ cell sorter (BD Biosciences, Madrid, Spain). FACS single-cell sorted RBCs were visualized in the IN Cell Analyzer 6000 Cell Imaging system (GE Healthcare, Little Chalfont, UK) (**Figure S1**). Each sample was resuspended in 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). Twenty-four fish samples were grouped in three pools of eight individuals for each condition (pmTFP1 or pmTFP1GVHSV) (**Figure 1**) and preserved at −80°C until cDNA library construction. Then, cDNA was directly produced from pooled lysed cells using SMART-Seq v4 Ultra Low Input RNA Kit (Clontech, Takara Bio) (26). Sequence reads are available at SRA-NCBI, SRA-NCBI Accession SRP133501. RNA-Seq library preparation and sequencing were carried out by STABVida Lda (Caparica, Portugal).

Proteome Analysis of Transfected RBCs

Ficoll-purified RBCs from 16 fish were transfected as described above with pmTFP1 or pmTFP1GVHSV (**Figure 1**). At six days post-transfection, RBCs were pelletized by centrifugation (1,600 rpm), the supernatant was removed, and the cell pellet was washed three times with PBS, digested, and cleaned-up/desalted as previously described (26). Samples were pooled in two pools of eight individuals for each condition (pmTFP1 or pmTFP1GVHSV) (**Figure 1**). Then, samples were subjected to liquid chromatography and mass spectrometry analysis (LC-MS) as previously described (26), except that High pH Reversed-phase Peptide Fractionation Kit (Pierce, Thermo Fisher Scientific Inc.) was used for pH reversed-phase peptide fractionation, and four peptide fractions were collected. Progenesis QI v4.0 (Nonlinear Dynamics, Newcastle, UK) was used to analyze differential protein expression according to the "between-subject design." 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 were used to estimate *P*-values and false discovery rates (FDR) at a quantitation 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 to be correctly identified. Only proteins having at least two peptide spectrum matches (PSMs) were considered in the quantitation.

Pathway Enrichment Analysis

Differentially expressed genes (DEGs) and differentially expressed proteins (DEPs) pathway enrichment analysis were performed using ClueGO (27) CluePedia (28), and Cytoscape (29). The GO Immune System Process, Reactome Pathway, and Reactome Reactions 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 Gotz (30).

RTG-2 Cell Line Immune Response to Conditioned Medium From Transfected RBCs

In order to evaluate the immune response elicited by GVHSV-transfected RBCs on RTG-2 cells, RTG-2 cell monolayers in 96-well plates were treated with CM from pmTFP1- or pmTFP1GVHSV-transfected RBCs. First, CM of transfected RBCs were collected at three and six days post-transfection, recovered by centrifugation (1,600 rpm), and filtered with 0.2 μm filters (Cultek). The CM was diluted 1/5 in MEM 10% FBS, and RTG-2 cell monolayers were treated with diluted CM for three days at 14°C. Finally, RTG-2 cell were stored at −80°C in lysis buffer until RNA extraction and RT-qPCR.

To evaluate the protection conferred by GVHSV-transfected RBC CM on RTG-2 cells against VHSV infection, pmTFP1- and pmTFP1GVHSV-transfected RBC CM was collected at three and six days post-transfection as described above. Then RTG-2 cell monolayers were pre-treated with the CM, diluted 1/5 and 1/125 in MEM 10% FBS, and incubated for 24 h at 14°C. Then, CM was removed and RTG-2 cell monolayers were infected with VHSV at a multiplicity of infection (MOI) of 10^{-2} in RPMI 2% FBS, for 2 h at 14°C. Medium was removed and fresh medium (RPMI 2% FBS) was added. The cells were incubated for an additional 24 h at 14°C. After that,

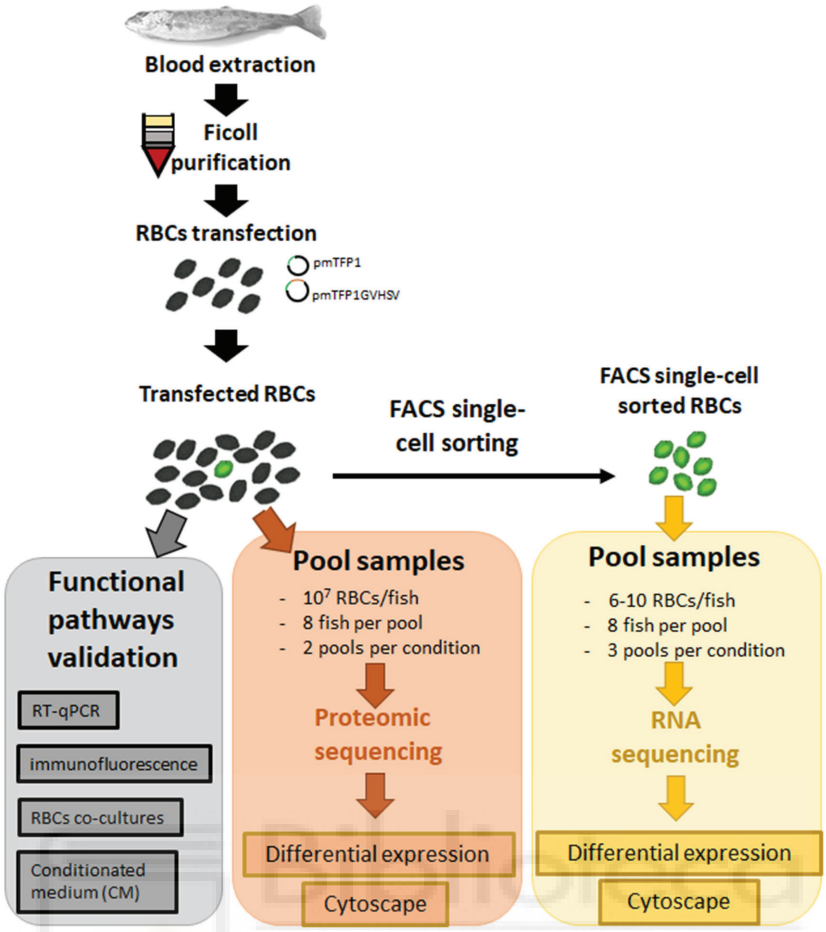

FIGURE 1 | General workflow of experimental steps from sample collection to data analysis.

VHSV infectivity was evaluated by means of focus forming units (FFU)/mL as previously described (9). N-VHSV antibody (2C9) was used as primary antibody. Immunofluorescence images were taken with the IN Cell Analyzer 6000 cell imaging system.

Co-cultures of Transfected RBCs With RTS11 Cells

Ficoll-purified RBCs were transfected as indicated above. Transfected RBCs were co-cultured with RTS11 cells using Transwell® polyester membrane cell culture inserts (0.4 μm pore size, Costar, Corning, Sigma-Aldrich) on 24-well plates for three days at 14°C. Then, RTS11 samples were stored at −80°C in lysis buffer until RNA extraction and RT-qPCR.

RNA Extraction, cDNA Synthesis, and RT-qPCR Gene Expression

RNA extraction, cDNA synthesis and RT-qPCR analyses were performed as previously described (8). Briefly, E.Z.N.A.® Total RNA Kit (Omega Bio-Tek, Inc., Norcross, GA) was used together with DNAse (TURBO™ DNase, Ambion, Thermo Fisher Scientific, Inc.) for RNA extraction. RNA was quantified with a NanoDrop® Spectrophotometer (Nanodrop Technologies, Wilmington, DE). After cDNA synthesis (31), RT-qPCR was performed using the ABI PRISM 7300 System (Applied Biosystems, Thermo Fisher Scientific, Inc.). Specific primers and probes are listed in **Table 1**. The eukaryotic 18S rRNA gene (Applied Biosystems, Thermo Fisher Scientific, Inc.) or the gene encoding EF1α were used as endogenous controls.

Immunofluorescence and Flow Cytometer Assays

Transfected RBCs were fixed, permeabilized, and incubated with primary and secondary antibodies as described in Nombela et al. (9). Flow cytometry was done using a FACS Canto II (BD Biosciences, Madrid, Spain) flow cytometer. RBC populations were selected by forward scatter (FSC) and side scatter (SSC) (**Figure S2**). Immunofluorescence images were taken with the IN Cell Analyzer 6000 cell imaging system.

TABLE 1 | Table of primers used in RT-qPCR.

Gene	Forward primer	Reverse primer	Probe	Reference or accession number
arrb2	GTGGAGCTGCCCTTTGTCTTA	TGAATGTGGGCGGGATATG	TGCACCCCAAACCCACAGAACTGC	NM_001171899.1
cat	TGCAAGACACCCCGTTCATA	TGGCGTGTACCACCCTCTCT		XM_021557350.1
dnm2	GTCAACAAGTCCATCAGGGATCT	CAACTCAGAATGGATGAAGTCTTTAGC		XM_021596596.1
ef1α	ACCCTCCTCTTGGTCGTTTC	TGATGACACCAACAGCAACA	GCTGTGCGTGACATGAGGCA	(32)
gvhsv	GGGCCTTCCTTCTACTGGTACTC	CGGAATCCCGTAATTTGGAAT	CTGTTGCTGCAAGGCGTCCCCT	(31)
ifit5	CCCTGCCCTCATCTTTCTTCT	CCCTCAATGACTCTGACAAGCA	CCAGCTTCGGCCTGTTTCTGTTCCA	AF483530.1
ifn1	ACCAGATGGGAGGAGATATCACA	GTCCTCAAACTCAGCATCATCTATGT	AATGCCCCAGTCCTTTTCCCAAATC	(8)
il10	CTGCTGGACGAAGGGATTCTA	TAAAGTCGTTGTTGTTCTGTGTTCTG	AAGTTCTATCTCGACACGGTGCTGCCC	NM_001245099.1
il12β	TGACAGCCAGGAATCTTGCA	GAAAGCGAATGTGTCAGTTCAAA	ACCCAACGACCAGCCTCCAAGATG	(33)
inos	TCAGAACCTCCTCCACAA	GTGTACTCCTGAGAGTCCTTT	GCACCGACAGCGTCTA	(33)
jak2	CCTGCTCTACGCCTCACAGATC	GCCAAGTCACGGTGGATGTA	CAAGGGCATGGACTACCTAGCGACCA	XM_021622657.1
mhcI	GACAGTCCGTCCCTCAGTGT	CTGGAAGGTTCCATCATCGT		(34)
mhcII	TGCCATGCTGATGTGCAG	GTCCCTCAGCCAGGTCACT	CGCCTATGACTTCTACCCCAAACAAAT	(35)
mx1-3	TGAAGCCCAGGATGAAATGG	TGGCAGGTCGATGAGTGTGA	ACCTCATCAGCCTAGAGATTGGCTCCCC	(36)
nkef	CGCTGGACTTCACCTTTGTGT	ACCTCACAACCGATCTTCCTAAAC		(8)
nup107	GCTGTCGCCTATTGTACGAGATG	TGAGCCTTCTTCTGAACTGAACTCT		XM_021564152.1
pkr	ACACCGCGTACCGATGTG	GGACGAACTGCTGCCTGAAT	CACCACCTCTGAGAGCGACACCACTTC	(8)
prdx6	GGACCCTGATGAGCTTGACAA	CTTATCTGGACCAATCACAAACACA		NM_001165132.2
rab7a	GTTGCGTGCTGGTGTTTGAC	ACTCGTCCCTCCAGCTGTCTAG	TGACCGCCCCCAACACCTTCAA	XM_021609589.1
rora	AGGTGGTGTTCATCAGGATGTG	CGTCGGTCCCAGCGTACTT	CGTGCCTTTGACTCTCAGAACAGCACC	XM_021608048.1
sec13	GCAGTGATCCAGGCACAGAA	CTGGGACTAGGATAGATGGTAGAAGTG	ATTCCACTCCTCCTCCTACCCCCACA	XM_021610740.1
socs1	GATTAATACCGCTGGGATTCTGTG	CTCTCCCATCGCTACACAGTTCC		(37)
sod1	GCCGGACCCCACTTCAAC	CATTGTCAGCTCCTGCAGTCA		(8)
trx	AGACTTCACAGCCTCCTGGT	ACGTCCACCTTGAGGAAAAC		(8)

Statistical Analysis

GraphPad Prism 6 (www.graphpad.com) software was used for statistical analysis. Flowing Software (www.flowingsoftware.com) was used to analyze flow cytometry experiments.

RESULTS

GVHSV Expression in Rainbow Trout RBCs

TFP1 (**Figure 2A**) and GVHSV (**Figure 2B**) expression in transfected RBCs was monitored through fluorescent microscopy. Perinuclear expression of GVHSV was observed in pmTFP1GVHSV-transfected RBCs (**Figure 2B**), which is in contrast to the nuclear and cytoplasmic expression observed in pmTFP1-transfected RBCs (**Figure 2A**).

Time-course and dose-response assays were performed to establish the optimal conditions of pmTFP1GVHSV transfection. RBCs achieved the maximum expression of the GVHSV gene at six days post-transfection with 4 μg per 1×10^6 RBCs evaluated by RT-qPCR (**Figures 2C,D**). These conditions were used for the following assays.

GVHSV gene expression in rainbow trout RBCs was compared with that in RTS11, another rainbow trout cell line. The RTS11 monocyte/macrophage-like cell line had higher levels of GVHSV gene expression at 24 h post-transfection (**Figure 2D**) than the RBCs and decreased over time. This is in contrast to pmTFP1GVHSV-transfected RBCs, which reached the maximum level of GVHSV expression at six days post-transfection. GVHSV gene expression levels were not significantly different between RBCs and RTS11 at three and six days post-transfection, although GVHSV gene expression was lower in RBCs than RTS11 at all-time points analyzed.

Also, GVHSV protein expression was lower in RBCs than RTS11 at three days post-transfection by flow cytometry (**Figures 2E,G**) compared to RTS11 (**Figures 2F, H**).

RNA Sequencing of FACS Single-Cell Sorted GVHSV-Expressing RBCs

In order to evaluate the immune response triggered by GVHSV DNA vaccine in RBCs, we analyzed the transcriptome of FACS single-cell sorted GVHSV-expressing RBCs, exclusively (**Figure 1**; **Figure S1**).

RNA-sequencing of FACS single-cell sorted GVHSV-expressing RBCs (compared with FACS single-cell sorted TFP1-expressing RBC) revealed 3249 DEGs (FDR < 0.05) from a total of 137,444 transcripts. Among these 3,249 DEGs, 1,786 were upregulated, and 1,463 were downregulated (**Table S1**). Functional pathway enrichment evaluation in FACS single-cell sorted GVHSV-expressing RBCs showed upregulation of GPCR downstream signaling and RORA-activates gene expression pathways using the Reactome Pathways Database (**Figure 3A**, **Table S2**), and the complement activation pathway using GO Immune Process Database (**Figure 3C**, **Table S3**). On the other

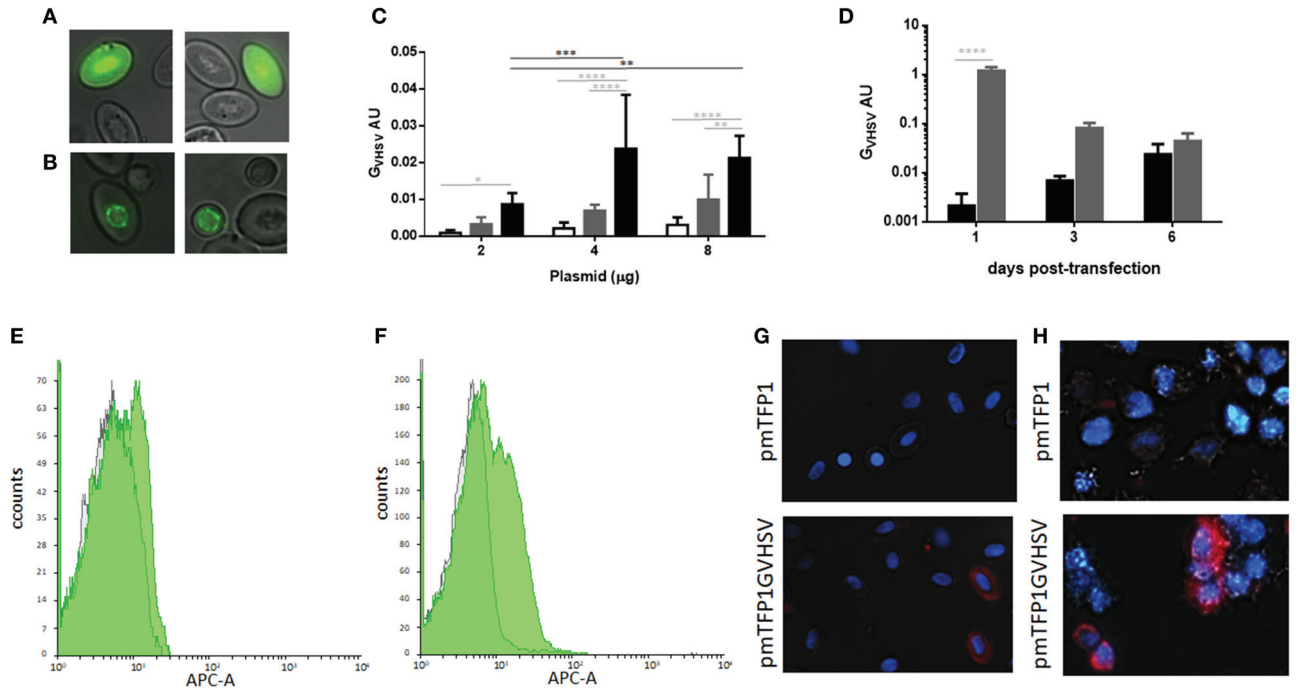

FIGURE 2 | Monitoring transfection of rainbow trout red blood cells (RBCs). Fluorescent micrographs of RBCs transfected with **(A)** pmTFP1 and **(B)** pmTFP1GVHSV at six days post-transfection at 14°C, monitored by teal fluorescent protein (TFP). Fluorescent images taken with the IN Cell 6000 imaging system, augmentation 60x. **(C)** RBC transfection with 2, 4, and 8 µg of plasmid pmTFP1GVHSV was confirmed by GVHSV gene RT-qPCR, at one day (white bars), three days (gray bars) and six days (black bars) post-transfection. The eukaryotic 18S rRNA gene was used as an endogenous control. Data are displayed as mean ± SD ($n = 3$). Two-way ANOVA with Tukey's multiple comparisons test was performed between plasmid concentrations (black lines and asterisks) and times post-transfection (gray lines and asterisks). **(D)** Time course of transfected RBCs (black bars) and transfected RTS11 (gray bars) with 4 µg of pmTFP1GVHSV at one, three and six days post-transfection monitored by GVHSV RT-qPCR. The eukaryotic 18S rRNA gene was used as an endogenous control. Data are displayed as mean ± SD ($n = 3$ for RBCs and $n = 2$ for RTS11). Two-way ANOVA with Sidak's multiple comparisons test was performed between cell types at the different times post-transfection. *, **, ***, and ****, represent the P values < 0.05, < 0.01, < 0.001, and < 0.0001, respectively. Overlay flow cytometry histogram of representative GVHSV immunostaining of **(E)** RBCs and **(F)** RTS11 cells transfected with 4 µg of pmTFP1 or pmTFP1GVHSV at three days post-transfection (gray histogram, pmTFP1-transfected; green histogram, pmTFP1GVHSV-transfected). Representative immunofluorescence of GVHSV intracellular immunostaining of **(G)** RBCs and **(H)** RTS11 cells transfected with 4 µg of pmTFP1 or pmTFP1GVHSV at three days post-transfection (protein [APC, red] and nuclei [DAPI, blue]). Fluorescence images were taken with 60 × magnification.

hand, transcriptional regulation by RUNX3 and eukaryotic translation elongation pathways appeared to be downregulated using the Reactome Pathways Database (**Figure 3B**, **Table S2**).

Among all DEGs identified, we identified modulation of genes related to interferon alpha/beta signaling in antiviral mechanisms. Particularly, genes encoding suppressor of cytokine signaling 3 (*socs3*); adenosine deaminase, RNA specific (*adar*); radical s-adenosyl methionine domain-containing 2 (*rsad2*); tripartite motif-containing 25 (*trim25*); and nucleoporins such as nucleoporin 58 (*nup58*), karyopherin subunit beta 1 (*kpnb1*), and nucleoporin 210 (*nup210*) were upregulated (**Table S1**). Conversely, interferon regulatory factor 1 (*irf1*), interferon alpha and beta receptor subunit 1 (*ifnar1*), Janus kinase 1 (*jak1*), and major histocompatibility complex class I (*mhcI*) genes were downregulated. We also found that several DEGs related to interleukin signaling were upregulated. These included interleukin 2 receptor subunit beta (*il2rb*), *socs3*, Janus kinase 2 (*jak2*), interleukin 16 (*il16*), interleukin 15 (*il15*), interleukin 12a (*il12a*), tumor necrosis factor (*tnf*), rar-related orphan receptor α (*rora*), and interleukin 8 (*il8*) (**Table S1**). The complement cascade was represented by the upregulation of genes encoding complement c3 (*c3*), carboxypeptidase b2 (*cpb2*), coagulation factor II, thrombin (*f2*), and complement c1q b chain (*c1qb*) (**Table S1**).

