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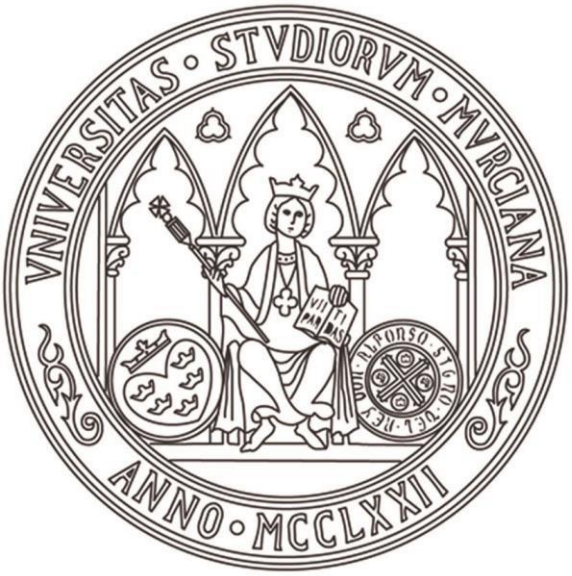
DINÁMICA DE LA RESPUESTA CITOTÓXICA MEDIADA POR LEUCOCITOS DE PECES FRENTE A BETANODAVIRUS

D. Miguel Ángel García Álvarez

2024



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DECLARACIÓN DE AUTORÍA Y ORIGINALIDAD DE LA TESIS PRESENTADA EN MODALIDAD DE COMPENDIO O ARTÍCULOS PARA OBTENER EL TÍTULO DE DOCTOR

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doctorando del Programa de Doctorado en

Biología Molecular y Biotecnología

de la Escuela Internacional de Doctorado de la Universidad Murcia, como autor/a de la tesis presentada para la obtención del título de Doctor y titulada:

Dinámica de la respuesta citotóxica mediada por leucocitos de peces frente a Betanodavirus/Cell-mediated cytotoxicity of fish leucocytes against Betanodavirus

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“Aceptaré la responsabilidad por lo que he hecho.

Si debo caer, cada vez me alzaré como un hombre mejor.”

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García-Álvarez, M.Á., González-Fernández, C., Esteban, M.Á., Cuesta, A., 2023. Molecular characterization of the cytotoxic and regulatory T cell coreceptor (CRTAM), and its ligand CADM1, in the European seabass and gilthead seabream. Fish Shellfish Immunol. 134, 108569. https://doi.org/10.1016/j.fsi.2023.108569

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Cervera, L., **García-Álvarez, M.Á.,** Valero, Y., González-Fernández, C., Arizcun, M., Chaves-Pozo, E., Esteban, M. A., Cuesta, A. "*Herramientas biotecnológicas para el estudio y control de nodavirus"*. Poster communication in "I *Congreso sobre Avance Europeo en Crecimiento Azul".* 7-8 June, 2023. Murcia, Spain.

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García-Álvarez, M.Á., Chaves-Pozo, E., Cuesta, A. "*Localización y distribución de células productoras de IgM en tejidos de lubinas infectadas por Nodavirus"*. Poster communication in "*XIX Congreso Nacional de Acuicultura".* 17-20 June, 2024. Las Palmas de Gran Canaria, Spain.

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ABBREVIATIONS INDEX

ADCC	Antibody-dependent cellular cytotoxicity
AMP	Antimicrobial peptide
APC	Antigen presenting cell
BCR	B-cell receptor
BFNNV	Barfin flounder nervous necrosis virus
BTLA	B and T-lymphocyte attenuator
CADM1	Cell adhesion molecule 1
CD	Cluster of differentiation
CHNV	Crucian carp hematopoietic necrosis virus
CMA	Concanamycin A
CMC	Cell-mediated cytotoxicity
CNS	Central nervous system
ConA	Concanavalin A
cp	Capsid protein
CRTAM	Cytotoxic and regulatory T cell molecule
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte-associated antigen 4
DC	Dendritic cell
dph	days post-hatching
dpi	days post-infection
EGTA	Ethyleneglycol-bis(β-aminoethyl)-tetraacetic acid
HK	Head-kidney
HKL	Head-kidney leucocytes
ICOS	Inducible costimulatory signal
ifi44	IFN-induced protein 44
ifit	IFN-induced proteins with tetratricopeptide repeats
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
irf3	Interferon regulatory factor 3
ISG	Interferon-stimulated genes
ITAM	Activation intracellular motifs
ITIM	Inhibitory intracellular motifs