Proteome Sequencing of GVHSV-Transfected RBCs

We evaluated the proteome of pmTFP1GVHSV-transfected RBCs, a sample composed of few GVHSV-expressing RBCs and mostly non–GVHSV-expressing RBCs, in order to evaluate the immune response of non–GVHSV-expressing RBCs to the signal triggered by GVHSV-expressing RBCs. Proteomic profiling identified 1,750 proteins (**Table S4**). After applying a filter of FDR < 0.001 and $[-1.5 < \text{Log}_2\text{fold change (FC)} > 1.5]$, for pmTFP1GVHSV-transfected RBCs compared to pmTFP1-transfected RBCs, 199 DEPs were identified, of which 75 were upregulated and 124 were downregulated (**Table S4**). ClueGO analysis using the Reactome Pathways

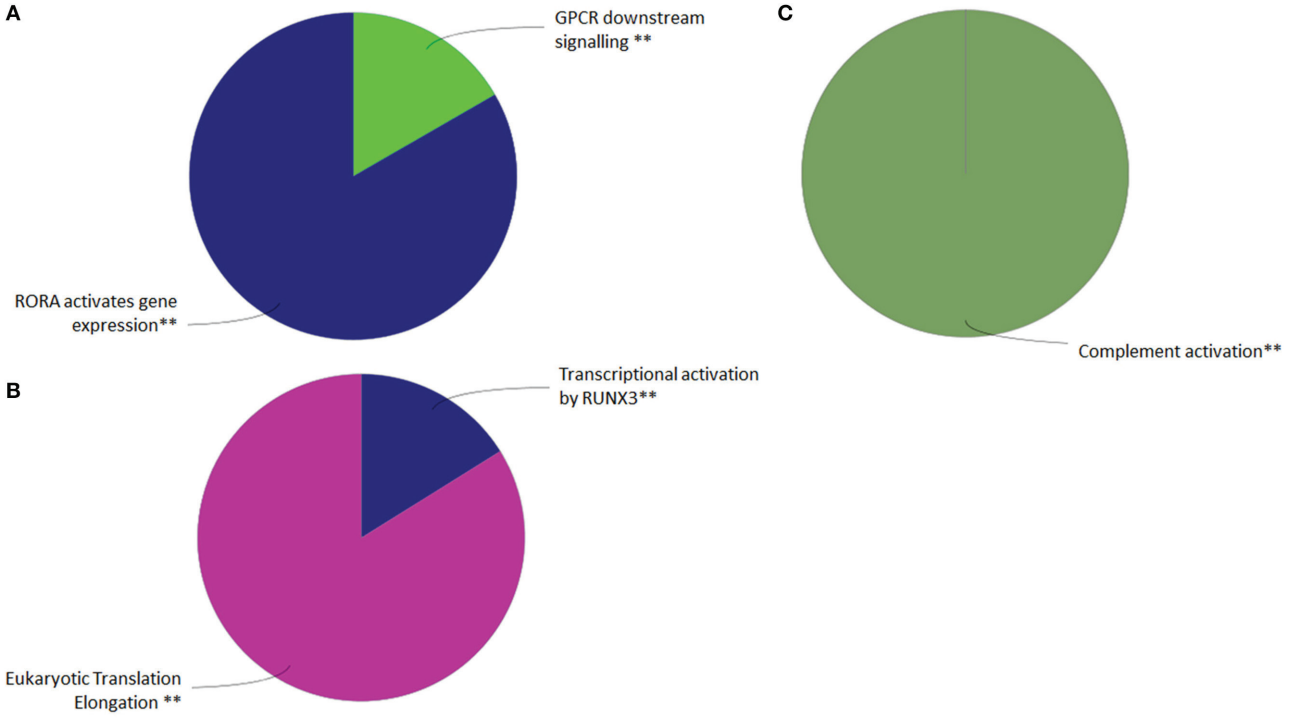

FIGURE 3 | Functional pathway analysis of differentially expressed genes (DEGs) in the FACS single-cell sorted GVHSV-expressing RBC transcriptome profile. DEGs in pmTFP1GVHSV with FDR *Q* value < 0.05 and fold change *P* value < 0.05. Overrepresented GO terms were identified by the Cytoscape ClueGO app with Reactome and GO Immune Process terms. The Reactome Pathways multilevel pie chart shows **(A)** upregulated and **(B)** downregulated overrepresented terms in pmTFP1GVHSV-expressing RBCs. The GO Immune Process multilevel pie chart shows **(C)** upregulated overrepresented terms in pmTFP1GVHSV-expressing RBCs. Asterisks denote GO-term significance (**$p < 0.01$).

Database revealed upregulated terms related to ISG15 antiviral mechanism, detoxification of reactive oxygen species (ROS), mRNA splicing, host interactions of HIV, CLEC7A signaling, interleukin1 family signaling, and FCERI-mediated NF-κβ factors in pmTFP1GVHSV-transfected RBCs (**Figure 4A**, **Table S5**). Conversely, downregulated terms in pmTFP1GVHSV-transfected RBCs appeared to be related to DNA replication and cell cycle regulation, Orc1 removal from chromatin, synthesis of PIPs at the plasma membrane, and COPI-mediated anterograde transport (**Figure 4B**, **Table S5**). ClueGO analysis using Reactome Reactions Database showed upregulated terms related to proteosomal cleavage of exogenous antigen, snRNP nuclear import and release, and formation of the AT-AC C complex in pmTFP1GVHSV-transfected RBCs (**Figure 4C**, **Table S6**). On the other hand, downregulated terms included degradation of ubiquitinated p27/p21 by the 26S proteasome, Orc1 phosphorylation by cyclin A/CDK2, association of phospo-L13a with GAIT element of ceruloplasmin mRNA and exocytosis of secretory granulate lumen proteins (**Figure 4D**, **Table S6**). ClueGO analysis using the GO Immune Process Database identified antigen processing and presentation of exogenous peptide terms in pmTFP1GVHSV-transfected RBCs (**Figure 4E**, **Table S7**).

Among the most upregulated DEPs in pmTFP1GVHSV-transfected RBCs (**Table S4**), we identified proteins related to: (i) the nuclear pore complex, such as importin-8 (IPO8), nuclear pore complex protein Nup107 (NUP107), and translocated promoter region nuclear basket protein (TPR); (ii) inhibitor of nuclear factor kappa-B kinase subunit alpha (CHUK), and (iii) protection against oxidative stress such as thioredoxin (TRX), peroxiredoxin 4 (PRDX4), superoxide dismutase 1 (SOD1), and thioredoxin like 1 (TXNL1).

Validation of Upregulated Pathways by RT-qPCR

Upregulated pathways in pmTFP1GVHSV-transfected RBCs were validated via RT-qPCR analysis. Genes and proteins were selected from each pathway for validation. For the GPCR-downstream signaling term (which was upregulated in the RNA sequencing results of FACS single-cell sorted GVHSV-expressing RBCs), we measured gene expression levels of the arrestin beta 2 (*arrb2*) gene, which was significantly upregulated, and the Janus kinase 2 (*jak2*) gene, which was significantly downregulated in our RT-qPCR results in contrast to RNA sequencing results (**Figure 5**). The *rora* gene, a representative gene of RORA-activates gene expression pathway, an overrepresented term in RNA sequencing results of FACS single-cell sorted GVHSV-expressing RBCs, was significantly upregulated in RT-qPCR results (**Figure 5**). For the detoxification of reactive oxygen species pathway (found to be upregulated in the proteome analysis of pmTFP1GVHSV-transfected RBCs), the superoxide

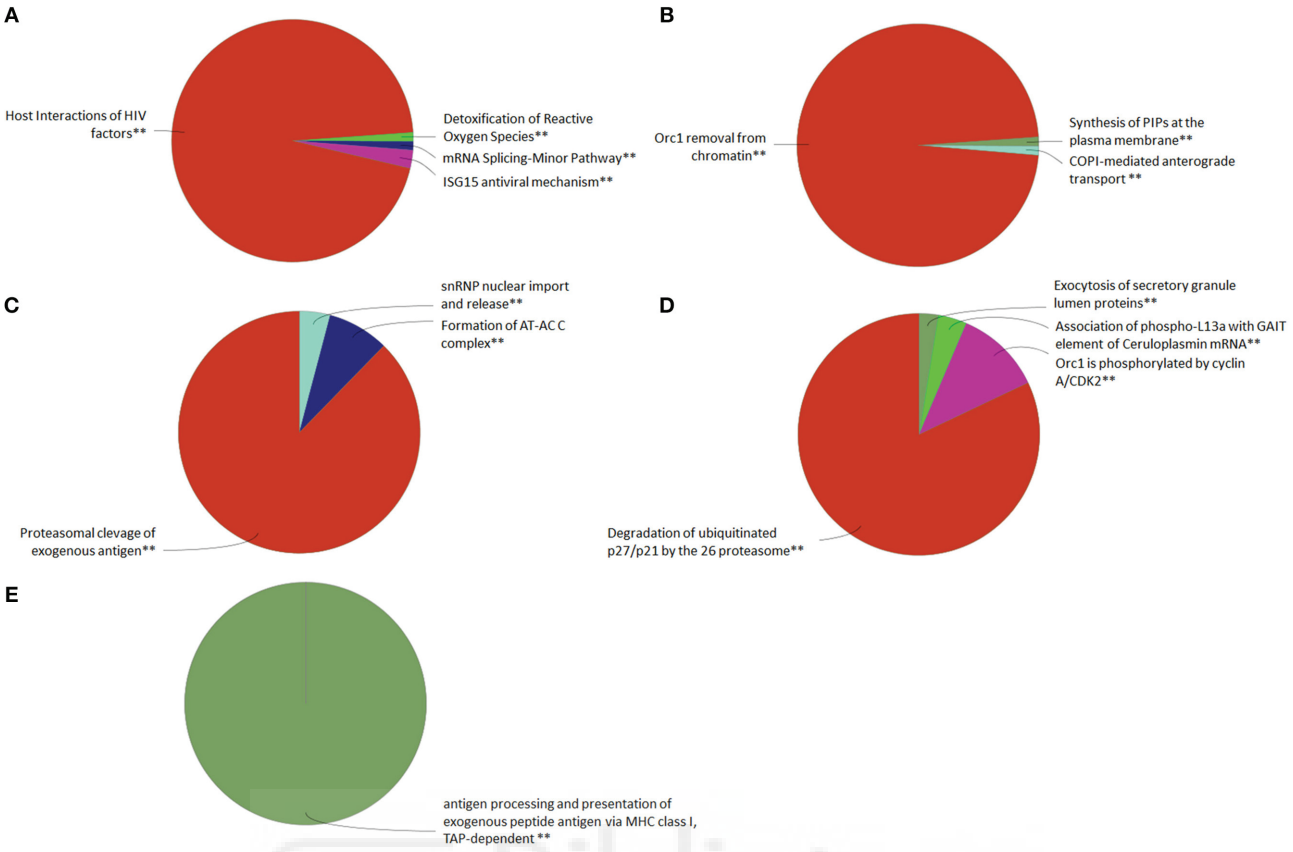

FIGURE 4 | Functional pathway analysis of differentially expressed proteins (DEPs) in the GVHSV-transfected RBC proteome profile. DEPs in pmTFP1GVHSV with ($-1.5 < \log_2$ fold change < 1.5) and an FDR *P*-value <0.001. Overrepresented GO terms were identified by the Cytoscape ClueGO app, with Reactome Pathways, Reactome Reactions and GO Immune Process terms. The Reactome Pathways multilevel pie chart shows **(A)** upregulated and **(B)** downregulated overrepresented terms in pmTFP1GVHSV-transfected RBCs. The Reactome Reactions multilevel pie chart shows **(C)** upregulated and **(D)** downregulated overrepresented terms in pmTFP1GVHSV-transfected RBCs. The GO Immune Process multilevel pie chart shows **(E)** upregulated overrepresented terms in pmTFP1GVHSV-transfected RBCs. Asterisks denote GO-term significance (**$p < 0.01$).

dismutase 1 (*sod1*), peroxiredoxin 6 (*prdx6*), natural killer enhancing factor (*nkef*), and thioredoxin (*trx*) genes were significantly upregulated (**Figure 5**). For the ISG15 antiviral mechanism pathway (which was upregulated in the proteome analysis of pmTFP1GVHSV-transfected RBCs), the nucleoporin 107 (*nup107*), interferon-induced protein with tetratricopeptide repeats 5 (*ifit5*), interferon-inducible Mx (*mx*), and interferon-inducible RNA-dependent protein kinase (*pkr*) genes were significantly upregulated. However, the interferon type 1 (*ifn1*) gene was significantly downregulated (**Figure 5**). For the antigen presentation of exogenous peptide pathways (upregulated in proteome analysis of pmTFP1GVHSV-transfected RBCs), the major histocompatibility complex class I (*mhcI*) and II (*mhcII*), SEC13 homolog-nuclear pore and COPII coat complex component (*sec13*), and dynamin 2 (*dnm2*) genes were significantly upregulated, but the RAB7A-member RAS oncogene family (*rab7a*) appeared to be slightly downregulated in contrast to proteomic results (**Figure 5**).

However, at a protein level, we confirmed the upregulation of interferon inducible Mx protein, and interleukins interleukin 1 beta (IL1β), interleukin 8 (IL8), and tumor necrosis factor alpha (TNFα) measured by flow cytometry (**Figures 6A, B**).

Protection Conferred by pmTFP1GVHSV-Transfected RBC CM on RTG-2 Cells

In order to evaluate the capacity of RBCs to propagate the immune response elicited by GVHSV to other cell types, we measured the protection conferred by pmTFP1GVHSV-transfected RBC CM to RTG-2 cells against VHSV infection. Treatment of RTG-2 cells with three or six days pmTFP1GVHSV-transfected RBC CM significantly decreased VHSV compared with pmTFP1-transfected RBC CM (**Figures 7A,B**, for three and six days transfected RBC CM, respectively).

To determine whether this protection was due to the stimulation of type 1 interferon signaling in RTG-2 cells, we evaluated the expression of *ifn1* and interferon-inducible *mx* genes in RTG-2 cells incubated with pmTFP1- or pmTFP1GVHSV-transfected RBC CM (**Figure 7C**). We

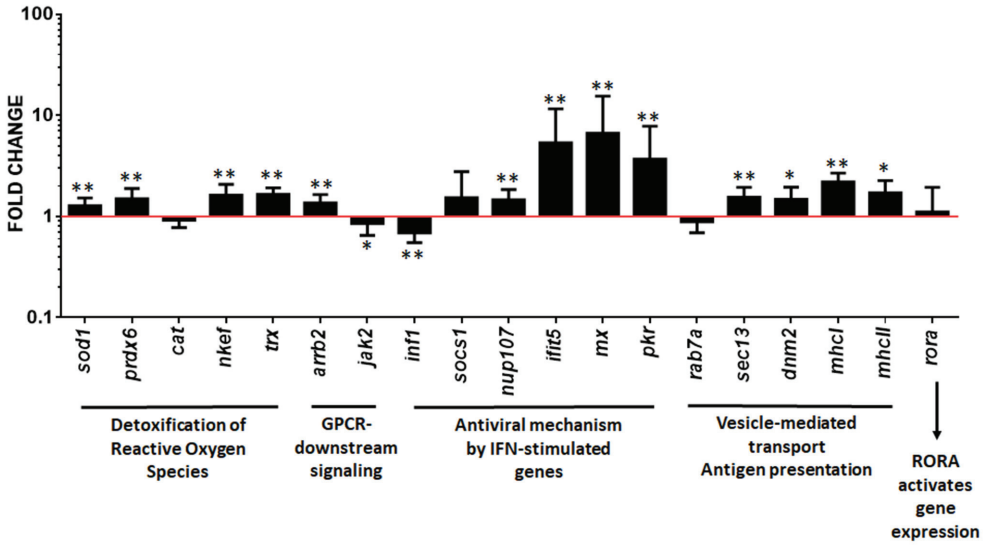

FIGURE 5 | Gene expression evaluation to validate functional pathway analysis of differentially expressed genes (DEGs) and differentially expressed proteins (DEPs) in GVHSV-transfected RBCs transcriptome and proteome profiles, respectively. RBCs were transfected with pmTFP1 and pmTFP1GVHSV plasmids for 6 days at 14°C. Afterwards, gene expression was evaluated by RT-qPCR. Data are displayed as mean ± SD ($n = 5$). The EF1α gene was used as an endogenous control. The Wilcoxon test was performed between pmTFP1GVHSV- and pmTFP1-transfected RBCs (control, red line). *and **represent *P*-values < 0.05 and < 0.01, respectively.

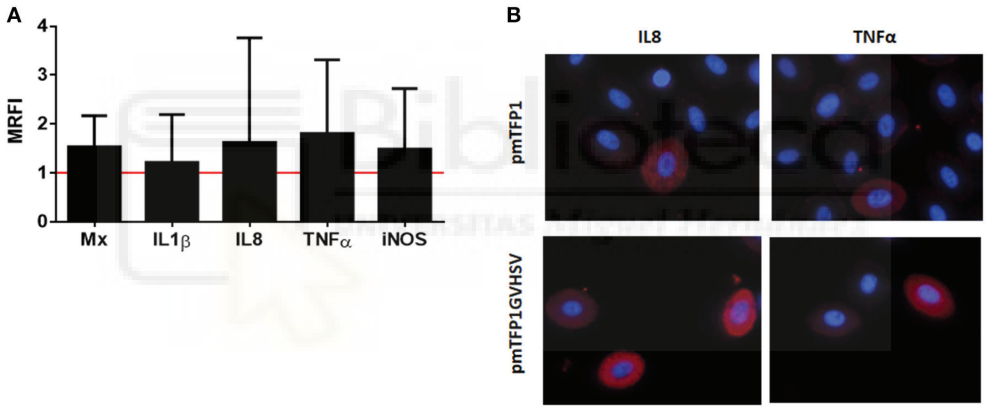

FIGURE 6 | Immune proteins expression in GVHSV-transfected RBCs. **(A)** Immune protein expression measured by flow cytometry and calculated by the formula MRFI (mean relative fluorescence intensity) = fluorescence in pmTFP1GVHSV- transfected RBCs MRFI (mean relative fluorescence intensity) = fluorescence in pmTFP1GVHSV- transfected RBCs /fluorescence in pmTFP1-transfected RBCs. **(B)** Representative immunofluorescence of pmTFP1- and pmTFP1GVHSV-transfected RBCs (protein [APC, red] and nuclei [DAPI, blue]). Fluorescence images were taken with 60× magnification.

observed significant upregulation of *ifn1* gene expression in RTG-2 cells treated with pmTFP1GVHSV-transfected RBC CM at six days post-transfection and of *mx* gene expression at three and six days post-transfection.

Crosstalk Between Transfected RBCs and RTS11

In order to evaluate whether pmTFP1GVHSV-transfected RBC CM could induce monocyte/macrophage differentiation, we co-incubated transfected RBCs with RTS11, a monocyte/macrophage-like cell line. At three days post-transfection, pmTFP1-and pmTFP1GVHSV-transfected RBCs were co-cultured with RTS11 cells for three days. Using RT-qPCR, we observed slight, but not significant, upregulation of RTS11 differentiation markers The slight upregulation of the interleukin 10 (*il10*) gene, a marker of M2 macrophages (38) was accompanied by a slight downregulation of interleukin 12 subunit beta (*il12β*) and inducible nitric oxide synthase (*inos*), which are markers of M1 macrophages (**Figure 8**).

DISCUSSION

Recent studies have implicated nucleated RBCs in the immune response to viral infections in fish, as these cells are able to

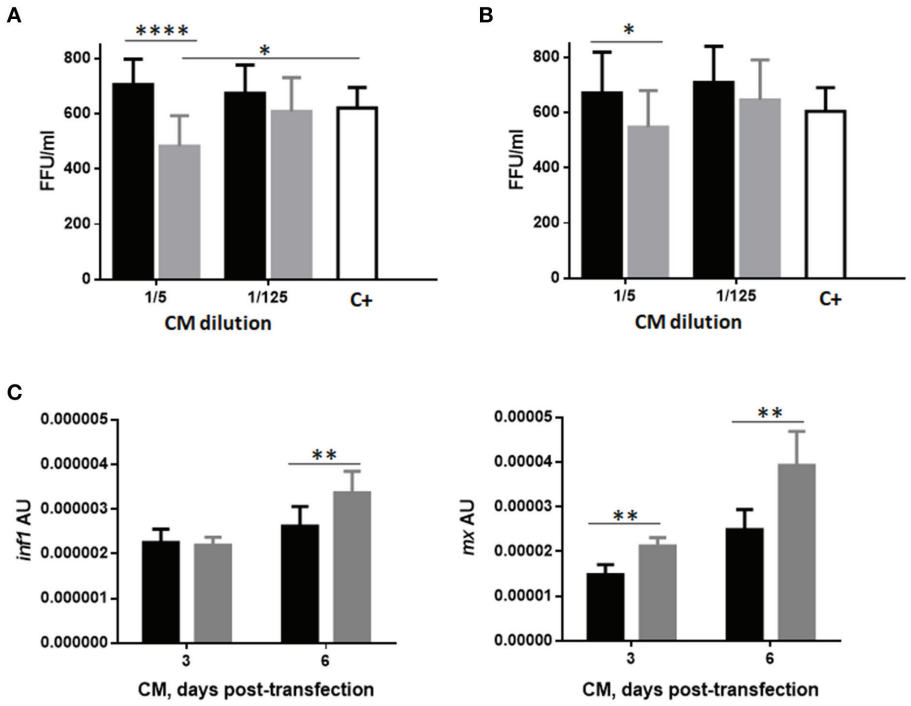

FIGURE 7 | Protection conferred by conditioned medium (CM) from GVHSV-transfected RBCs against VHSV infection in RTG-2 cells. RTG-2 cells pretreated with CM from RBCs transfected with pmTFP1 (black bars) and pmTFP1GVHSV (gray bars) plasmids (diluted 1/5 and 1/125 in MEM 10% FBS), at three **(A)** and six **(B)** days post-transfection. Cells were then infected with VHSV (MOI 1×10^{-2}) for 24h at 14°C. The positive control is non-pretreated RTG-2 cells infected with VHSV. VHSV infectivity was evaluated by means of focus forming units (FFU)/mL. FFUs were stained with 2C9 antibody against the N protein of VHSV. Data are displayed as mean ± SD ($n = 3$). The Mann Whitney test was performed between treatments at each dilution. **(C)** *ifn1* and *mx* gene expression in RTG-2 cells quantified by RT-qPCR after treatment with CM from pmTFP1- (black bars) and pmTFP1GVHSV- (gray bars) transfected RBCs (diluted 1/5 in MEM 10% FBS) at three and six days post-transfection. The eukaryotic 18S rRNA gene was used as an endogenous control. Data are displayed as mean ± SD ($n = 3$). The Mann Whitney test was performed between conditions. *, **, and ****, represent *P*-values < 0.05, < 0.01, and < 0.0001, respectively.

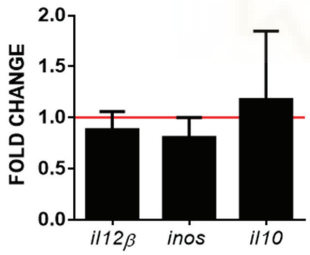

FIGURE 8 | M1 and M2 macrophage markers in RTS11 cells co-cultured with GVHSV-transfected RBCs. Rainbow trout RBCs transfected with pmTFP1 or pmTFP1GVHSV for three days at 14°C. Afterwards, transfected RBCs were co-cultured with the RTS-11 cell line at 14°C for three days. Then, the *il12β*, *inos*, and *il10* gene expression profiles were quantified by RT-qPCR. Gene expression was normalized against eukaryotic 18S rRNA and compared to control cells (RTS11 co-cultured with pmTFP1-transfected RBCs, red line) (fold-change). Data represent the mean ± SD ($n = 6$). A Wilcoxon test was performed between both conditions.

actively transcribe and transduce signaling molecules in response to viral attack (2). Moreover, although DNA vaccines are mainly delivered via intramuscular injection, non-nucleated RBCs are thought to be promising drug and vaccine carriers (39–42) by eliciting humoral immune responses comparable or superior to those obtained via the subcutaneous vaccination route (39). In this study our aim was to elucidate the role of nucleated RBCs in the immune response to DNA vaccines in order to explore their usefulness in improving immune response to DNA vaccines in fish.

As far as we know, this is the first report of fish nucleated RBCs expressing the antigen encoded by a DNA vaccine *in vitro*. Besides, we have not found any report showing nucleated RBCs expressing the protein encoded by a DNA vaccine *in vivo*. GVHSV-transfected RBCs showed a characteristic perinuclear expression of GVHSV protein that appeared in the perinuclear region of stressed RBCs or on the membrane of shape-shifted RBCs (shRBC) (33). shRBCs are small, round cells with a thin membrane derived from RBCs subjected to stressful conditions. Translation of the GVHSV gene into protein and translocation to the cell membrane induces RBC transformation into stressed RBC and shRBC (33). Like RBCs, shRBCs have been shown to participate in roles related to immune response and homeostasis (33).

Transcriptome profiling of single-cell sorted GVHSV-expressing RBCs demonstrated the capacity of RBCs to modulate the expression of genes related to innate and adaptive

immune responses in response to the DNA vaccine. Among the pathways upregulated in single-cell sorted GVHSV-expressing RBCs, GPCR-downstream signaling and RORA activates gene expression were the most represented. GPCRs constitute a large protein family of receptors that detect molecules outside the cell and activate internal signal transduction pathways (43). GPCRs are known to play a role in inflammation and are widely targeted in drug discovery (44). Immune cells such as monocytes and macrophages express a large number of GPCRs for classic chemoattractants and chemokines (44, 45). These receptors are critical for phagocyte migration and accumulation at sites of inflammation, where these cells can intensify inflammation or contribute to its regulation (44, 45). Among the molecules found downstream of GPCRs, β-arrestins are known to be key regulators of GPCR signaling through interaction with the Iκβα component of the NF-κβ signaling complex (44, 45). Consistent with these effects on signaling, knockdown of ARRB2 has been described to enhance the expression of the NF-κβ target proteins IL6 and IL8 in response to proinflammatory stimulus (46). After RNA sequencing and RT-qPCR, we observed that the *arrb2* gene was significantly upregulated in GVHSV-transfected RBCs. We also observed that the IL1β, IL8, and TNFα proteins were slightly upregulated in response to GVHSV transfection. However, the *il8* and *tnfα* genes appeared to be highly upregulated in single-cell sorted GVHSV-expressing RBCs, and the *il6* gene appeared to be highly downregulated. In this regard, it would be interesting to study the implication of β-arrestins in RBCs regulation of the proinflammatory response. Considering the RORA activates gene expression pathway (upregulated in single-cell sorted GVHSV-expressing RBCs), it has been described that RORA is a nuclear receptor highly expressed in Th17 cells that regulates differentiation of Th17 cells (47). Moreover, RORA depletion has been reported to attenuate cytokine production (48) and has thus demonstrated its involvement in inflammatory responses. The signaling paradigms of GPCRs and RORA in inflammatory regulation and immune cell differentiation in nucleated RBCs remain to be studied and are part of our ongoing research.

Interestingly, GVHSV-transfected RBC CM could switch RTS11 monocyte/macrophage differentiation markers, upregulating the *il10* gene [a marker of M2 macrophages (38)] and downregulating the *il12β* and *inos* genes [markers of M1 macrophages (38)]. M1 macrophages are known to be activated by LPS and IFNγ and secrete high levels of IL12 and low levels of IL10. On the other hand, M2 macrophages are alternatively activated by certain cytokines such as IL4, IL10, or IL13 and produce high levels of IL10 and TGFβ and low levels of IL12. Their function is implicated in constructive processes like wound healing and tissue repair and in anti-inflammatory responses. The cytokines or molecules secreted by GVHSV-transfected RBCs responsible for inducing M2 macrophages markers in RTS11 are unknown and should be further investigated.

Genes related to the complement pathway were also overrepresented in single-cell sorted GVHSV-expressing RBCs. The complement system is an essential part of the innate immune response and acts as a connection between innate and acquired immunity [reviewed in Nesargikar et al. (49)]. The complement system is known to mediate responses to inflammatory triggers, leading to clearance of foreign cells through pathogen recognition, opsonization and lysis (50). On the other hand, genes and proteins related to proteosomal cleavage of exogenous antigen and antigen presentation of exogenous peptides were also upregulated in GVHSV-transfected RBCs (via MHCI or MHCII pathways), indicating that RBCs may have the capacity to present DNA vaccine antigens as has been recently reported (26). Further research is needed to determine whether RBCs are functionally capable of inducing T cell activation upon antigen presentation on their membrane.

Proteomic sequencing of GVHSV-transfected RBCs, a sample containing both few GVHSV-expressing RBCs and non–GVHSV-expressing RBCs, revealed the upregulation of ISG15 antiviral mechanisms. ISG15 is a member of the ubiquitin-like (UBL) family. ISG15 conjugates with several target proteins in a process termed ISGylation. Hundreds of target proteins have been identified in ISGylation. Among them, several proteins that are part of antiviral signaling pathways, such as Mx1 or PKR, have been identified as targets for ISGylation (51). Upregulation of the ISG15 antiviral mechanism pathway was confirmed by gene expression analysis of effector molecules within the pathway such as *mx, pkr*, and *ifit5*. The Mx, PKR, and IFIT proteins are known interferon-inducible antiviral effectors (51). Surface expression of the GVHSV protein by GVHSV-transfected cells has been reported to be a major mechanism of interferon induction (52), and VHSV infection and GVHSV vaccination have been demonstrated to induce ISGs such as *isg15* (53) and *mx* (16, 17). However, *ifn1* appeared to be downregulated in GVHSV-transfected RBCs by RT-qPCR and in single-cell sorted GVHSV-expressing RBCs by RNA sequencing. Also, RNA sequencing data showed the downregulation of genes related to interferon alpha/beta signaling such as *irf1*, *ifnar1,* and *jak1*. However, VHSV has been reported to induce *ifn1* downregulation in rainbow trout RBCs (8). These differences between *ifn1* and ISG gene expression could be due to the effort of the immune system to maintain homeostasis or to the differential regulation of these genes. Alternatively, it has been reported that Mx induction could be independent of interferon in HIV infection (54, 55). In addition, infectious salmon anemia (ISA) virus could trigger *mx* and *isg15* stimulation but not *ifn1* gene expression, suggesting ISG stimulation independent of interferon (56). Despite the fact that *ifn1* gene expression was downregulated in GVHSV-transfected RBCs, the IFN protein, which could be differently expressed to *inf* gene, or other cytokines or molecules secreted by GVHSV-transfected RBCs, were able to stimulate *ifn1* and *mx* gene expression as well as induce protection against VHSV infection in RTG-2 cells.

Another interesting pathway identified during the proteomic profiling of GVHSV-transfected RBCs was detoxification of reactive oxygen species (ROS). Gene expression of antioxidant enzymes such as *sod1*, *nkef, prdx6*, and *trx* appeared to be upregulated in GVHSV-transfected RBCs. This mechanism has

been reported in rainbow trout RBCs exposed to VHSV (8), where protective antioxidant enzymes were implicated in the response of RBCs to the induction of ROS after viral exposure. However, in this study, it is important to note that the antigen GVHSV encoded by this DNA vaccine is able to induce ROS signaling and homeostasis.

In summary, rainbow trout nucleated RBCs were able to induce immune responses to the DNA vaccine and send signals to neighboring cells or other cell types. This reveals a new approach to explore the function of RBCs in the complex teleost immune system and could prompt development in the field of vaccination with RBCs as targets or carrier cells for immunostimulation. Future studies will be focused on the molecules of interest produced by GVHSV-expressing RBCs in order to identify future vaccination targets.

ETHICS STATEMENT

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). Besides, 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.

AUTHOR CONTRIBUTIONS

SP-M performed experiments, analyzed data, and wrote the manuscript. IN and VC performed experiments. SC and MM performed proteomic sequencing. LM provided valuable antibodies for the experiments. MO-V conceived ideas, oversaw the research, and co-wrote the manuscript. VC and JC contributed to the preparation of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02477/full#supplementary-material

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PUBLICACIÓN 3

TÍTULO: Potential role of rainbow trout erythrocytes as mediators in the immune response induced by a DNA vaccine in fish

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Article

Potential Role of Rainbow Trout Erythrocytes as Mediators in the Immune Response Induced by a DNA Vaccine in Fish

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Abstract:

In recent years, fish nucleated red blood cells (RBCs) have been implicated in the response against viral infections. We have demonstrated that rainbow trout RBCs can express the antigen encoded by a DNA vaccine against viral hemorrhagic septicemia virus (VHSV) and mount an immune response to the antigen *in vitro*. In this manuscript, we show for the first time the role of RBCs in the immune response triggered by DNA immunization of rainbow trout with glycoprotein G of VHSV (GVHSV). Transcriptomic and proteomic profiles of RBCs revealed genes and proteins involved in antigen processing and presentation of exogenous peptide antigen via MHC class I, the Fc receptor signaling pathway, the autophagy pathway, and the activation of innate immune response. On the other hand, GVHSV-transfected RBCs could induce proliferation in autologous leukocytes *in vitro* and also induce specific antibodies against VHSV in the serum of rainbow trout immunized with GVHSV *in vivo*. In this study, rainbow trout RBCs actively participated in the innate and adaptive immune response after DNA vaccination. Based on our findings, we suggest the use of RBCs as target cells or carriers for future novel vaccine strategies.

Keywords: rainbow trout; erythrocytes; red blood cells; GVHSV; transcriptome; proteome; antigen presentation

1. Introduction

Nucleated red blood cells (RBCs) are involved in the fish immune response by expressing cytokines [1-7] and acting as immune cell mediators against viral pathogens [5]. Nucleated RBCs can carry and respond to a DNA vaccine encoding glycoprotein G of viral hemorrhagic septicemia virus (GVHSV) to modulate the expression of genes related to type 1 interferon (IFN1), antioxidant enzymes, and antigen presentation genes and provide protection to other cell types against VHSV infection and crosstalk with other cell lines *in vitro* [7].

Currently, DNA vaccination is one of the best strategies to prevent viral diseases in fish because it triggers effective protection [8]. In the case of *Novirhabdovirus*, a genus of virus responsible for high economic losses in aquaculture [9], only glycoprotein G has shown effectiveness as a DNA vaccine [10,11]. DNA vaccination is known to stimulate cellular and humoral immune responses [12], but the mechanisms and cells involved in this protection are still unknown and must be studied further in order to improve the effectiveness of DNA vaccines.

In recent decades, mammalian RBCs, which are not nucleated, have been proposed as possible drug and vaccine carriers [13-16] because of their capacity to induce effective immune responses comparable to traditional vaccination [13]. Similarly, nucleated RBCs may act as vaccine carriers or immunomodulatory cells because they have demonstrated the ability to carry and generate an immune response to DNA vaccine *in vitro* [7].

DNA vaccines directed to antigen-presenting cells (APCs) have demonstrated improved humoral and cellular responses compared to non-targeted vaccines [17-19]. Considering that fish RBCs have been proposed to behave as atypical APCs [20], the strategy of targeting DNA vaccines or immunostimulants to RBCs represents a new approach in the field of fish prophylaxis.

Taking everything into account, including the fact that the role of nucleated RBCs in the immune response has gained interest recently, our aim was to explore the currently unknown role of rainbow trout RBCs in the context of *in vivo* DNA vaccination. In this study, we show for the first time the role of rainbow trout RBCs in the global host immune response to a DNA vaccine. Our results show that rainbow trout RBCs can modulate their transcriptome and proteome in response to a DNA vaccine encoding GVHSV. In addition, these RBCs can act as cell mediators of the immune response to activate antigen presentation, blood and head kidney immune cell signaling, and hematopoiesis. RBCs transfected *in vitro* with GVHSV can boost leukocyte proliferation, increasing the number of cells and leukocyte-specific markers. Moreover, reinfusion of autologous GVHSV-transfected RBCs *in vitro* induced VHSV-specific antibodies *in vivo*.

2. Material and Methods

2.1. Animals

Rainbow trout (*Oncorhynchus mykiss*) of approximately 7 cm to 10 cm (for transcriptomic and proteomic assays) and 20 cm to 25 cm (for RBC reinfusion assays) were obtained from a VHSV-free commercial farm (PISZOLLA S.L., CIMBALLA FISH FARM, Zaragoza, Spain). Fish were maintained at the University Miguel Hernandez (UMH) facilities at 14°C and fed daily with a commercial diet (Skretting, Burgos, Spain). Prior to experiments, fish were acclimatized to laboratory conditions for 2 weeks.

2.2. DNA immunization

For transcriptomic and proteomic analyses, juvenile rainbow trout (7 cm to 10 cm) were anesthetized with tricaine (tricaine methanesulfonate, Sigma-Aldrich, Madrid, Spain) (40 mg/L) and injected intramuscularly (im) with 10 μg of plasmid pmTFP1 (Allele Biotechnology, ABP-FP-TCNCS), which encodes teal fluorescent protein 1 (mTFP1) [21], as a control (referred to as TFP1), or pmTFP1GVHSV, which encodes mTFP1 fused to the C-terminus of GVHSV (GenBank accession A10182.1) [22] (referred to as GVHSV) in 50 μL of phosphate buffered saline (PBS) using insulin syringes (NIPRO, Bridgewater, NJ, USA). At 14 days post immunization (dpi), fish were sacrificed by overexposure to tricaine (0.3 g/L), and peripheral blood and head kidney organs were recovered. Sample collection time point was selected based on previous *gvhsv* gene transcripts expression monitorization in blood and head kidney (data not shown) and in the bibliography [12,23].

For the GVHSV-transfected RBC reinfusion assay, adult rainbow trout (20 cm to 25 cm) were anesthetized with 40 mg/L tricaine and reinfused intravenously (iv) with previously extracted autologous peripheral blood RBCs (PB-RBCs) (15 x 106 cells) that were GVHSV-transfected *in vitro* as previously described [7]. For *in vitro* transfection of RBCs, Ficoll-purified PB-RBCs were transfected by electroporation with 4 μg of GVHSV plasmid per 106 cells using the Neon™ Transfection System (Life Technologies, Thermo Fisher Scientific, Inc). Fish were immunized with im or iv injection of 4 μg GVHSV for immunization controls. At 30 dpi, blood was drawn from the caudal vein and left overnight at 4°C to separate the serum from cell pellet.

2.3. Transcriptome analysis of RBCs

Thirty-two fish (16 for TFP1 injection divided into 2 groups of 8 fish and 16 for GVHSV injection divided into 2 groups of 8 fish) were immunized as described above. Peripheral blood and head kidney organs were sampled at 14 dpi in RPMI-1640 medium (Dutch modification) (Gibco, Thermo Fischer Scientific Inc, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) gamma irradiated (Cultek, Madrid, Spain), 1 mM pyruvate (Gibco), 2 mM L-glutamine (Gibco), 50 μg/mL gentamicin (Gibco), 2 μg/mL fungizone (Gibco), 100 U/mL penicillin (Sigma-Aldrich), and 100 μg/mL streptomycin (Sigma-Aldrich). Samples were stained with 500 nM SYTO RNASelect (Molecular Probes) for 20 min at room temperature as recommended by the manufacturer. Then, head kidney RBCs (HK-RBCs) and PB-RBCs were FACS single-cell sorted using BD FACSJazz™ cell sorter (BD Biosciences, Madrid, Spain) based on SYTO RNASelect staining, which separates RBC populations based on their RNA staining fluorescence compared to other blood or head kidney cells (Supplementary Figure S1). Approximately 102 HK-RBCs and 106 PB-RBCs of each individual were sorted and visualized in the IN Cell Analyzer 6000 Cell

Imaging system (GE Healthcare, Little Chalfont, UK) to verify purity ≥99.99% (Supplementary Figure S1).

Each sample was resuspended in lysis buffer (Clontech, Takara Bio, Mountain View, CA, USA) and RNase Inhibitor (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) as indicated in [7] and then grouped in 2 pools of 8 individuals for each condition (TFP1 or GVHSV) and organ (HK-RBCs or PB-RBCs) (Figure 1). Samples were 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). RNA-Seq library preparation and sequencing were carried out by STABVida Lda (Caparica, Portugal) as previously described [24]. Sequence reads are available at SRA-NCBI, SRA-NCBI Accession SRP133501.

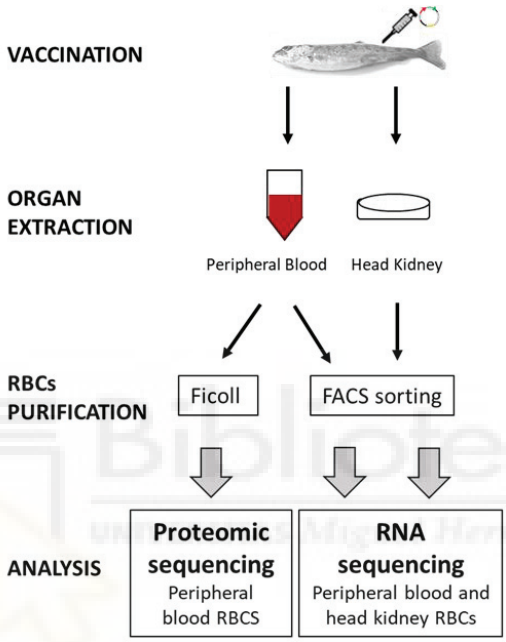


Figure 1. General workflow of experimental steps from sample collection to data analysis.

2.4. Proteome analysis of RBCs

Thirty-two fish were immunized as described above: 16 fish were im injected with TFP1 and 16 fish were im injected with GVHSV, both divided into 2 groups of 8 fish. Peripheral blood was extracted at 14 dpi, and PB-RBCs were purified by 2 density gradient centrifugations (1600 rpm, Ficoll 1.007; Lymphoprep, Reactiva, Sigma-Aldrich) as previously described [1]. The 99.9% purity of RBCs was estimated by optical microscopy (Supplementary Figure S1). Cells were pelletized by centrifugation (1600 rpm, 5 min), the supernatant was removed, and the cell pellet was washed 3 times with PBS. The pellet was then digested, cleaned-up/desalted, and pooled into 2 pools of 8 individuals for each condition (TFP1 or GVHSV) (Figure 1). Samples were subjected to liquid chromatography and mass spectrometry analysis (LC-MS) as previously described [7,24].

2.5. Pathway enrichment analysis

Pathway enrichment analysis of differentially expressed genes (DEGs) and differentially expressed proteins (DEPs) were performed using Cytoscape version 3.6.1 [25] with ClueGO version 2.5.0 [26] and CluePedia version 1.5.0 [27] plugins. The GO Biological Process and GO Immune System Process Databases were used with *P*-value ≤0.05 and *P*-value ≤0.5, respectively, and Kappa score of 0.4 as threshold values. STRING v11 (http://string.embl.de/) [28] software was

used to analyze protein-protein interaction (PPI) networks, with a medium confidence score threshold of 0.4. The Homo sapiens model organism was used for ClueGO and STRING analyses. Genes and proteins were identified by sequence homology with Homo sapiens using Blast2GO version 4.1.9 Gotz [29] as previously described [24].

2.6. RNA extraction, cDNA synthesis, and RT-qPCR gene expression

RNA was isolated using E.Z.N.A. Total RNA Kit (Omega Bio-Tek, Inc., Norcross, GA) following the manufacturer's instructions together with DNAse (TURBO DNase, Ambion, Thermo Fisher Scientific, Inc) and quantified with a NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA synthesis and RT-qPCR were performed using the ABI PRISM 7300 System (Applied Biosystems, Thermo Fisher Scientific, Inc) as previously described [1]. Specific primers and probes are listed in Table 1. The gene encoding EF1α was used as an endogenous control.