KIR	Killer-cell immunoglobulin-like receptors
LFA-1	Leucocyte-function-associated antigen-1
LPS	Lipopolysaccharide
mAB	Monoclonal antibody
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
MLR	Mixed leucocytes reactions
MMC	Melanomacrophage center
mx	Mixovirus (influenza) resistance protein
NCC	Non-specific cytotoxic cell
NCCRP-1	Non-specific cytotoxic cell receptor protein 1
NILT	Novel immunoglobulin-like transcripts
NITR	Novel immune-type receptors
NK	Natural killer
NLR	Nucleotide-oligomerization domain-like receptor
NNV	Nervous necrosis virus
PAMP	Pathogen-associated molecular patterns
PBL	Peripheral blood leucocyte
PCA	Principal component analysis
PD-1	Programmed cell death-1
PHA	Phytohaemagglutinin
PRR	Pathogen recognition receptors
RAG	Recombination activating gene
rdrp	RNA-dependent RNA polymerase
RGNNV	Red spotted grouper nervous necrosis virus
RIG-I	Retinoic acid-inducible gene I
RLR	Retinoic acid-inducible gene I like receptors
SJNNV	Striped jack nervous necrosis virus
TCR	T-cell receptor
Th	T helper cell
TIGIT	T cell immunoglobulin and ITIM domain
Tim-3	T cell immunoglobulin-3
TLR	Toll-like receptor

TNF	Tumor necrosis factor
TNV	Turbot nodavirus
TPNNV	Tiger puffer nervous necrosis virus
VER	Viral encephalopathy and retinopathy
VHSV	Viral haemorrhagic septicaemia virus
VLR	Variable lymphocyte receptors
zap	Zeta chain of T cell receptor associated protein kinase

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CHAPTER IV

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SUMMARY

The immune system of animals is tasked with protecting organisms against a multitude of external pathogens. To prevent entry, colonization and subsequent spread throughout the body, numerous biochemical and cellular processes are initiated, all part of the innate and adaptive immune systems. The study models for this work are (i) fish, which exhibit an immune system akin to that of mammals, encompassing most cellular and humoral components; and (ii) viruses, the most lethal pathogens. Fish have several immunological mechanisms for eliminating virus-infected cells. While the interferon (IFN) pathway is the most studied, the cell-mediated cytotoxicity (CMC), carried out by non-specific cytotoxic cells (NCCs), natural killer (NK) and $CD8^+$ T lymphocytes (CTLs) (CD, cluster of differentiation), is also very relevant but scarcely evaluated. In the Mediterranean aquaculture sector, European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) represent the most extensively farmed and commercialized species. However, both species face several viruses that cause substantial economic losses annually. Among these, nervous necrosis virus (NNV; classified into RGNNV, SJNNV, BFNNV and TPNNV genotypes) causes high mortality in European sea bass whereas gilthead seabream is a resistant species to most of the isolates and may act as a reservoir of traditional genotypes, although natural reassortant genotypes cause mortalities in this species. Therefore, in this Doctoral Thesis, we aimed to delve into the dynamic interaction between European sea bass or gilthead seabream leucocytes and NNV, deepening the CMC mechanisms.

Due to the appearance in recent years of natural outbreaks in gilthead seabream hatcheries of reassortant NNV genotypes that cause high mortality rates, in **Chapter I,** we first focused on evaluating the effect of RGNNV/SJNNV and SJNNV/RGNNV natural reassortant genotypes under laboratory conditions. To achieve this goal, larvae at 37 and 86 days post-hatching (dph) were subjected to immersion infection with RGNNV/SJNNV or SJNNV/RGNNV genotypes. Then, we evaluated the time course of mortality, viral replication, modulation of immune genes and expression of antimicrobial peptides. We found that larvae at both stages were vulnerable to both NNV infection, with the RGNNV/SJNNV reassortant genotypes demonstrating higher pathogenicity and the youngest larvae showing the highest susceptibility. Furthermore, analysis of mRNA levels for the capsid protein (*cp*) and RNA-dependent RNA polymerase (*rdrp*) viral genes revealed that both reassortant genotypes could replicate, with RGNNV/SJNNV showing elevated viral gene levels. Notably, viral particle recovery for both genotypes was