Table 1. List of primers and probes used.

Gene	Forward primer	Reverse primer	Probe	Reference or accession number
ccl13	CCTCTTCAACAA GTGGTTTCTCTCA	AGAAGGGTCAACACAA AATGTCTTC		NM_001160689.1
cd8	GAC TGC TGG CTG TGG CTT CC	CCC CGG AGC TGC CAT TCT		[30]
cd83	TTGGCTGATGAT TCTTTCGATATC	TGCTGCCAGGAG ACACTTGT	TCCTGCCCAATG TAACGGCTGTTG	[31]
dnm2	GTCAACAAGTCC ATCAGGGATCT	CAACTCAGAATGGATG AAGTCTTTAGC		[7]
ef1α	ACCCTCCTCTTGG TCGTTTC	TGATGACACCAACAGC AACA	GCTGTGCGTGAC ATGAGGCA	[32]
gabarap	CCTCATCCATCC ATTT TTACCTCTT	ATTCAACCGAAATCCC C ATCT	TCTGAATTTTATT TG CCTCCGGGTCTC C	[20]
gvhsv	GGGCCTTCCTTCT ACTGGTACTC	CGGAATCCCGTAATTTG GAAT	CTGTTGCTGCAA GGCGTCCCCT	[33]
hck	CCATCTCCACTG GCCCTACA	TACCCTCATAGTCATAC AGTGCGATAG		XM_021567092.1
ifit5	CCCTGCCCTCAT CTTTCTTCT	CCCTCAATGACTCTGAC AAGCA	CCAGCTTCGGCC TGTTTCTGTTCCA	[7]
igm	AAAGCCTACAAG AGGGAGACCGAT	AGAGTTATGAGGAAGA GTATGATGAAGGTG	CTCGTGTTGACTG ACTGTCCATGCA GCAAC	[34]
il8	AGAGACACTGA GATCATTGCCAC	CCCTCTTCATTTG TTGTTGGC	TCCTGGCCCTCC TGACCATTACTG AG	[35,36]
irf8	CCGAGGAGGAG CAGAAGAGTAA AAG	GCGGCATTGAAAGAAC CCAT		[37]
mhcI	GACAGTCCGTCC CTCAGTGT	CTGGAAGGTTCCATCAT CGT		[38]

mhcII	TGCCATGCTGAT GTGCAG	GTCCCTCAGCCAGGTCA CT	CGCCTATGACTT CTACCCCAAACA AAT	[39]
mx1-3	TGAAGCCCAGGA TGAAATGG	TGGCAGGTCGATGAGT GTGA	ACCTCATCAGCC TAGAGATTGGCT CCCC	[40]
pax5	ACGGAGATCGGA TGTTCCTCTG	GATGCCGCGCTGTAGT AGTAC		[41]
pkr	ACACCGCGTACC GATGTG	GGACGAACTGCTGCCT GAAT	CACCACCTCTGA GAGCGACACCAC TTC	[1]
tcr	AGCACCCAGACT GCCAAGCT	GAGGAGCCCTGGAACT CCA	TCT TCA TCG CTA AGA GTA CCT TCT ATG GCC TGG T	[30]
ulk1	CTTCTGCTGCTGG GTCTTCTG	GGTGACGGAAGAACTC CTCAAA	CGAAACCACAAG GACCGCATGGA	[20]
wipi1	CAAAGACATGAA GCTG CTGAAGA	GGTTCACAGAGAGGGC ACAGA	CTCAACACGCCC CACAA CCCCT	XM_021581280

154

155 *2.7. Coculture of transfected RBCs with white blood cells*

156 Ficoll-purified RBCs from peripheral blood were transfected with pmTFP1 or pmTFP1GVHSV
157 plasmid as indicated previously [7]. Transfected RBCs were cocultured with autologous Ficoll-
158 purified white blood cells (WBCs) from peripheral blood on 96-well plates for 7 days at 14°C at a
159 ratio of 105 RBCs/105 WBCs per well. WBCs (cocultured with untransfected RBCs) treated with
160 phytohemagglutinin-L (Sigma-Aldrich) (PHA-L) were used as a positive control of lymphocyte
161 proliferation because PHA-L is a well-known lymphocyte proliferation compound [42-44]. After
162 7 days of coculture, WBCs proliferation was evaluated via cell counting and RT-qPCR of
163 lymphocyte cell markers. For cell counting, cells were stained with Hoechst (Sigma-Aldrich) and
164 counted using the IN Cell Analyzer 6000 workstation 3.7.2 software (GE Healthcare, Little
165 Chalfont, UK). WBC proliferation was calculated using the formula: ((nº of cell nuclei in WBCs &
166 treated RBCs−nº of cell nuclei in untreated RBCs)/(nº of cell nuclei in WBCs & control RBCs−nº of
167 cell nuclei in untreated RBCs))×100. WBCs cocultured with TFP1-transfected RBCs were used as
168 control. RBCs untreated and no cocultured were used as negative control. For RT-qPCR, samples
169 were stored at -80°C in lysis buffer until RNA extraction and RT-qPCR analysis.

170

171 *2.8. Enzyme-linked immunosorbent assay (ELISA)*

172 Serum was collected from immunized or reinfused fish at 30 dpi as indicated above. After
173 centrifugation for 15 min at 3500 rpm, serum was stored at -20°C until used. Negative serum was
174 collected from unimmunized fish, and positive serum was collected from VHSV-challenged
175 survivors. VHSV-specific IgM antibodies were measured by ELISA as previously described [45]
176 with minor modifications. Briefly, 96-well plates were coated with concentrated VHSV (0.5
177 μg/well in PBS) and dried overnight at 37°C. Then, immunized fish serum dilutions ranging from
178 1/30 to 1/240 diluted in PBS with 0.05% Tween (Sigma-Aldrich) and 0.5% bovine serum albumin
179 (BSA) (Sigma-Aldrich) were applied to each well and incubated for 2 hours at room temperature
180 (RT). Plates were washed 3 times with PBS-0.05% Tween. Then, plates were incubated with
181 primary monoclonal antibody against trout IgM (1G7) [46] diluted 1/200 in PBS-0.05% Tween for
182 90 min at RT. Plates were washed 3 times with PBS-0.05% Tween and then incubated with rabbit

anti-mouse conjugated with peroxidase (RAM-Po) (Sigma-Aldrich) diluted 1/500 in PBS-0.05% Tween for 1 hour at RT. Finally, plates were washed 3 times with PBS-0.05% Tween and incubated with the 1-Step ultra TMB-ELISA (Thermo Fisher Scientific, Inc.) as substrate for the peroxidase reaction for 20 to 30 min at RT. Absorbance was measured at 450 nm in an Eon microplate reader (BioTek, Winooski, VT, USA).

2.9. Statistical analysis

GraphPad Prism 6 software (www.graphpad.com) was used for statistical analysis.

2.10. Ethics statement

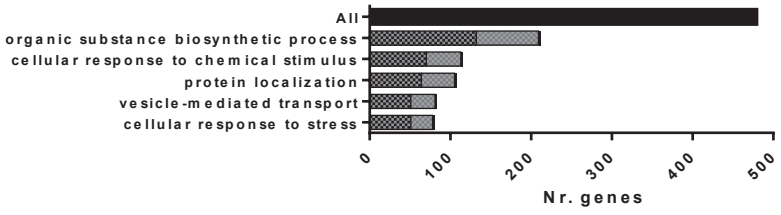
Experimental protocols and methods relating to experimental animals were reviewed and approved by the Animal Welfare Body and the Research Ethics Committee at the University Miguel Hernandez (approval number 2014.205.E.OEP; 2016.221.E.OEP) and by the competent authority of the Regional Ministry of Presidency 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.

3. Results

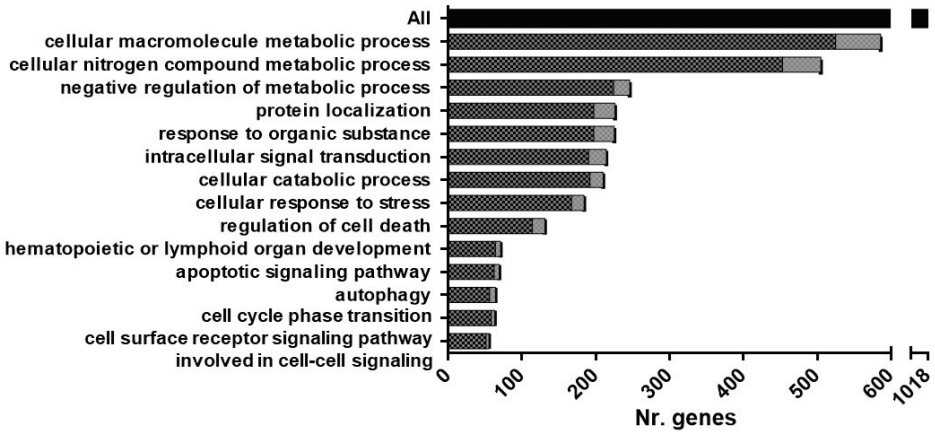
3.1. RNA sequencing of HK-RBC from GVHSV immunized rainbow trout

Transcriptome profiling of HK-RBCs that were FACS single-cell sorted from GVHSV-immunized individuals (Figure 1) identified 479 DEGs (false discovery rate [FDR] <0.05); 287 were upregulated and 192 were downregulated when compared to HK-RBCs from TFP1-injected individuals (Supplementary Table S1). *gvhsv* gene transcripts were detected, but not significantly (FDR >0.05), in HK-RBCs from GVHSV-immunized individuals.

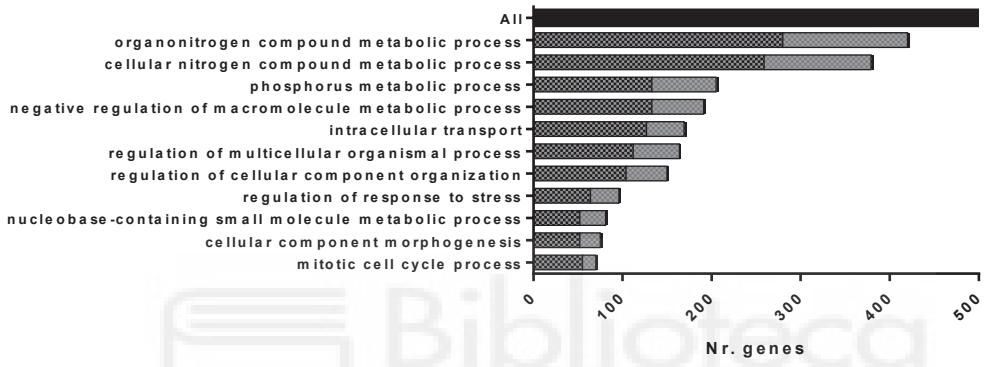
Functional pathway enrichment analysis of DEGs in HK-RBCs from GVHSV-immunized individuals using the GO Biological Process Database revealed overrepresentation of the following categories: organic substance biosynthetic process, cellular response to chemical stimulus, protein localization, vesicle-mediated transport, and cellular response to stress (Figure 2A) (Supplementary Table S2 and S3). Among the identified cellular response pathways, we were particularly interested in chemokines C-X-C motif chemokine receptor 4 (*cxcr4*) (log2 fold change [FC] = 13.01), C-C motif chemokine receptor 9 (*ccr9)* (log2 FC = 13.07), C-C motif chemokine ligand 25 (*ccl25*) (log2 FC = 8.63), and C-C motif chemokine ligand 13 (*ccl13*) (log2 FC = 8.97), all of which are involved in leukocyte chemotaxis.



(**A**)



(**B**)

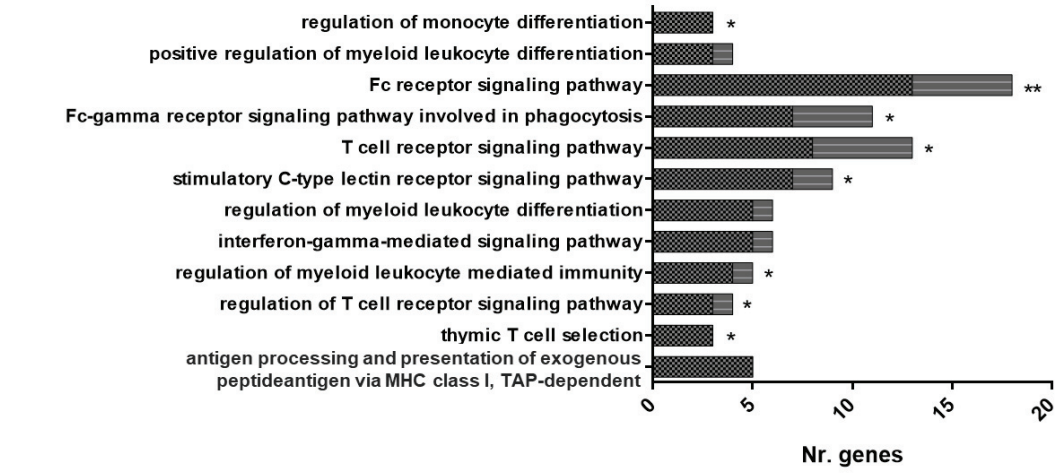


(**C**)

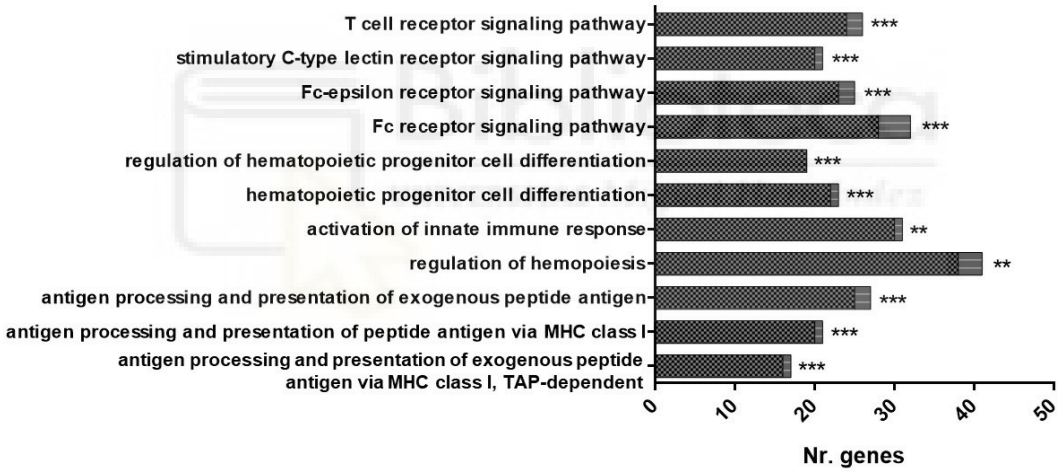
Figure 2: Overrepresented functional pathways in RBCs from GVHSV-immunized fish. Main overrepresented GO Biological Process Database terms were identified by the Cytoscape ClueGO. A) Overrepresented pathways in HK-RBC transcriptome profile. B) Overrepresented pathways in PB-RBC transcriptome profile. C) Overrepresented pathways in PB-RBC proteome profile. Black squares represent upregulated genes or proteins, and gray squares represent downregulated genes or proteins identified in each GO term. The black bar represents the total number of genes or proteins with FDR <0.05 and FC *P*-value <0.05. All GO terms overrepresented were statistically significant with *P*-value <0.05.

Functional pathway enrichment analysis of DEGs in HK-RBCs from GVHSV-immunized individuals using the GO Immune System Process Database revealed overrepresentation of the following categories: antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent; regulation of T cell receptor signaling pathway; thymic T cell selection; regulation of myeloid leukocyte differentiation; T cell receptor signaling pathway; stimulatory C-type lectin receptor signaling pathway; regulation of myeloid leukocyte mediated immunity; Fc receptor signaling pathway; and interferon-gamma-mediated signaling pathway (Figure 3A,D) (Supplementary Table S4). Among the DEGs overexpressed in GVHSV-immunized fish, we were particularly interested in the Fc fragment of IgG receptor Ia (*fcgr1a*) (log2 FC = 10.79) and hematopoietic cell kinase (*hck*) (log2 FC = 5.97), which are implicated in the Fc receptor signaling pathway; TNF superfamily member 11 (*tnfsf11*) (log2 FC = 8.43), which is involved in regulation of myeloid leukocyte differentiation; and C-C motif chemokine receptor 7 (*ccr7*) (log2 FC = 9.67), which is involved in the T cell receptor signaling pathway (Supplementary Table S5). Moreover,

protein-protein interaction (PPI) network analysis of genes overrepresented in GVHSV-immunized HK-RBC pathways using the GO Immune System Process Database demonstrated high interaction between the identified genes and corroborated the functional pathway enrichment analysis results (Figure 4A).



(A)



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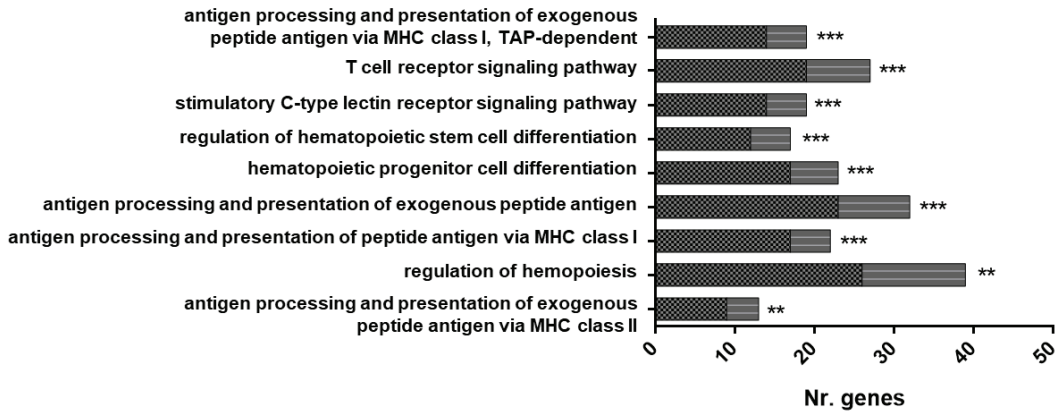
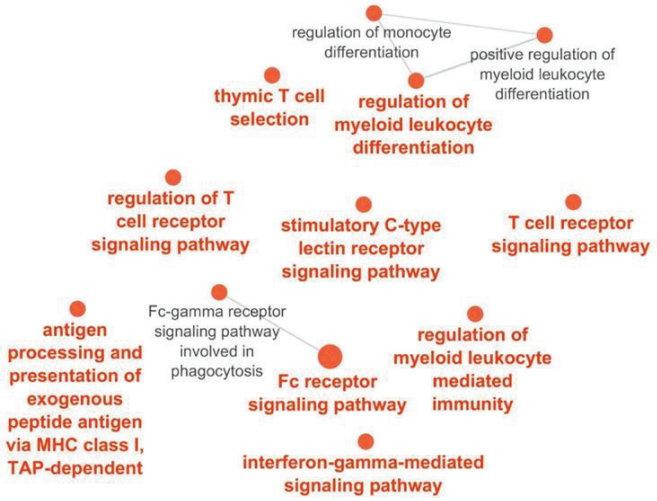
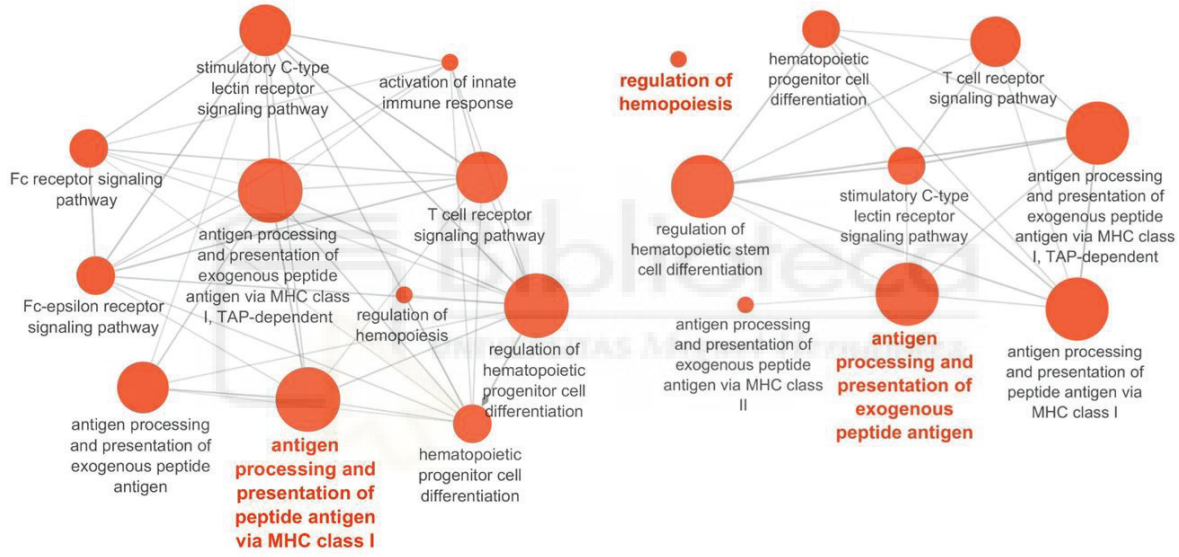


(C)



(**D**)



(**E**) (**F**)

Figure 3: Overrepresented functional pathways in RBCs from GVHSV-immunized fish. Overrepresented GO Immune System Process terms were identified by the Cytoscape ClueGO. A) Overrepresented pathways in HK-RBC transcriptome profile. B) Overrepresented pathways in PB-RBC transcriptome profile. C) Overrepresented pathways in PB-RBC proteome profile. Black squares represent upregulated genes or proteins, and gray squares represent downregulated genes or proteins identified in each GO term. Asterisks denote GO-term significance: * *P*-value < 0.05, ** *P*-value < 0.01 and *** *P*-value < 0.001. Overrepresented terms in the GO Immune System Process pathway network are shown in the (D) HK-RBC transcriptome profile, (E) PB-RBC transcriptome profile, and (F) PB-RBC proteome profile. Each node represents a GO term from an immune system process. Node size shows GO term significance (*P*-value); a smaller *P*-value is represented by a larger node size. Edges between nodes indicate the presence of common genes; a thicker line implies a larger overlap. The most significant GO term for each group is labeled.

3.2. RNA sequencing of PB-RBCs from GVHSV-immunized rainbow trout

The transcriptome profile of FACS single-cell sorted PB-RBCs from GVHSV-immunized individuals (Figure 1) identified 1018 DEGs (FDR <0.05); 892 were upregulated and 126 were downregulated when compared to PB-RBCs from TFP1-immunized fish (Supplementary Table

S6). *gvhsv* gene transcripts were detected, but not significantly (FDR >0.05), in PB-RBCs from GVHSV-immunized individuals.

Functional pathway enrichment analysis of PB-RBCs from GVHSV-immunized individuals using the GO Biological Process Database revealed overrepresentation of the following processes: cellular macromolecule metabolic process, cellular nitrogen compound metabolic process, negative regulation of metabolic process, protein localization, response to organic substance, intracellular signal transduction, cellular catabolic process, cellular response to stress, regulation of cell death, hematopoietic or lymphoid organ development, apoptotic signaling pathway, autophagy, and cell surface receptor signaling pathway involved in cell-cell signaling (Figure 2B) (Supplementary Table S7). Among the genes identified, we were particularly interested in the WD repeat domain, phosphoinositide interacting 1 (*wipi1*) (log2 FC = 6.69), GABA type A receptor-associated protein (*gabarap*) (log2 FC = 5.12), and unc-51 like autophagy activating kinase 1 (*ulk1*) (log2 FC = 6.63), which are involved in the autophagy pathway; BCL2-like 1 (*bcl2l1*) (log2 FC = 3.93), BCL2-associated athanogene 3 (*bag3*) (log2 FC = 5.97), BCL2-associated athanogene 5 (*bag5*) (log2 FC = 5.25), BCL2-interacting protein 3 (*bnip3*) (log2 FC = 4.78), superoxide dismutase 1 (*sod1*) (log2 FC = 5.37), and superoxide dismutase 2 (*sod2*) (log2 FC = 6.22), which are involved in the apoptosis signaling pathway and specifically the negative regulation of apoptosis and the antioxidant response; TNF superfamily member 11 (*tnfsf11*) (log2 FC = 7.16), cytokine receptor-like factor 1 (*crlf1*) (log2 FC = 7.08), suppressor of cytokine signaling 3 (*socs3*) (log2 FC = 6.95), lymphocyte cytosolic protein 1 (*lcp1*) (log2 FC = 5.58), TNF receptor-associated protein 1 (*trap1*) (log2 FC = 4.31), and TNF receptor-associated factor 2 (*traf2*) (log2 FC = 6.78), which are involved in intracellular signal transduction; and interferon regulatory factor 8 (*irf8*) (log2 FC = 6.83) and suppressor of cytokine signaling 5 (*socs5*) (log2 FC = 6.81), which participate in hematopoietic or lymphoid organ development (Supplementary Table S8).

Functional pathway enrichment analysis of PB-RBCs from GVHSV-immunized individuals using the GO Immune System Process Database revealed upregulation of antigen processing and presentation of peptide antigen via MHC class I (Figure 3B,E) (Supplementary Table S9). Within the DEGs overexpressed in these pathways, we were particularly interested in the following genes: beta-2-microglobulin (*b2m*) (log2 FC = 7.37), calnexin (*canx*) (log2 FC = 4.42), TAP binding protein-like (*tapbpl*) (log2 FC =13.99), and genes related to the proteasome, such as proteasome subunit alpha 3 (*psma3*) (log2 FC = 7.17) and proteasome subunit alpha 7 (*psma7*) (log2 FC = 6.28) (Supplementary Table S10). Moreover, the PPI network of genes overrepresented in GVHSV-immunized PB-RBC pathways in the GO Immune System Process Database demonstrated high interaction between the identified genes (Figure 4B).

3.3. Proteome sequencing of PB-RBC from GVHSV-immunized rainbow trout

The proteome profile of Ficoll-purified PB-RBCs from GVHSV-immunized fish identified 848 DEPs (FDR <0.05); 573 proteins were upregulated and 275 proteins were downregulated compared to PB-RBCs from TFP1-immunized fish (Supplementary Table S11). GVHSV protein was not detected in PB-RBCs from GVHSV-immunized individuals.

Functional pathway enrichment analysis of PB-RBCs GVHSV-immunized individuals using the GO Biological Process Database revealed overrepresentation of the following processes: organonitrogen compound metabolic process, cellular nitrogen compound metabolic process, phosphorus metabolic process, negative regulation of macromolecule metabolic process, intracellular transport, regulation of multicellular organismal process, regulation of cellular

component organization, regulation of response to stress, nucleobase-containing small molecule metabolic process, cellular component morphogenesis, and mitotic cell cycle process (Figure 2C) (Supplementary Table S12). Within the category of regulation of response to stress and intracellular pathways, we detected overexpression of several nucleoporins, such as nucleoporin 107 (NUP107) (log2 FC = 5.44), nucleoporin 155 (NUP155) (log2 FC = 3.64), nucleoporin 43 (NUP43) (log2 FC = 1.65), nucleoporin 133 (NUP133) (log2 FC = 1.72), nucleoporin 85 (NUP85) (log2 FC = 4.00), and nucleoporin 88 (NUP88) (log2 FC = 3.34). We found particularly interesting the identification of NLR family CARD domain-containing 3 (NLRC3) (log2 FC = 3.77), which is involved in the regulation of cellular component organization and in the regulation of response to stress (Supplementary Table S13).

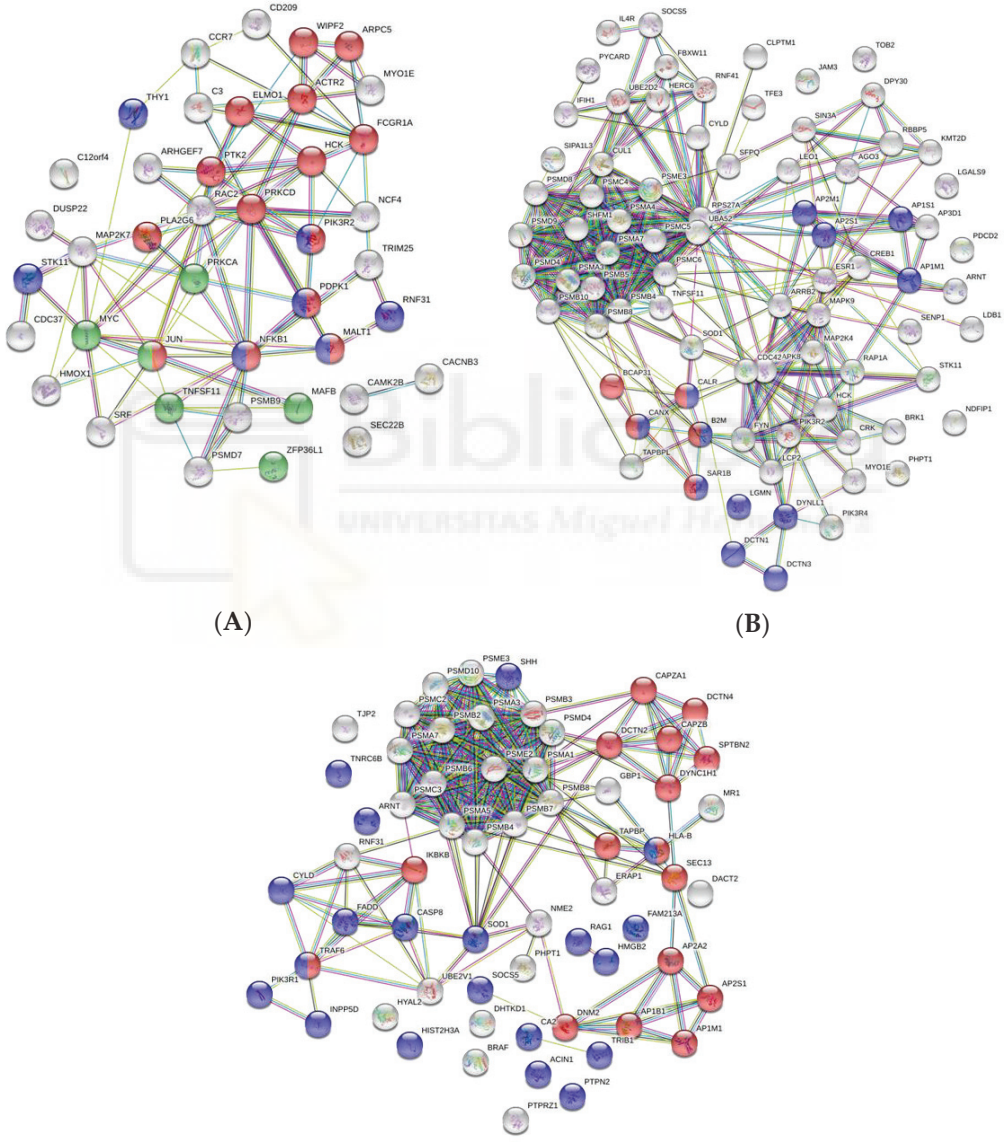


Figure 4: PPI network of the set of genes/proteins identified in overrepresented GO Immune System Process terms. PPI networks were constructed using STRING software. A) PPI network of identified genes from the HK-RBC transcriptome profile. B) PPI network of identified genes from the PB-RBC transcriptome profile. C) PPI network of identified proteins from the PB-RBC proteome profile. Nodes represent proteins, while edges denote the interactions between 2 proteins. Network edge line thickness indicates the strength of data support. The PPI enrichment *P*-value was <1.0 -16 for the 3 networks represented. Red nodes denote proteins implicated in A) FC receptor signaling pathway

(GO:0038093), B) antigen processing and presentation of peptide antigen via MHC class I (GO:0002474), and C) antigen processing and presentation of exogenous peptide antigen (GO:0002478). Blue nodes denote proteins implicated in A) T cell receptor signaling pathway (GO:0050852), B) antigen processing and presentation of exogenous peptide antigen (GO:0002478), and C) regulation of hemopoiesis (GO:1903706). Green nodes denote proteins implicated in the regulation of myeloid leukocyte differentiation (GO:0002761).

Functional pathway enrichment analysis of PB-RBCs from GVHSV-immunized individuals using the GO Immune System Process Database revealed overrepresentation of the following pathways: antigen processing and presentation of exogenous peptide antigen via MHC class I (MHCI), TAP dependent, or via MHC class II (MHCII) and regulation of hematopoiesis (Figure 3C,F) (Supplementary Table S14). Within the pathways related to antigen presentation, we found particularly interesting the presence of proteins such as major histocompatibility complex, class I, B (HLA-B) (log2 FC = 1.76) and TAP binding protein (TAPBP) (log2 FC = 2.11) for antigen processing and presentation of exogenous peptide antigen via MHCI, TAP-dependent, as well as dynamin 2 (DNM2) (log2 FC = 1.59), dynein cytoplasmic 1 heavy chain 1 (DYNC1H1) (log2 FC = 3.48), and SEC13 homolog, nuclear pore and COPII coat complex component (SEC13) (log2 FC = 2.85) for antigen processing and presentation of exogenous peptide antigen via MHCII (Supplementary Table S15). Furthermore, we highlight certain proteins overexpressed in PB-RBCs from GVHSV-immunized individuals, such as major histocompatibility complex, class I-related (MR1) (log2 FC = 4.98), interleukin 12 receptor subunit beta 2 (IL12RB2) (log2 FC = 3.56), tripartite motif-containing 25 (TRIM25) (log2 FC = 3.58), tripartite motif-containing 35 (TRIM35) (log2 FC = 2.65), interferon-induced protein 35 (IFI35) (log2 FC = 2.05), interferon-induced protein 44-like (IFI44L) (log2 FC = 3.71), and novel immune-type receptor 9 *nitr9* (log2 FC = 4.09) (Supplementary Table S11). The PPI network of proteins overrepresented in GVHSV-immunized PB-RBC pathways in the GO Immune System Process Database demonstrated high interaction between the identified genes (Figure 4C).

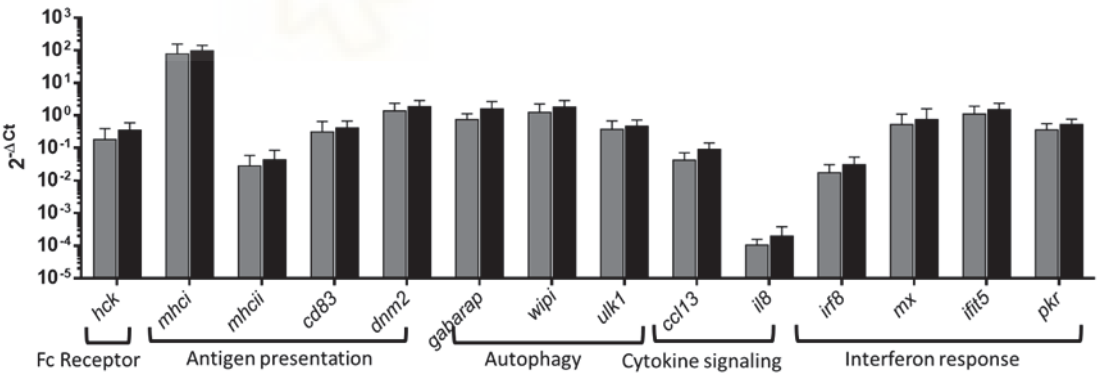


Figure 5: RT-qPCR analysis of overrepresented pathways. PB-RBCs were purified from rainbow trout immunized with TFP1 or GVHSV at 14 dpi. Gene expression was evaluated by RT-qPCR. Data are displayed as mean ± standard deviation (SD) (n=6). The *ef1α* gene was used as an endogenous control. The Mann-Whitney test was performed to compare PB-RBCs between GVHSV- and TFP1-immunized fish.

3.4. Overrepresented pathways RT-qPCR analysis

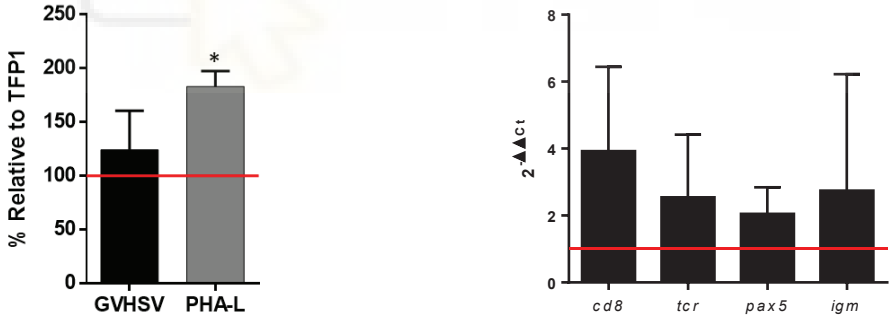
Overrepresented pathways in PB-RBCs and HK-RBCs from GVHSV-immunized fish were validated via RT-qPCR of Ficoll-purified PB-RBCs at 14 dpi. For the Fc receptor signaling pathway (overrepresented in the transcriptome profile of HK-RBC), we measured the *hck* gene expression level, which was upregulated, although without statistical significance (Figure 5).

Forthe antigen presentation pathways (which were overrepresented in the transcriptome analysis of PB-RBC and HK-RBC and in the proteome analysis of PB-RBCs), major histocompatibility complex I (*mhcI*), major histocompatibility complex II (*mhcII*), *dnm2*, and cluster of differentiation 83 (*cd83*) genes were upregulated, although without statistical significance (Figure 5). For the autophagy pathway, the *gabarap*, *ulk1*, and *wipi1* genes had increased expression, but without statistical significance (Figure 5). For the cytokine signaling pathway, the *ccl13* and C-X-C motif chemokine ligand 8 (*il8*) genes were upregulated, but without statistical significance. For the interferon response pathway, interferon regulatory factor 8 (*irf8*), interferon-induced protein with tetratricopeptide repeats 5 (*ifit5*), dsRNA-activated protein kinase R (*pkr*), and interferon-inducible Mx (*mx*) gene expression levels were upregulated, but again without statistical significance (Figure 5). Separately, *gvhsv* gene transcripts were hardly detected (over 35 of 40 Cts) in PB-RBCs from GVHSV immunized individuals.

3.5. Leukocyte proliferation

GVHSV-transfected RBCs cocultured with autologous WBCs from peripheral blood resulted in the proliferation of WBCs compared to WBCs cocultured with TFP1-transfected RBCs, as observed by enumeration of cell nuclei (Figure 6A). As a positive control, coculture of WBCs with untransfected RBCs and stimulation with PHA-L (a well-known lymphocyte mitogen) resulted in greater proliferation of WBCs compared to other conditions (Figure 6A).

The expression of certain genes related to T cells (cluster of differentiation 8 [*cd8*] and T-cell receptor [*tcr*]) and B cells (paired box gene 5 [*pax5*] and IgM membrane [*igm*]) was upregulated in WBCs cocultured with GVHSV-transfected RBCs compared to WBCs cocultured with TFP1-transfected RBCs (Figure 6B).

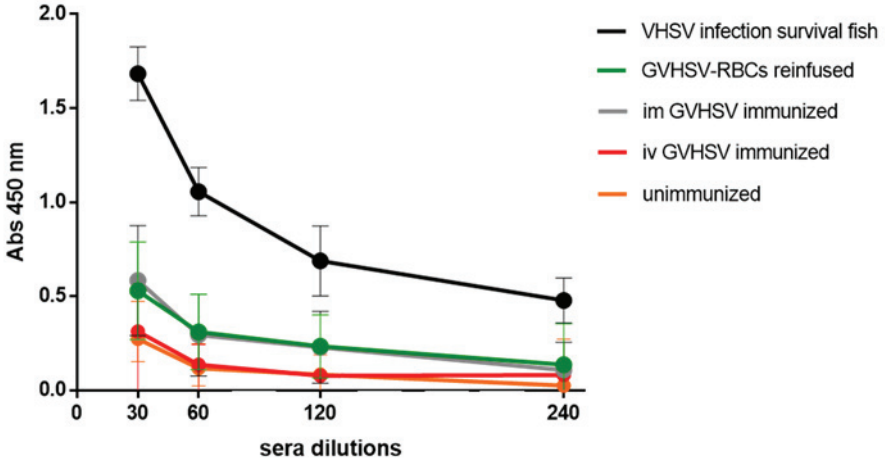


(**A**) (**B**)

Figure 6: WBC proliferation after coculture with GVHSV-transfected RBCs. RBCs and WBCs were purified from the peripheral blood of rainbow trout. WBCs were cocultured with autologous TFP1-transfected RBCs (control), RBCs and PHA-L (positive control), or GVHSV-transfected RBCs. A) WBC proliferation was measured after 7 days as a percentage of fluorescent nuclei (Hoechst stain) using the IN Cell Analyzer and calculated using the following formula: ((nº of cell nuclei in WBCs & treated RBCs−nº of cell nuclei in untreated RBCs)/(nº of cell nuclei in WBCs & control RBCs−nº of cell nuclei in untreated RBCs))×100. Data are displayed as mean ± SD (n=4). Data are shown relative to the TFP1 condition (control, red line). B) WBC gene expression of lymphocyte cell markers was measured at 7 days in cocultures of WBCs and GVHSV- or TFP1-transfected RBCs. Gene expression was evaluated by RT-qPCR. Data are displayed as mean ± SD (n=4). The *ef1α* gene was used as anendogenous control. Data shown are relative to the TFP1 condition (control, red line). A Kruskal-Wallis with Dunn's multiple comparisons test was performed between each condition and the control condition (WBCs cocultured with TFP1-transfected RBCs). **P*-value<0.05.

401 *3.6. Antibody detection in GVHSV-RBCs reinfusion/immunization*

402 VHSV-specific IgM was detected in the serum of individuals iv reinfused/immunized with
403 autologous RBCs transfected *in vitro* with GVHSV at 30 dpi (Figure 7), reaching the same level of
404 antibodies as individuals im immunized with GVHSV DNA vaccine (Figure 7). Anti-VHSV
405 antibodies were not detected in individuals iv immunized with GVHSV DNA vaccine (Figure 7),
406 which resulted in same levels of absorbance as the negative control.



407

408 **Figure 7: Serum VHSV-specific antibody detection in GVHSV-transfected RBCs-**
409 **reinfused/immunized fish.** Serum dilution from: i) im GVHSV-injected fish, ii) iv GVHSV-injected
410 fish, and iii) iv GVHSV-transfected RBCs-reinfused/immunized fish. Serum obtained from VHSV-
411 challenged survivor trout was used as a positive control, and serum from unimmunized, uninfected
412 fish was used as a negative control. Anti-VHSV antibodies were detected by absorbance readings at
413 450 nm. Results are expressed as mean ± SD of absorbance (n=4), performed in triplicate.