detected exclusively in the 37 dph larvae, with a predominance of RGNNV/SJNNV. Subsequently, immune genes related to inflammation, antiviral defence and CMC were evaluated, revealing different expression patterns for the two genotypes and larval ages. In 37 dph larvae, high mortality and viral replication of RGNNV/SJNNV appeared to be due to a general and time-decreasing tendency in the transcription of IFN and CMC-related genes whilst the lower viral replication of SJNNV/RGNNV might be due to the increasing state of immunity. However, in 86 dph larvae, the increased transcription of the antiviral *mx* gene might explain the low mortalities observed. Therefore, the resistance of the larvae was related, partially, to the increase in the transcription of the markers of the CMC response.

In **Chapter II,** we focused on the characterization of a well-known co-receptor of active T lymphocytes in mammals, named cytotoxic and regulatory T cell co-receptor (CRTAM), and its ligand, the cell adhesion molecule 1 (CADM1), in the European sea bass and gilthead seabream. We have evaluated their transcriptional levels and modulation in response to NNV *in vivo* infection and during the CMC activity. Firstly, we conducted a bioinformatical analysis, confirming the presence of one *crtam* gene and two *cadm1* genes, named *cadm1a* and *cadm1b*. The identified putative proteins displayed the structure seen in mammals. Thus, CRTAM contains two immunoglobulin (Ig) domains whereas CADM1, both a and b, variants exhibit three Ig domains in the extracellular region. Furthermore, synteny and phylogenetic analysis underscored their good conservation across vertebrate evolution for the three proteins. At the transcriptional level, *crtam, cadm1a* and *cadm1b* were all constitutively expressed in naïve tissues, with *crtam* and *cadm1a* predominantly found in immune-related organs such as the spleen, thymus and head-kidney (HK), whereas *cadm1b* was mainly expressed in the brain. The *in vitro* analysis revealed that concanavalin A (ConA) and phytohemagglutinin (PHA), two well-established T cell mitogens, elevated the mRNA levels of *crtam* and *cadm1a* in European sea bass head-kidney leucocytes (HKLs), respectively. By contrast, in seabream HKLs, no genes suffered regulation by any treatment. Subsequently, upon NNV infection, an up-regulation in the expression of *crtam* and *cadm1a* was observed in the brain and HK of both species, manifesting earlier in seabream compared to European sea bass, and explaining the resistance of seabream against NNV. Moreover, these genes were also up-regulated during the innate CMC response in seabream, a pattern not seen in sea bass. Taking all the data together, the findings suggest a closer association of CRTAM with

innate cytotoxicity in seabream and with specific T cell-mediated cytotoxicity in European sea bass.

Thus, considering the involvement of European sea bass T lymphocytes against NNV, **Chapter III** consisted of the study of perforin (PRF) in that species, one of the main effector molecules of the CMC response, produced mainly by CTLs. Therefore, we aimed to identify the *prf* gene, its transcriptional level and its modulation upon an *in vivo* NNV infection. Firstly, we performed an *in silico* study that revealed four *prf* genes within European sea bass, denominated *prf1.2, prf1.3, prf1.5* and *prf1.9*. All predicted PRF proteins conserved the characteristic domains found in its human counterpart, exhibiting the membrane attack complex/PRF (MACPF), at the N-terminal end, and the C2 domain. All *prf* genes showed constitutive and widespread tissue expression, being the *prf1.9* gene predominant in immune-related tissues such as the thymus and spleen. Moreover, the *in vitro* stimulation of HKLs with PHA generated an increase in *prf1.2, prf1.5* and *prf1.9* gene expression. Additionally, during innate CMC responses to xenogeneic target cells, HKLs showed increased transcription of *prf1.2* and *prf1.9* genes. Furthermore, the *in vivo* infection evidenced the up-regulation of all genes in HK, brain and gonad with the exception of the *prf1.3* gene. Lastly, the application of a polyclonal antibody against PRF1.9 led us to identify an increase in PRF1.9^{+} cells in HK, brain and gonad of NNV-infected fish. Overall, the evidence suggests that all *prf* genes, with the notable exception of *prf1.3*, are implicated in the immunity of European sea bass and possibly in the CMC response, with PRF1.9 playing the most important role.