414

415 **4. Discussion**

416 Currently, DNA vaccination is one of the most effective approaches to prevent viral diseases in
417 aquaculture [8]. DNA vaccines encoding the glycoprotein G gene have demonstrated to be highly
418 effective against fish rhabdoviruses [10,11]. In this study, we determined the role of RBCs in the
419 context of GVHSV DNA vaccination and propose RBCs as mediators of the immune response
420 triggered by GVHSV DNA vaccine.

421 Fish RBCs are nucleated cells, and as such, they are able to respond at transcript and protein
422 levels to a stimulus. Further, fish RBCs have been implicated in the immune response against
423 different viruses [1-3,6,47]. Recently, our laboratory found that rainbow trout RBCs are able to
424 carry a DNA vaccine and respond to the encoded antigen *in vitro* [7]. Transcriptome profiling of
425 GVHSV-expressing RBCs revealed gene expression changes related to G-protein coupled
426 receptor (GPCR)-downstream signaling, complement activation, and RAR-related
427 orphanreceptor α (RORA) [7]. On the other hand, proteomic profile functional network analysis
428 of GVHSV-transfected RBCs revealed overexpression of proteins involved in the interferon-
429 stimulated gene 15 (ISG15) antiviral mechanisms, detoxification of reactive oxygen species,
430 antigen presentation of exogenous peptides, and the proteasome [7].

431 In the present work, the role of RBCs from blood and head kidney tissues of immunized fish was
432 investigated through transcriptomic and proteomic analyses. Rainbow trout head kidney is the
433 major hematopoietic organ in fish [48] and is the location in which phagocytosis, antigen

processing, and B cell maturation and differentiation occur [49]. In this regard, we aimed to determine the role of RBCs within the head kidney WBCs, where the main innate and adaptive immune responses to DNA vaccination occur.

Transcriptomic sequencing of FACS single-cell sorted HK-RBCs from GVHSV-immunized fish revealed the overrepresentation of pathways related to cellular response to chemical stimulus and stress using the GO Biological Process Database. These pathways were also overrepresented in the transcriptome and proteome profile of PB-RBCs. Genes related to responses to cellular stress have been also reported to be modulated in RBCs from blood of Piscine orthoreovirus (PRV)-challenged Atlantic salmon [6]. In the transcriptome profile of HK-RBCs within these pathways, we highlighted the overexpression of the genes *cxcr4*, *ccl13*, *ccl25*, and the *ccl25* receptor *ccr9*, which are all involved in mammalian leukocyte chemotaxis [50-52]. The presence of *ccl25*/*ccr9* in mammalian intestine has been widely discussed, particularly the involvement of these genes in the development and trafficking of T cells [53]. In teleosts, the presence of *ccl25*/*ccr9* has been reported mainly in the gut, but also in hematopoietic tissue such as the thymus, spleen, or head kidney [54,55]. The CCL25/CCR9 system has been described as highly conserved throughout vertebrates and recruits homing T cells after oral vaccination in fish [54]. In addition, upregulation of *ccl25*/*ccr9* was found after parasitic infection of fish [55]. The role of the CCL25/CCR9 system in the RBC immune response has not been investigated yet and represents an open field of study. On the other hand, it has been reported that RBCs from peripheral blood of PRV-challenged individuals down-regulated the expression of *cxcr4b* and *ccl13* genes [6], in contrast to what we observed in HK-RBCs from GVHSV-immunized individuals.

Functional pathway analysis of the HK-RBC transcriptome profile from GVHSV-immunized fish using the GO Immune System Process Database revealed overrepresentation of the antigen processing and presentation of exogenous peptide antigen via the MHCI, TAP-dependent pathway. This pathway was also overrepresented in the transcriptomic and proteomic PB-RBC profile from GVHSV-immunized fish. Commonly, MHCI is characterized by endogenous antigen presentation from the degradation of intracellular pathogens and presentation to CD8+ T lymphocytes for their clearance [56]. MHCI plays an important role in the defense against viruses [57]. Transcriptomic analysis of PRV-infected RBCs [6] revealed upregulation of genes related to antigen presentation via MHCI. Also, rockbream iridovirus-infected RBCs [47] revealed upregulation of antigen processing and presentation of exogenous peptide antigen via MHCI, TAP-dependent pathway. This process suggests the presentation of exogenous peptides in MHCI molecules through TAP transportation, which is known as crosspresentation [58-61]. In this process, exogenous peptides are presented on the cell surface together with MHCI molecules through transport via the TAP pathway through the cytosol [62-64]. Recently, proteomic profiling of *in vitro* GVHSV-transfected rainbow trout RBCs detected upregulation of antigen processingand presentation of exogenous peptide antigen via the MHCI, TAP-dependent pathway [7]. Antigen presentation of exogenous peptide antigen via MHCI was one the main pathways overrepresented in HK-RBCs and PB-RBCs; overexpression was confirmed at both the transcriptomic and proteomic levels. The presentation of exogenous peptide antigen via MHCI has been especially described for professional APCs [65,66].

Antigen presentation of exogenous peptide via MHCII was also overrepresented in the functional pathway analysis of the PB-RBC proteome from GVHSV-immunized fish. Genes and proteins related to proteosomal cleavage of exogenous antigen and antigen presentation of exogenous peptides have been reported to be upregulated in GVHSV-transfected RBCs, indicating that RBCs

could have the capacity to present DNA vaccine antigens via MHCI or MHCII [7]. Also, MHCII gene and protein expression in nucleated RBCs have been recently reported [7,20,24,67]. Currently, MHCII is undergoing functional evaluation in these cells.

Another remarkable pathway overrepresented in the HK-RBC transcriptomic profile was the Fc receptor signaling pathway. The molecular signaling triggered by the union of the immunoglobulin Fc regions with Fc receptors mediates cellular responses that are fundamental in the immune response [68]. HCK, which was upregulated in HK-RBCs from GVHSV-immunized individuals, is a member of the Src family of tyrosine kinases. This family plays an important role in the regulation of innate immune responses [69]. Scr family tyrosine-protein kinases of hematopoietic origin have been suggested to be potential transducers in the activation of monocytes/macrophages [70], participants in the regulation of myeloid cell migration [71], and players in neutrophil activation and recruitment [72]. In contrast, RBCs from PRV-challenged Atlantic salmon have been reported to downregulate *hck* gene expression [6].

Lymphocyte signaling is an important issue to consider in DNA vaccination strategies to improve efficacy [18]. Pathways such as the regulation of myeloid leukocyte differentiation, T cell receptor signaling, regulation of myeloid leukocyte-mediated immunity, and the thymic T cell selection pathways were overrepresented in the HK-RBC transcriptomic profiling, suggesting crosstalk between RBCs and WBCs in the fish head kidney. The T cell receptor signaling pathway was also overrepresented in both the transcriptome and proteome of PB-RBCs. Nucleated and non-nucleated RBCs may be inducers of T cell proliferation and contribute to the immune system through crosstalk with leukocytes [73,74]. Crosstalk between rainbow trout RBCs and other cell types has been also reported. VHSV-exposed RBCs cocultured with TSS, a stromal cell line from rainbow trout spleen, resulted in the upregulation of IFN in both cell types [1]. IFN crosstalk was also observed in RBCs cocultured with conditioned medium from the rainbow trout gonad-2 (RTG-2) cell line previously exposed to VHSV [1]. GVHSV-transfected RBCs *in vitro* induced *ifn1* and *mx* gene expression and protected against VHSV infection in RTG-2 cells, in addition to inducing differentiation markers in the rainbow trout monocyte/macrophage-like cell line RTS11 [7]. The present study showed that GVHSV-transfected RBCs induced WBC proliferation *in vitro*, suggesting that RBCs can stimulate T cells and B cells. However, the role of nucleated RBCs in antigen presentation and crosstalk with WBCs requires additional study.

Functional pathway analysis using the GO Biological Process Database revealed overrepresentation of the autophagy pathway in the PB-RBC transcriptome from GVHSV-immunized fish. Autophagy is a natural, conserved, and self-digestive catabolic process that can be critical for cell survival under stressful conditions, such as viral infection [75,76]. In fish, autophagy has been implicated in viral infections either facilitating [77,78] or inhibiting virus replication [79]. Recently, autophagy has been described in nucleated RBCs as a mechanism for defense against viruses [20,80], and the GVHSV protein is known to be involved in autophagy following immunization with DNA vaccine [22]. In the present study, we identified a correlation between GVHSV DNA vaccination and autophagy in rainbow trout RBCs. The apoptotic signaling pathway, specifically the negative regulation of apoptosis, was overrepresented in PB-RBCs from GVHSV-immunized individuals. Apoptosis and autophagy play critical roles in maintaining cell homeostasis and are involved in immune system regulation [81]. Overrepresentation of the apoptosis pathway was also detected in the transcriptomic profile of rock bream RBCs after RBIV infection [47], and apoptosis has been described for RBCs under oxidative stress [82]. On the other hand, an antioxidant response has been reported *in vitro* in

VHSV-infected RBCs and GVHSV-transfected RBCs to likely counteract the oxidative stress triggered by the virus and DNA vaccine [1,7].

The reinfusion/immunization of fish with RBCs transfected *in vitro* with GVHSV DNA vaccine revealed the presence of specific antibodies against VHSV in the serum, reaching the same levels of specific antibodies induced by conventional intramuscular GVHSV DNA vaccination. The idea of RBCs as vaccine carriers has been previously explored in non-nucleated RBCs [13-16], and RBCs have demonstrated their capacity to induce a humoral response [13]. We have previously demonstrated that rainbow trout nucleated RBCs can respond to and express GVHSV DNA vaccine *in vitro*. In this study, we demonstrated that RBCs can mount an innate immune response in response to a DNA vaccine *in vivo*, and moreover, they can induce a humoral immune response.

The use of cytokine genes as vaccine adjuvants has been shown to improve IgM titer, lymphocyte proliferation, and virus protection in glycoprotein G DNA vaccination of rainbow trout [83]. The use of type I interferon as a DNA vaccine adjuvant has also been shown to improve protection against virus, augmentation of antibody response, and migration of B and CD8 T cells [84]. Nucleated RBCs are able to upregulate interferon and interferon-inducible genes and proteins [1-7]. This link between the innate and adaptive immune responses triggered by RBCs implicates these cells as potential targets for DNA vaccination. Moreover, the involvement of HK-RBCs and PB-RBCs from GVHSV-immunized individuals in antigen presentation of exogenous peptide antigen via MHCI, as well as the capacity of PB-RBCs to induce WBC proliferation and the ability of GVHSV-transfected PB-RBCs to induce a humoral immune response, lead us to suggest that RBCs may behave as an APC-like.

The concept of atypical or no professional APCs has been previously explored. Some cells, such as mast cells, basophils, eosinophils, innate lymphoid cells [85], and neutrophils [86], have been classified as atypical APCs. According to Kambayashi and Laufer [85], atypical APCs differ from professional APCs (ie, dendritic cells, B cells, and macrophages) in their non-constitutive expression of MHCII molecules and the incapacity (or unknown capacity) to prime naïve CD4+T cells [85]. Studies in neutrophils revealed that these cells can express MHCII and costimulatory molecules under activated/stimulatory conditions, present antigen to CD4+ T cells, crosstalk with other leukocyte populations, respond by synthesizing cytokines, and link the innate and adaptive immune response, among other functions [86]. Nucleated red blood cells share several of these qualities with neutrophils. Rainbow trout RBCs can upregulate MHCII under stimulatoryconditions, such as GVHSV transfection [7]. In the present study, we detected mRNA expression of MHCII and the overrepresentation of antigen processing and presentation of exogenous peptide via MHCI and II at a proteomic level in PB-RBCs from GVHSV-immunized individuals. Additionally, RBCs can engage in crosstalk with other cell types by releasing cytokines under a stimulus *in vivo*, as we show in this manuscript, and *in vitro*, as previously described [1,7]. Thus, RBCs may participate in part of the humoral response as DNA vaccine carriers. The findings described here have led us to suggest nucleated RBCs as potential atypical APCs. Cassatella and colleagues compared neutrophils (atypical APCs) with professional APCs and suggested that the high number of atypical APCs found in the immunization site could compensate for their lower capacity for antigen presentation compared with professional APCs [86]. As such, and considering the high number of RBCs present in an organism and their participation in the innate and adaptive immune responses triggered by DNA immunization *in vivo*, nucleated RBCs may be ideal target cells for adjuvant/vaccination strategies.

Supplementary materials:

Table S1: List of DEGs identified in HK-RBCs from GVHSV-immunized individuals, by transcriptomic sequencing.

Table S2: GO Biological Process terms identified in HK-RBCs from GVHSV-immunized individuals, by transcriptomic sequencing.

Table S3: List of GO Biological Process terms and associated DEGs identified in HK-RBCs from GVHSV-immunized individuals, by transcriptomic sequencing.

Table S4: GO Immune System Process terms identified in HK-RBCs from GVHSV-immunized individuals, by transcriptomic sequencing.

Table S5: List of GO Immune System Process terms and associated DEGs identified in HK-RBCs from GVHSV-immunized individuals, by transcriptomic sequencing.

Table S6: List of DEGs identified in PB-RBCs from GVHSV-immunized individuals, by transcriptomic sequencing.

Table S7: GO Biological Process terms identified in PB-RBCs from GVHSV-immunized individuals, by transcriptomic sequencing.

Table S8: List of GO Biological Process terms and associated DEGs identified in PB-RBCs from GVHSV-immunized individuals, by transcriptomic sequencing.

Table S9: GO Immune System Process terms identified in PB-RBCs from GVHSV-immunized individuals, by transcriptomic sequencing.

Table S10: List of GO Immune System Process terms and associated DEGs identified in PB-RBCs from GVHSV-immunized individuals, by transcriptomic sequencing.

Table S11: List of DEPs identified in PB-RBCs from GVHSV-immunized individuals, by proteomic sequencing.

Table S12: GO Biological Process terms identified in PB-RBCs from GVHSV-immunized individuals, by proteomic sequencing.

Table S13: List of GO Biological Process terms and associated DEPs identified in PB-RBCs from GVHSV-immunized individuals, by proteomic sequencing.

Table S14: GO Immune System Process terms identified in PB-RBCs from GVHSV-immunized individuals, by proteomic sequencing.

Table S15: List of GO Immune System Process terms and associated DEPs identified in PB-RBCs from GVHSV-immunized individuals, by proteomic sequencing.

Figure S1: FACS single-cell sorting of HK-RBCs and PB-RBCs. A) Representative dotplot and histogram showing selected population for FACS single-cell sorted HK-RBCs using BD FACSJazz™ cell sorter. B) 102 purified HK-RBCs and C) 106 purified PB-RBCs stained with SYTO RNASelect and purified by FACS using BD FACSJazz™ cell sorter for transcriptome analysis. Brightfield and FITC images were taken at 10× magnification. D) RBCs after Ficoll gradient purification for proteome analysis. The brightfield image was taken at 20× magnification. Images were taken with the IN Cell Analyzer 6000 Cell Imaging system.

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TÍTULO: Fish red blood cells modulate immune genes in response to bacterial inclusion bodies made of TNFα and a G-VHSV fragment

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Fish Red Blood Cells Modulate Immune Genes in Response to Bacterial Inclusion Bodies Made of TNFα and a G-VHSV Fragment

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Fish Red-Blood Cells (RBCs) are nucleated cells that can modulate the expression of different sets of genes in response to stimuli, playing an active role in the homeostasis of the fish immune system. Nowadays, vaccination is one of the main ways to control and prevent viral diseases in aquaculture and the development of novel vaccination approaches is a focal point in fish vaccinology. One of the strategies that has recently emerged is the use of nanostructured recombinant proteins. Nanostructured cytokines have already been shown to immunostimulate and protect fish against bacterial infections. To explore the role of RBCs in the immune response to two nanostructured recombinant proteins, TNFα and a G-VHSV protein fragment, we performed different *in vitro* and *in vivo* studies. We show for the first time that rainbow trout RBCs are able to endocytose nanostructured TNFα and G-VHSV protein fragment *in vitro*, despite not being phagocytic cells, and in response to nanostructured TNFα and G-VHSV fragment, the expression of different immune genes could be modulated.

Keywords: erythrocytes, red blood cells, bacterial inclusion bodies, TNFα, VHSV glycoprotein G, immune response

INTRODUCTION

Fish red blood cells (RBCs) are nucleated cells that contain organelles in their cytoplasm unlike those of mammals (1). Apart from their well-known role in gas exchange, recently a set of new biological roles for nucleated RBCs related to the immune response have been reported. Nucleated RBCs are able to phagocytose and act as antigen presenting cells (2, 3). They can respond to different pathogen associated molecular patterns (PAMPs), modulate leukocyte activity, release cytokine-like factors (4, 5) and lately they have been implicated in the response to viral infections [reviewed in Nombela and Ortega-Villaizan (6)].Considering all of these findings, the potential role of RBCs in the immune system of fish takes on a new, interesting perspective.

To date, one of the best strategies for preventing and controlling viral diseases in aquaculture is DNA vaccination. However, it remains unclear which mechanisms are responsible for this protection (7). The search for new, safe and effective vaccines has become a priority in this field. Among fish viral diseases, viral hemorrhagic septicaemia (VHS) is a lethal infectious fish disease

caused by viral hemorrhagic septicaemia virus (VHSV), which affects over 50 species of fish, freshwater and marine, in the northern hemisphere (8).

As an alternative to overcome the safety problems associated to live attenuated or DNA vaccines, bacterial inclusion bodies (IBs) nanostructured recombinant proteins have been presented as a new option for vaccination (9). IBs are *per se* strong stimulants of the fish immune system and have a set of characteristics which make them an attractive alternative: they are mechanically stable, production is scalable and cost-effective, they are non-toxic biomaterials and are composed of recombinant proteins. The latter means they are an adaptable prototype, which could be a good platform for vaccination against a wide range of diseases (9, 10). Such nanostructured recombinant proteins have already been shown to protect fish against bacterial infection (9).

In this paper, we show for the first time the response of rainbow trout RBCs *in vitro* and *in vivo* to two different nanostructured recombinant proteins, recombinant rainbow trout tumor necrosis factor alpha protein ($IB^{TNF\alpha}$) and recombinant fragment 16 of the glycoprotein G of VHSV (11) ($IB^{frg16G-VHSV}$). In response to recombinant protein IBs, RBCs were able to modulate the expression of interferon related genes, the myxovirus resistance (*mx*) gene and genes related to antigen presentation (cluster of differentiation 83 [*cd83*], major histocompatibility class I [*mhcI*] and major histocompatibility class II [*mhcII*]). Genes related to antioxidant response (natural killer enhancing factor [*nkef*] and glutathione S-transferase pi 1 gene [*gstp1*] and cytokines (interleukin 1β [*il1β*], interleukin 12β [*il12β*], interleukin 6 [*il6*], interleukin 2 [*il2*], and interleukin 8 [*il8*]) were also modulated. Interestingly, $IB^{TNF\alpha}$ mostly down-regulated *in vitro* and *in vivo* immune genes expression in RBCs meanwhile $IB^{frg16G-VHSV}$ mainly showed an up-regulation trend.

MATERIALS AND METHODS

Production of IBs, Purification, Quantification , and Fluorescent Labeling

Nanostructured proteins were produced in *E. coli* following the method described in Torrealba et al. (9) and Thwaite et al. (12). In short, *E. coli* transformed with the plasmid of interest was cultured in LB with the appropriate antibiotic and recombinant protein expression was induced at OD_{550nm} 0.5–0.8 with 1 mM IPTG (Panreac, Barcelona, Spain). IBs were isolated after 3 h additional incubation at 37°C via enzymatic and mechanical disruption of the cells according to Torrealba et al. (10), followed by sterility monitoring (12). Purified nanoparticles, named here $IB^{frg16G-VHSV}$, $IB^{TNF\alpha}$ and IB^{iRFP} [an inclusion body made of a non-immunogenic phytochrome-based near infra-red fluorescent protein (iRFP) with the excitation/emission maxima at 690/713 nm (13)], were stored at −80°C until use. Quantification was performed by western blot using an anti-His-tag antibody (Genscript, Piscataway, NJ, USA) and calculating the protein concentration from a standard curve using Quantity One software (Biorad, Hercules, CA, the USA). For flow cytometry or confocal microscopy, $IB^{frg16G-VHSV}$ and $IB^{TNF\alpha}$ were conjugated with fluorescent Atto-488 NHS ester (Sigma-Aldrich) following manufacturer's instructions.

Animals

Juvenile rainbow trout (*Oncorhynchus mykiss*) were obtained from a commercial farm (Piszolla S.L., Cimballa Fish Farm, Zaragoza, Spain), and maintained at the University Miguel Hernandez (UMH) facilities at 14°C, fed daily with a commercial diet (Skretting, Burgos, Spain). Prior to experiments, fish ware acclimatized to laboratory conditions for 2 weeks. Separately, adult rainbow trout were maintained at the Universitat Autònoma de Barcelona (UAB) at 17 ± 1°C, fed daily with a commercial diet. The number of individuals used in each experiment is indicated by an "n" in each figure legend.

Cell Cultures

Rainbow trout RBCs were obtained from peripheral blood of fish sacrificed by overexposure to tricaine (tricaine methanesulfonate, Sigma-Aldrich) (0.3 g/L). Peripheral blood was sampled from the caudal vein using insulin syringes (Nipro, Bridgewater, NJ, USA) as previously described (14). RBCs were purified by two consecutive density gradient centrifugations (7,206 g, Ficoll 1.007; Sigma-Aldrich). Purity of RBCs of 99.9% was estimated by optical microscopy (**Figure S1**). Purified RBCs were cultured with RPMI-1640 medium (Dutch modification) (Gibco, Thermo Fischer Scientific Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) 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 penicillin and 100 μg/mL streptomycin (Sigma-Aldrich) at a density of 10^6 cells/mL at 14°C.

Uptake of $IB^{TNF\alpha}$ and $IB^{frg16G-VHSV}$ by RBCs

RBCs cultures were treated with fluorescent $IB^{TNF\alpha}$ or $IB^{frg16G-VHSV}$ at different concentrations and uptake was analyzed by flow cytometry using a FACSCanto™ cytometer (BD Biosciences, Madrid, Spain) (10.000 total events), at different times post-treatment. For dose-response evaluation, IBs at concentrations of 10, 20 and 50 μg/mL were added to RBCs cultures for 24 h. For time-course experiments, RBCs were treated with 80 μg/mL $IB^{TNF\alpha}$ or 160 μg/mL $IB^{frg16G-VHSV}$ for 6, 24 and 48 h. After incubation with IBs, the medium was removed and RBCs were washed with phosphate-buffered saline (PBS). RBCs were then resuspended in 200 μL of RPMI 2% FBS for flow cytometry analysis.

In addition, confocal microscopy was performed to evaluate the uptake of IBs by RBCs. RBCs were incubated with 80 μg /mL of $IB^{TNF\alpha}$ or 160 μg /mL of $IB^{frg16G-VHSV}$ for 24 h. Then, medium was removed and RBCs were washed as indicated above. The RBC nucleus was labeled with 10 μg/mL Hoechst (Sigma-Aldrich) and RBC membrane was stained with 5 μg/mL of CellMask (Thermo Fischer Scientific). Images were taken with a Zeiss LSM 700 microscope (Zeiss, Oberkochen, Germany) and analyzed with Imaris Software v8.2.1 (Bitplane, Zurich, Switzerland).

RBCs Immune Response After *in vitro* Treatment With IB$^{TNF\alpha}$ or IB$^{frg16G\text{-}VHSV}$

RBCs were treated *in vitro* with 50 μg/mL of each IB for 24 h. IB^{iRFP} was used as a control. After treatment, RBCs were resuspended in TRK lysis buffer (Omega Bio-Tek Inc., Norcross, GA, USA) and stored at −80°C until RNA extraction.

RBCs Immune Response After *in vivo* Treatment With IB$^{TNF\alpha}$ or IB$^{frg16G\text{-}vhsv}$

Juvenile rainbow trout (15–20 g) were treated by intravenous injection in caudal vein with 50 μL of IBs (5.5 mg/kg) or 50 μL of PBS. At 24 and 48 h post-injection fish were sacrificed by overexposure to tricaine. Peripheral blood was sampled as described above and resuspended in RPMI 10% FBS. Then, RBCs were Ficoll-purified as explained above. Purified RBCs were either resuspended in TRK lysis buffer and stored at −80°C until RNA extraction or fixed for immunofluorescence and flow cytometry, as described below.

In order to track the presence of IBs *in vivo*, IB$^{TNF\alpha}$ was monitored in peripheral blood and head kidney from IB$^{TNF\alpha}$ intravenously injected in caudal vein of rainbow trout by means of fluorescent microscopy using IN Cell Analyzer 6,000 Cell Imaging system (GE Healthcare, Little Chalfont, UK). Blood was extracted 3 h post-injection as described above. Head kidney was aseptically removed, placed in 24 well plates with RPMI 10%

TABLE 1 | List of primers and probes used.

Gene	Forward primer	Reverse primer	Probe	Reference or accession number
tlr3	ACTCGGTGGTGCTGGTCTTC	GAGGAGGCAATTTGGACGAA	CAAGTTGTCCCGCTGTCTGCTCCTG	(14)
tlr9	CCTGCGACACTTCCTGGTTT	GCCAGTGGTAAGAAGGAGGATCT	CAGACTTCCTGCGTGCCGGCC	(15, 16)
ifn1	ACCAGATGGGAGGAGATATCACA	GTCCTCAAACTCAGCATCATCTATGT	AATGCCCCAGTCCTTTTCCCAAATC	(14)
mx1-3	TGAAGCCCAGGATGAAATGG	TGGCAGGTCGATGAGTGTGA	ACCTCATCAGCCTAGAGATTGGCTCCCC	(16)
il15	TACTATCCACACCAGCGTCTGAAC	TTTCAGCAGCACCAGCAATG	TTCATAATATTGAGCTGCCTGAGTGCCACC	(14)
nkef	CGCTGGACTTCACCTTTGTGT	ACCTCACAACCGATCTTCCTAAAC		(14)
gstp1	CCCCTCCCTGAAGAGTTTTGT	GCAGTTTCTTGTAGGCGTCAGA		(14)
hepcidin	TCCCGGAGCATTTCAGGTT	GCCCTTGTTGTGACAGCAGTT		(14)
trx	AGACTTCACAGCCTCCTGGT	ACGTCCACCTTGAGGAAAAC		(14)
il6	ACTCCCCTCTGTCACACACC	GGCAGACAGGTCCTCCACTA	CCACTGTGCTGATAGGGCTGG	(17)
il12β	TGACAGCCAGGAATCTTGCA	GAAAGCGAATGTGTCAGTTCAAA	ACCCAACGACCAGCCTCCAAGATG	(17)
tnfα	AGCATGGAAGACCGTCAACGAT	ACCCTCTAAATGGATGGCTGCTT	AAAAGATACCCACCATACATTGAAGCAGATTGCC	(18)
il8	AGAGACACTGAGATCATTGCCAC	CCCTCTTCATTTGTTGTTGGC	TCCTGGCCCTCCTGACCATTACTGAG	(17, 19)
il1β	GCCCCCAACCGCCTTA	CAGTGTTTGCGGCCATCTTA	ACCTTCACCATCCAGCGCCACAA	(17)
il2	GTTGCAGCATTGGCCTGTT	TGTTCTCCTTATCAATCGTCTTTTGT	CAACACCACATCAGCATGACTGCCAC	NM_001164065.2
cd83	TTGGCTGATGATTCTTTCGATATC	TGCTGCCAGGAGACACTTGT	TCCTGCCCAATGTAACGGCTGTTGA	(20)
mhcI	GACAGTCCGTCCCTCAGTGT	CTGGAAGGTTCCATCATCGT		(21)
mhcII	TGCCATGCTGATGTGCAG	GTCCCTCAGCCAGGTCACT	CGCCTATGACTTCTACCCCAAACAAAT	(22)

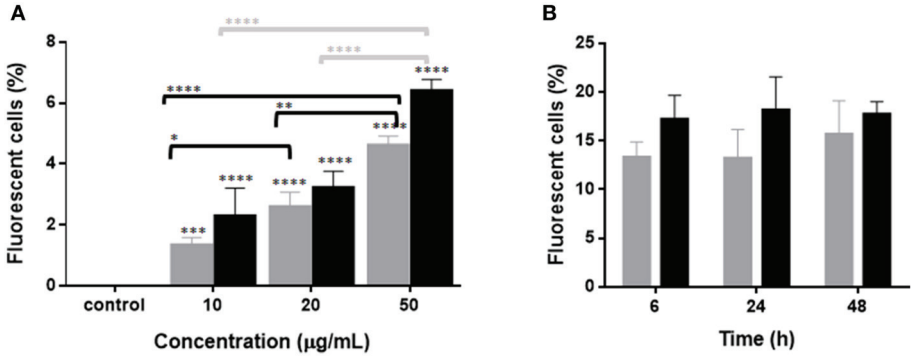

FIGURE 1 | Uptake of IB$^{TNF\alpha}$ and IB$^{frg16G-VHSV}$ by RBCs *in vitro*. **(A)** Dose-response of RBCs incubated 24 h with 10–50 μg/mL IB$^{frg16G-VHSV}$ (gray bars) or IB$^{TNF\alpha}$ (black bars). **(B)** Time course monitoring of RBCs incubated 6, 24, and 48 h with 160 μg/mL IB$^{frg16G-VHSV}$ (gray bars) or 80 μg/mL IB$^{TNF\alpha}$ (black bars). Data represent mean ± SD ($n = 4$). Two-way Anova and Dunnett's multiple comparisons test was performed between all conditions and control (untreated cells) and among concentrations. *, **, ***, ****P-value < 0.05, 0.01, 0.001, and 0.0001, respectively.

FBS and disaggregated with a Pasteur pipette and passed through a Falcon 40 μm nylon cell strainer (BD Biosciencies) using a plunger of a 5 ml syringe.

RNA Isolation, cDNA Synthesis, RT-qPCR, and Gene Expression Analysis

RBCs total RNA was extracted as previously described (14) using E.Z.N.A.® Total RNA Kit (Omega Bio-Tek Inc.). DNAse treatment was performed in order to eliminate residual genomic DNA using TURBO™ DNase (Ambion, Thermo Fischer Scientific Inc.). Then cDNA synthesis and RT-qPCR was performed as described in Nombela et al (14). Primers and probes used are listed in **Table 1**. Gene expression was analyzed by means of the $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ (23) using 18S rRNA (Applied Biosystems, Thermo Fischer Scientific Inc.) as endogenous gene. Principal component analysis (PCA) and clustering heatmap of immune-gene expression data ($2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$) were performed using Clustvis software (24). For PCA, unit variance scaling was applied to rows and singular value decomposition (SVD) with imputation was used to calculate principal components. For clustering heatmap, columns were collapsed by taking mean inside each group, rows were centered, and unit variance scaling was applied to rows; then, imputation was used for missing value estimation; and, both rows and columns were clustered using correlation distance and average linkage.

Immunofluorescence Assays

Purified RBCs were fixed as previously described (14), using 4% paraformaldehyde (PFA; Sigma-Aldrich) and 0.008% glutaraldehyde (GA, Sigma-Aldrich) in RPMI

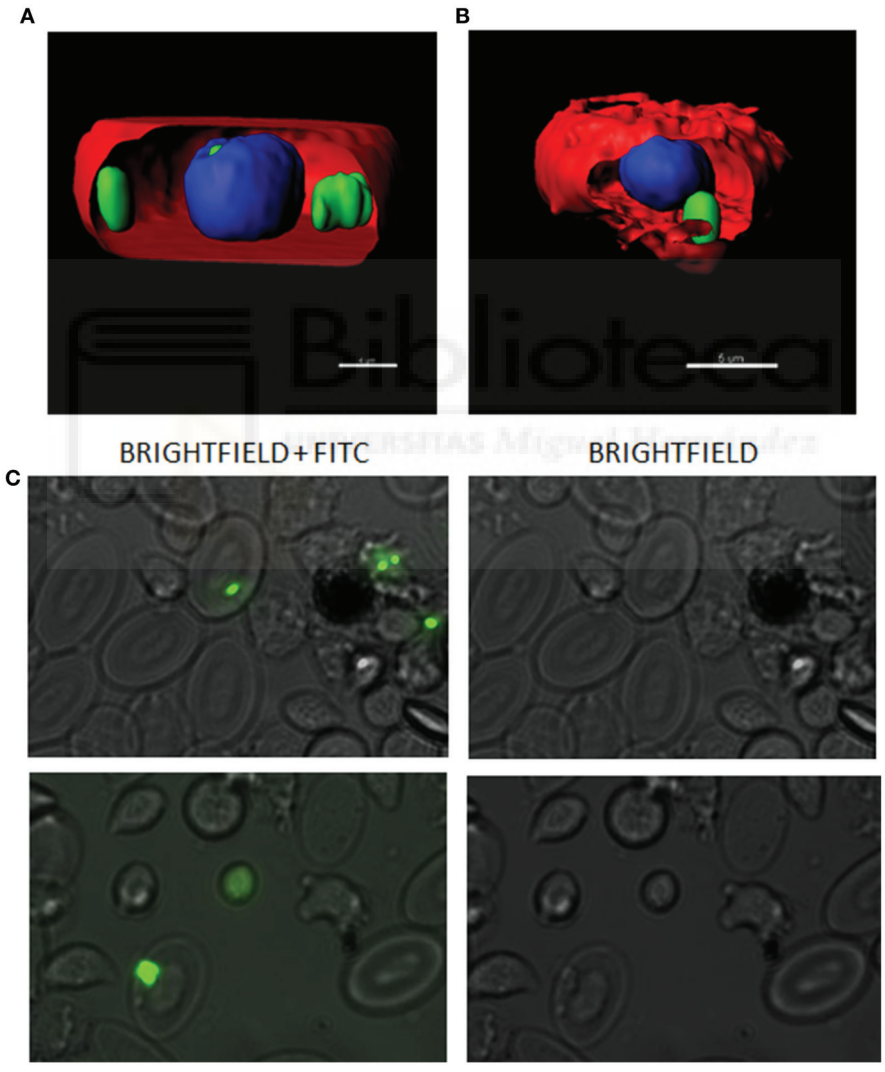

FIGURE 2 | Confocal microscopy images digitalized using z-stack. RBCs incubated with **(A)** 80 μg/mL $IB^{TNF\alpha}$ or **(B)** 160 μg/mL $IB^{frg16G-VHSV}$ for 24 h. IBs are showed in green, cell membrane (CellMask™) in red and nucleus (Hoechst-stained) in blue. **(C)** IBs monitorization *in vivo* in cells obtained from head kidney of rainbow trout injected intravenously with 5.5 mg/kg of $IB^{TNF\alpha}$, 3 h post- injection. Representative bright-field and FITC microscopy images taken with 40× magnification.

medium. Anti-MX (25, 26) and anti-IL8 (27) were used as primary antibodies and goat-CF™647 anti-mouse IgG (H+L) and goat-CF™647 anti-rabbit IgG (H+L) antibodies (Sigma-Aldrich) were used as secondary antibodies. Nuclear staining was performed with 1 μg/mL of 4′-6-Diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Images were captured in an IN Cell Analyzer 6000 Cell Imaging system. Flow cytometry was carried out in a FACSCanto™ flow cytometer.

Software and Statistics

Graphpad Prism 6.01 (www.graphpad.com) was used for statistics and graphic representation. 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/). Principal component analysis (PCA) and clustering of gene expression analysis was performed using ClustVis software (https://biit.cs.ut.ee/clustvis/) (24).

RESULTS

Uptake of IB$^{TNF\alpha}$ and IB$^{frg16G\text{-}VHSV}$ by RBCs

In order to evaluate the interaction between RBCs and IBs, we performed a dose-response and time-course evaluation by means of flow cytometry. According to our results, all IB concentrations assayed showed uptake or attachment to RBCs, which increased with IB concentration (**Figure 1A**). The percentage of IB positive cells ranged from 5 to 7% at 50 μg/mL after 24 h incubation. Time course evaluation at 6, 24, and 48 h showed no differences in IB load in RBCs (**Figure 1B**) indicating that the maximum IB internalization or attachment occurred by 6 h of incubation. However, the time course was carried out with a higher dose and up to 17% of fluorescent positive cells were detected. This was maximum percentage uptake achieved under our experimental conditions. The level of uptake of IB$^{TNF\alpha}$ by RBCs was observed to be higher than IB$^{frg16G-VHSV}$ when comparing the same concentration of both IBs (**Figure 1A**). IB uptake was confirmed by confocal 3D images, which showed the internalization of IB$^{TNF\alpha}$ (**Figure 2A**) and IB$^{frg16G-VHSV}$ (**Figure 2B**) in the cytosol of RBCs.

The presence of IB$^{TNF\alpha}$ in RBCs was monitored *in vivo* in peripheral blood and head kidney cells by fluorescent microscopy using intravenously injected IB$^{TNF\alpha}$. In blood, few RBCs were found to carry the IB$^{TNF\alpha}$ (data not shown); however, RBCs carrying IB$^{TNF\alpha}$ were easily found in head kidney cells extracts (**Figure 2C**).

Immune Response of RBCs Induced After Exposure to IB$^{TNF\alpha}$ or IB$^{frg16G\text{-}VHSV}$ *in vitro*

To explore the immune response triggered by IBs in RBCs *in vitro*, RBCs were treated with 50 μg/mL of IB$^{TNF\alpha}$, IB$^{frg16G-VHSV}$ or IB^{iRFP} and RNA was extracted at 24 h post-treatment. IB$^{TNF\alpha}$ tended to down-regulate the genes tested in RBCs at 24 h post-treatment. This down-regulation was statistically significant in genes related to antigen presentation (*cd83*, *mhcI*) and antioxidant gene *gstp1*. On the other hand, only the antioxidant *trx* gene was significantly up-regulated in IB$^{frg16G-VHSV}$ treated RBCs at 24 h post-treatment (**Table 2**).

TABLE 2 | Immune-gene expression analysis of RBCs stimulated *in vitro* with 50 μg/mL of IB^{iRFP}, IB$^{TNF\alpha}$ and IB$^{frg16G-VHSV}$ at 24 h post-treatment.

	IB$^{TNF\alpha}$		IB$^{frg16G-VHSV}$	
	Mean	**SD**	**Mean**	**SD**
mx	0.902	0.157	1.013	0.199
il15	0.943	0.288	1.181	0.414
cd83	0.782***	0.042	0.918	0.101
mhcI	0.794*	0.138	0.899	0.145
mhcII	0.965	0.235	1,270	0.428
nkef	1.106	0.753	1.067	0.943
gstp1	0.785**	0.105	1.254	0.588
trx	1.070	0.179	1.289**	0.316
tlr3	0.866	0.163	0.887	0.198
tlr9	0.814	0.656	0.907	0.623

*RBCs were Ficoll-purified and treated with IBs. 24 h post-treatment gene expression was analyzed by RT-qPCR, $2^{-\Delta\Delta Ct}$ method, normalized to the endogenous gene eukaryotic 18S, and relative to control cells (treated with IB^{iRFP}). Data represent mean fold change± SD (n = 4). Mann-Whitney test was performed between each condition and control cells. *, **, ***P-value < 0.05, 0.01, and 0.001 respectively.*

In order to analyse the gene expression of RBCs in response to each treatment as a whole, multivariate analyses of the gene expression data matrix were performed. A principal component analysis (PCA) plot of the gene expression profile showed a differentiated population of RBCs treated with IB$^{TNF\alpha}$ or IB$^{frg16G-VHSV}$ compared to IB^{iRFP} (**Figure 3A**). This is also appreciable in the clustering heatmap (**Figure 3B**), where the mean values of molecular (gene expression) signatures are clustered. The heatmap data matrix visualizes the values in the cells by the use of a color gradient which gives an overview of the largest and smallest values in the matrix (24).

Immune-Gene and Protein Expression Modulation in RBCs From Peripheral Blood After *in vivo* Treatment With IB$^{TNF\alpha}$ or IB$^{frg16G\text{-}VHSV}$

Rainbow trout were intravenously injected to evaluate the immune response triggered by IBs in RBCs of peripheral blood *in vivo*. RBCs were sampled at 24 and 48 h post-injection. In general, the results showed, as *in vitro*, a down-regulatory trend in the gene expression of IB$^{TNF\alpha}$ treated individuals compared to IB^{iRFP} treated individuals. It should be noted that *cd83* was significantly down-regulated at 24 h post-injection (**Figure 4A**), as occurred *in vitro*. On the other hand, *il6* was significantly up-regulated at 24 h post-injection. Further, *tlr9*, *ifn1*, *il1β*, *il2*, *mhcII* and *nkef* genes were significantly down-regulated at 48 h post-injection (**Figure 4B**). In contrast, IB$^{frg16G-VHSV}$ treated individuals showed an up-regulatory trend at both 24 and 48 h post-injection, compared to IB^{iRFP}, with significant up-regulation of cytokines *il2* and *il6,* and antioxidant gene *nkef* at 24 h post-injection, and of *tlr3*, interferon inducible

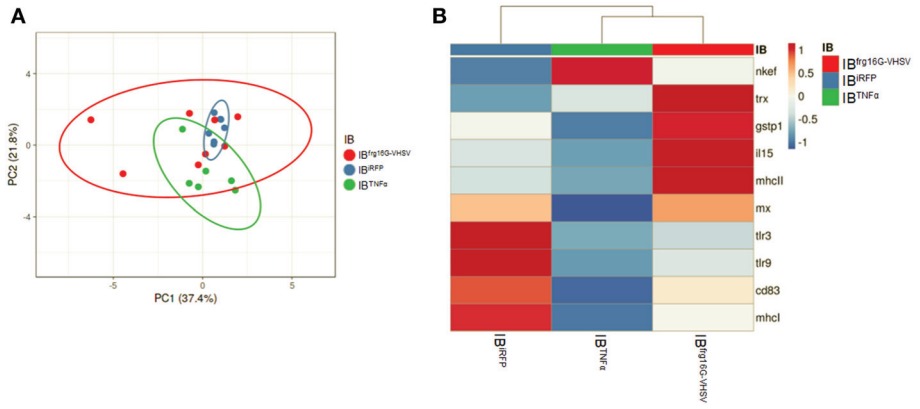

FIGURE 3 | Principal component analysis (PCA) of immune-gene expression evaluation of RBCs stimulated *in vitro* with 50 μg/mL of IB^{iRFP}, $IB^{TNFα}$, or $IB^{frg16G-VHSV}$, at 24 h post-treatment. **(A)** PCA plot of molecular (gene expression data, $2^{-\Delta\Delta Ct}$) signatures of IB^{iRFP}, $IB^{TNFα}$, or $IB^{frg16G-VHSV}$ treated samples, at 24 h post-treatment. Ellipses and shapes show clustering of the samples. **(B)** Heatmap of molecular (gene expression data, $2^{-\Delta\Delta Ct}$) signatures of IB^{iRFP}, $IB^{TNFα}$, or $IB^{frg16G-VHSV}$ treated samples. Annotations on top of the heatmap show clustering of the samples, mean values. PCA plot and heatmap were performed using Clustvis software. Heatmap data matrix visualizes the values in the cells using a color gradient which gives an overview of the largest and smallest values in the matrix.

mx, *cd83*, and *mhcII* at 48 h post-injection (**Figures 4A,B**, **Table S1**). However, *mx* gene appeared down-regulated at 24 h post-injection. Separately, most of the genes were up-regulated with all the treatments in comparison with PBS-injection.

The gene expression profile PCA plot depicted differentiated populations for RBCs from individuals treated with $IB^{TNFα}$ or $IB^{frg16G-VHSV}$ compared to IB^{iRFP} (**Figures 5A**, **6A**, for 24 and 48 h post-injection, respectively), which was also observed in the clustering heatmap (**Figures 5B**, **6B**, for 24 and 48 h post-injection respectively). In addition, at 48 h post-injection, MX and IL8 protein levels, evaluated by means of flow cytometry, showed an increment, but not statistically significant, in MX (**Figures 7A,C**) and IL8 (**Figures 7B,D**) in RBCs from rainbow trout treated with $IB^{frg16G-VHSV}$ in relation to PBS-injected or the other IBs assayed. This result correlates with the *mx* gene expression at 48 h *in vivo*. On the other hand, the protein levels of MX and IL8 in RBCs from $IB^{TNFα}$ treated rainbow trout were slightly lower than IB^{iRFP} and PBS-injected individuals (only showing statistical significance for MX between $IB^{TNFα}$ and IB^{iRFP} treatments), which is consistent with the down-regulatory trend observed in $IB^{TNFα}$ treated RBCs *in vivo* and *in vitro* at the transcriptional level. Moreover, in whole peripheral blood, a similar tendency was observed in MX protein expression, although more pronounced in this case. Note, however, for IL8 protein levels, we did not observe any difference among groups (**Figures 8A,B**).

DISCUSSION

Recently, IBs have been reported as new alternatives in fish prophylaxis as immunostimulants or adjuvants (10), thus potentially serving as a new platform for vaccine delivery. The uptake of IBs has been reported in rainbow trout macrophages (RT-HKM) and zebrafish liver cells (ZFL). In both cell types IBs made with cytokines stimulate the innate immune response (9). Moreover, IBs made with fish viral antigens have evoked an anti-viral innate immune response in ZFL and RT-HKM (12). However, the immune response of nucleated RBCs to nanostructured cytokine or viral antigen IBs has not been tested until now. Nucleated RBCs are the main cell in the blood and recently have been endorsed as immune cells mediators (6, 28). In this work we show that the uptake or attachment of IBs by rainbow trout RBCs occurred in approximately 7% of cells counted. This contrasts to the near 40% and 80% reported for ZFL and RT-HKM, respectively, at same concentration (50 μg/mL) of $IB^{TNFα}$ (9). RBCs endocytosed both the IBs tested here reaching their maximum level at 6 h post-treatment, in contrast to RT-HKM and ZFL cell lines, which reached their maximum uptake at 24 h post-treatment *in vitro* (10). Besides, monitorization of $IB^{TNFα}$ *in vivo* demonstrated its presence on/in RBCs from head-kidney 3 h post-injection. The mechanism by which RBCs endocytose IBs is unknown. It may occur via the micropinocytosis endocytic pathway, as proposed for mammalian cells (29).

Significantly, with this work, we add to the growing body of data demonstrating nucleated RBCs can exercise a role in the immune response. RBCs are able to respond to virus (6, 14, 30), produce cytokines when exposed to stimuli (17), and endocytose pathogens (2). Here we show for the first time rainbow trout RBCs evoke an immune response to IBs made of cytokine TNFα and viral protein frg16G-VHSV *in vitro* and *in vivo*. We demonstrate this response at protein and transcript level. Rainbow trout Ficoll-purified RBCs treated with IBs *in vitro* and RBCs Ficoll-purified from blood extracts from IB-intravenously injected individuals modulated the expression of genes related to antigen presentation, cytokines and other genes involved in the immune response. PCA clearly clustered the RBCs' immune-gene expression profiles for each treatment.

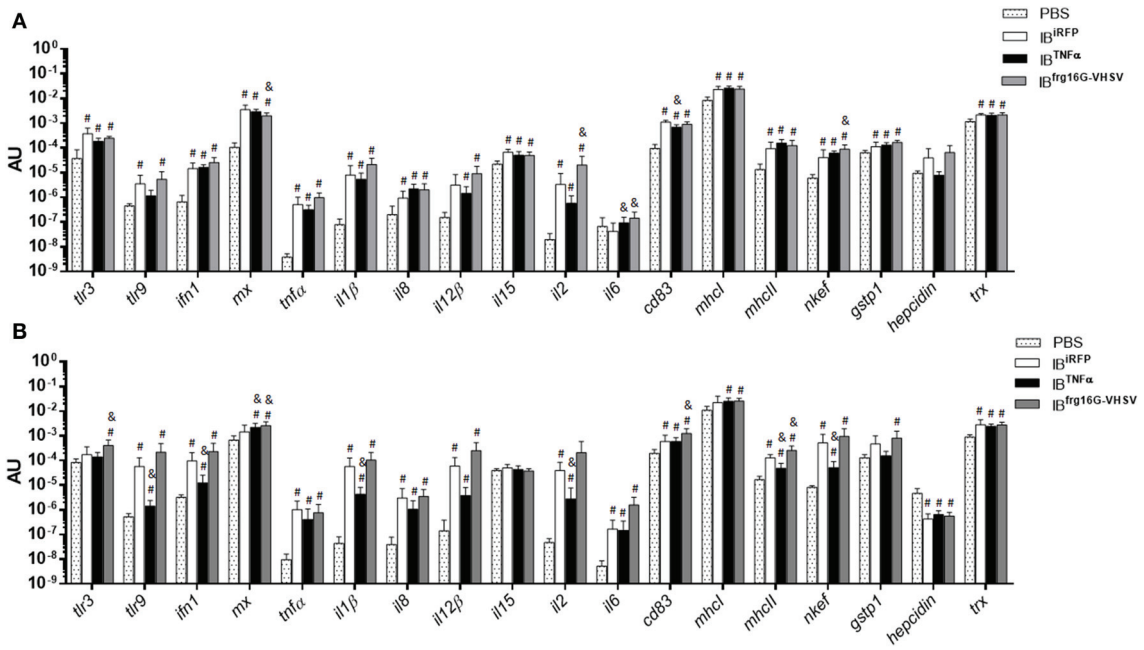

FIGURE 4 | Immune-gene expression analysis of RBCs from rainbow trout injected intravenously with PBS, IB^{iRFP}, $IB^{TNF\alpha}$, or $IB^{frg16G-VHSV}$ at 24 and 48 h post-injection. Rainbow trout of 15–20 g were injected with 5.5 mg/kg of IB intravenously. Blood was extracted and RBCs Ficoll-purified 24 h **(A)** and 48 h **(B)** post-injection. Gene expression was analyzed by RT-qPCR, $2^{-\Delta Ct}$ method, with endogenous gene eukaryotic 18S rRNA. Data represent mean AU (arbitrary units) ± SD ($n = 4$). Mann-Whitney test was performed between each condition and control (treated with PBS or IB^{iRFP}). #*P*-value < 0.05, compared to PBS; & *P*-value < 0.05, compared to IB^{iRFP}.

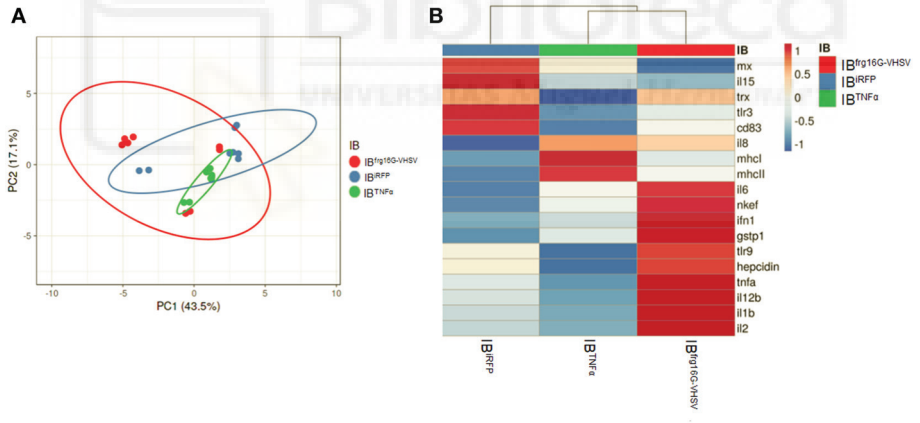

FIGURE 5 | Principal component analysis (PCA) of immune-gene expression evaluation of RBCs from rainbow trout injected intravenously with IB^{iRFP}, $IB^{TNF\alpha}$, or $IB^{frg16G-VHSV}$, at 24 h post-injection. **(A)** PCA plot of molecular (gene expression data, $2^{-\Delta Ct}$) signatures footprint of IB^{iRFP}, $IB^{TNF\alpha}$, or $IB^{frg16G-VHSV}$ treated samples, at 24 h post-injection. Ellipses and shapes show clustering of the samples. **(B)** Heatmap of gene expression ($2^{-\Delta Ct}$) signatures of IB^{iRFP}, $IB^{TNF\alpha}$, or $IB^{frg16G-VHSV}$ treated samples. Annotations on top of the heatmap show clustering of the samples mean values. PCA plot and heatmap was performed using Clustvis software. Heatmap data matrix visualizes the values in the cells using a color gradient which gives an overview of the largest and smallest values in the matrix.

As regards TNFα, RBCs from $IB^{TNF\alpha}$-treated rainbow trout individuals showed a down-regulatory trend for genes related to TNFα signaling such as *tlr9*, *tnfα*, *il1β*, *il12β*, and *il2* genes transcripts, *in vivo*, at 24 and 48 h post-injection, compared to fish injected with the non-immunogenic protein IB^{iRFP}. It is known that TNFα is a cytokine involved in the regulation of immune cells and inflammation. It is mainly produced by monocytes and macrophages along with additional producers including B and T lymphocytes, NK cells, polymorphonuclear leukocytes, and eosinophils in response to bacterial toxins, inflammatory products, and other invasive stimuli (31). Recently, nucleated RBCs have been also reported to modulate TNFα

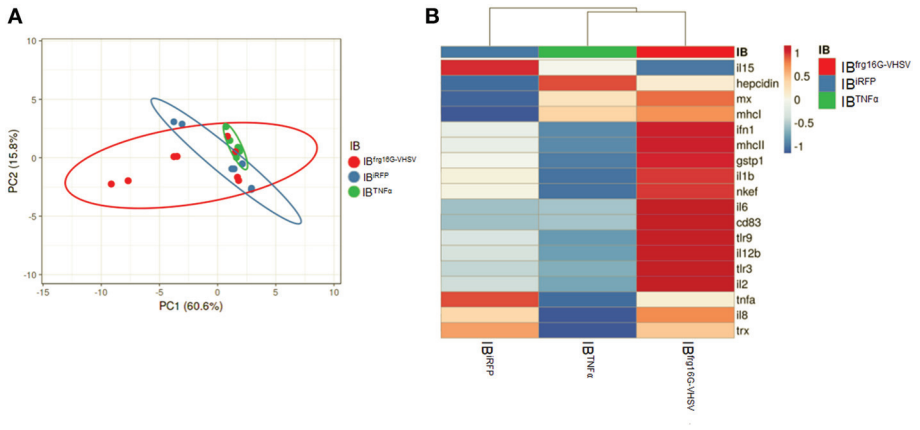

FIGURE 6 | Principal component analysis (PCA) of immune-gene expression evaluation of RBCs from rainbow trout injected intravenously with IB^{iRFP}, $IB^{TNF\alpha}$, or $IB^{frg16G-VHSV}$, at 48 h post-injection. **(A)** PCA plot of molecular (gene expression data, $2^{-\Delta Ct}$) signatures of IB^{iRFP}, $IB^{TNF\alpha}$, or $IB^{frg16G-VHSV}$ treated samples, at 48 h post-injection. Ellipses and shapes show clustering of the samples. **(B)** Heatmap of molecular (gene expression data, $2^{-\Delta Ct}$) signatures of IB^{iRFP}, $IB^{TNF\alpha}$, or $IB^{frg16G-VHSV}$ treated samples. Annotations on top of the heatmap show clustering of the samples mean values. PCA plot and heatmap was performed using Clustvis software. Heatmap data matrix visualizes the values in the cells using a color gradient which gives an overview of the largest and smallest values in the matrix.

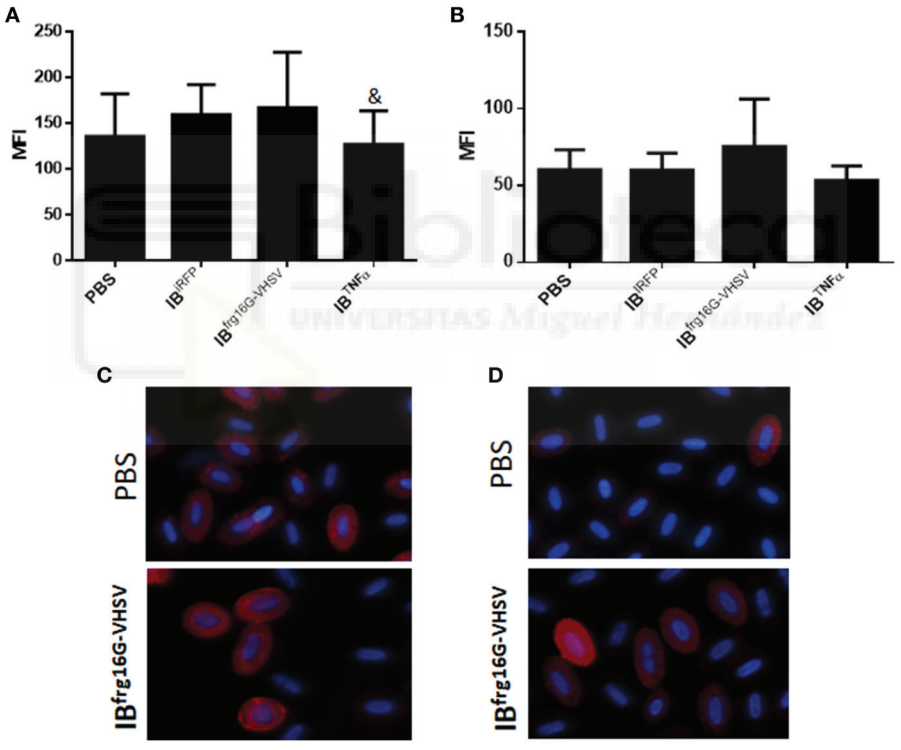

FIGURE 7 | Protein expression analysis of RBCs from rainbow trout injected intravenously with 5.5 mg/kg of IB^{iRFP}, $IB^{TNF\alpha}$, $IB^{frg16G-VHSV}$ or PBS at 48 h post-injection. **(A)** Interferon related protein MX and **(B)** chemokine IL8 Mean Fluorescence Intensity (MFI) measured by flow cytometry. Data represent mean ± SD ($n = 4$). Mann-Whitney test was performed between each condition and control cells (treated with PBS or IB^{iRFP}). &P-value < 0.05, compared to IB^{iRFP}. Representative immunofluorescence images of RBCs stained with **(C)** anti-MX and **(D)** anti-IL8, taken with 60× magnification. Protein stain in red, DAPI (blue) for nuclei stain.

protein in response to IPNV virus exposure (30). Here we observed that RBCs exposed to $IB^{TNF\alpha}$ down-regulated the inflammatory response at 24 and 48 h post-treatment. TNFα is a pleiotropic cytokine with a diverse range of biological actions. TNF family members are known to represent a "double-edged sword," having both beneficial and detrimental activities

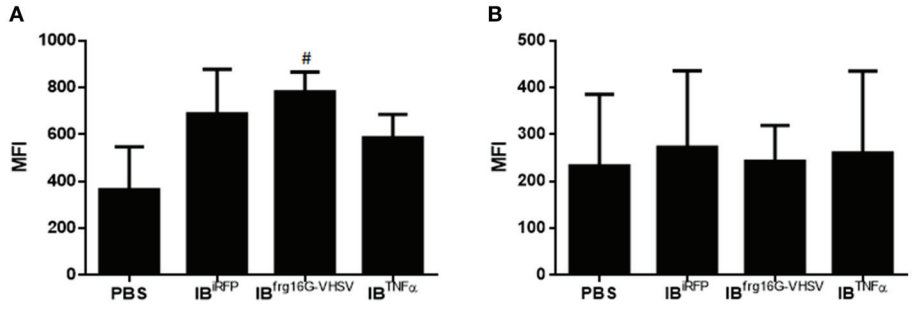

FIGURE 8 | Protein expression analysis of total blood samples from rainbow trout injected intravenously with 5.5 mg/kg of IB^{iRFP}, $IB^{TNF\alpha}$, $IB^{frg16G-VHSV}$, and PBS at 48 h post-injection. **(A)** Interferon related protein MX and **(B)** chemokine IL8 Mean Fluorescence Intensity (MFI) measured by flow cytometry. Data represent mean ± SD ($n = 4$). Mann-Whitney test was performed between each condition and control cells (treated with PBS or IB^{iRFP}). #P-value < 0.05, compared to PBS.

(32). Systemic exposure to recombinant TNFα would cause a shock similar to septic shock syndrome (31). Further, TNFα inhibition of IFNγ-induced IL12 production exerts mechanisms by which TNFα and IL12 cytokines can elicit anti-inflammatory and repair functions, tightly modulated by positive and negative feedback signals for optimal immunity without manifested inflammation (33). Another important observation is that fish recombinant TNFα has been reported to regulate the expression of endothelial cells TLRs, including TLR9, but had negligible effects on macrophages (34). Therefore, taking into account that nucleated RBCs are the most abundant cell type in peripheral blood, it would make sense that RBCs were equipped to modulate inflammation in response to a systemic exposure to TNFα. Moreover, in the $IB^{TNF\alpha}$ injected group, genes related to antigen presentation, *cd83* and *mhcII*, were also down-regulated at 24 and 48 h, respectively. As well, RBCs treated *in vitro* with $IB^{TNF\alpha}$ down-regulated the expression of *cd83* and *mhcI* 24 h post-treatment. TNFα has been reported to modulate IFNγ-induced MHC class II expression in a cell type-specific mode (35). Therefore, TNFα treatment augments or blocks MHC class II induction depending on the cell type and cellular differentiation state (35). *mhcII* and *cd83* gene expression has been previously reported for rainbow trout RBCs (3, 36) and chicken RBCs (37). However, this is the first report that shows the regulation of *cd83* and *mhcII* gene transcripts in response to an immunostimulant.

On the other hand, RBCs from rainbow trout injected with $IB^{frg16G-VHSV}$ showed an up-regulatory trend for most of the genes, specifically interleukins *il2* and *il6*, and antioxidant enzyme *nkef* were significantly up-regulated, compared to IB^{iRFP}, at 24 h post-injection. This is probably due to the effort of RBCs to compensate the inflammatory response triggered after the first treatment stimulus. Then, 48 h post-injection, the Type 1 IFN and antigen presentation responses were increased, since *tlr3*, *mx*, *cd83,* and *mhcII* genes transcripts were significantly up-regulated, compared to IB^{iRFP}. MX protein production was consistent with gene expression levels.

G-VHSV is known to induce the expression of *ifn1* and *mx* (25, 38, 39). Peptides derived from G-VHSV have also demonstrated their efficacy to induce type 1 IFN response (25, 26, 39). It is also noteworthy that $IB^{frg16G-VHSV}$ triggered the up-regulation of *mhcII* and *cd83* gene expression in rainbow trout RBCs, thus endowing them the characteristics of antigen presenting cells (APCs). CD83 and MHCII are principally produced by professional APCs to process antigens and induce T cell priming. However, recently, the concept of non-professional APCs is emerging (40). These atypical APCs up-regulate the expression of MHC and related molecules under certain stimuli. However, there is not enough evidence about their functionality priming T cells (40).

Bacterial lipopolysaccharide has been reported to stimulate the innate immune response of RBCs *in vitro* (28). Bacterial IBs, which contain remnants of endotoxin, are therefore considered immunostimulants *per se* (41), which is shown by the global increment in the immune response of RBCs from rainbow trout injected with IB^{iRFP} compared to PBS-injection. This, added to the utilization of IBs as delivery platforms to administrate cytokines, coadjuvants, or antigens, makes them a good candidate for future vaccines. In this context, RBCs have shown their ability to mount or modulate and immune-response to IBs made of cytokine TNFα and the viral protein frg16G-VHSV.

All these considerations provide a new perspective on the role and potential use of RBCs. Given the large amount of RBCs in the organism and their rapid distribution throughout the body they could be a promising target cell for the presentation or delivery of IBs or other types of vaccine carriers.

ETHICS STATEMENT

Experimental protocols and methods of the experimental animals at the UMH 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. All experimental procedures of

the experimental animals at the UAB were approved by the Human and Animal Experimentation Ethics Committee of the Universitat Autònoma de Barcelona (Reference 1533) and were done in strict accordance with the recommendations of the European Directive (2010/63/EU) on the protection of animals used for scientific purposes.

AUTHOR CONTRIBUTIONS

SP-M performed experiments, analyzed data, and wrote the manuscript. RT performed experiments and contributed to manuscript preparation. JC contributed to $IB^{frg16G-VHSV}$ construction. LM provided valuable antibodies for the experiments. NR conceived ideas, oversaw the research and contributed to manuscript preparation. MO-V conceived ideas, oversaw the research and co-wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01055/full#supplementary-material

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