Due to the presence of active T lymphocytes and cytotoxic molecules in the immune system of the NNV-susceptible European sea bass, coupled with the comparatively low innate cytotoxic response of sea bass HKLs to NNV-infected cells, in **Chapter IV** we decided to investigate the adaptive CMC of HKLs from NNV-infected European sea bass. For this purpose, we used as target cells the DLB-1 cell line, derived from the brain of the same species, infected by RGNNV or SJNNV/RGNNV reassortant genotypes as target cells, considering they show similar viral capsid. Moreover, we also examined the genes underlying this mechanism. Our results showed low and unaltered innate cytotoxic activity of HKLs through the infection time. In contrast, the CMC against both NNV-infected target cells evidenced a progressive increase from 7 to 30 days post-infection (dpi), with a noteworthy peak at 15 dpi. Strikingly, this CMC activity was abrogated if the target DLB-1 cells were infected with different NNV genotypes. Thus,

these data underscored the specificity of the cytotoxic response and pointed towards the involvement of CTLs, possibly in a major histocompatibility complex (MHC)-I-matched manner, considering that effector and target cells share various MHC-I alleles. At the transcriptomic level, we found an up-regulation of genes associated with the two main cytotoxic pathways of CTLs, the perforin/granzyme and Fas/FasL routes, as well as genes involved in apoptotic death.

1.INTRODUCTION

Over the last 150 years, the human population has tripled, reaching 8 billion people in 2022. In this context, where food demand has similarly increased, aquaculture has emerged during the last decades as a solution to supply human needs with high quality animal protein. The rise in marine food products has been possible thanks to the growth of the global aquaculture sector, while fishing activity has been decreasing year after year, having been surpassed by aquaculture activity in 2022, providing 57% of all aquatic food available for human consumption (FAO, 2024). Globally, Asian countries are the main producers, accounting for 91.5% of production, followed by America (3.6%) and Europe (2.9%) (APROMAR, 2023). Spain, due to its geographical location, was the main aquaculture producer country of Europe in 2023, with the 24% of total production. Within Spain, the Region of Murcia is the community with the highest production, with gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) being two of the most cultivated species with high economic importance (APROMAR, 2023).

However, the increasing demand for aquaculture production brings with it problems associated with the intensive farming of aquatic species. The high stocking densities in confined spaces can lead to high stress on the animals, increasing their susceptibility to infection by pathogens and the development of associated diseases, resulting in high economic losses (Ashley, 2007). Therefore, it is necessary to delve deeper into the study of the immune system and its different defence mechanisms to develop effective strategies that prevent infections in facilities and improve fish welfare.

1 Immune system of teleost fish

The evolution of living beings has been significantly influenced by the development of the immune system, a complex network that protects organisms against several pathogens and distinguishes between “self” and “altered self” or “non-self” molecules. This system is categorized into innate and adaptive immune systems (Mitchell and Aspinall, 2008), with innate immunity appearing around 600 million years ago, where a wide array of physical, humoral, and cellular mechanisms are responsible for defending against pathogens (Litman *et al*., 2005; Mitchell and Aspinall, 2008) (Fig. 1). Conversely, the adaptive immune system emerged millions of years later (between 500-450 million years ago) in the first vertebrates, with the jawless vertebrate group possessing variable lymphocyte receptors (VLRs), which are clonally expressed in independent cell

populations, akin to mammalian T and B lymphocytes (Guo *et al*., 2009; Pancer *et al*., 2004). However, the most notable similarity with higher vertebrates is observed in the jawed vertebrate clade, as numerous key genes of the mammalian adaptive immune system have been identified, including the T-cell receptor (TCR), B-cell receptor (BCR), recombination activating genes 1 and 2 (RAG1 and RAG2) and genes associated with major histocompatibility complex I and II (MHC-I and MHC-II) (Cannon *et al*., 2004; Smith *et al*., 2019).

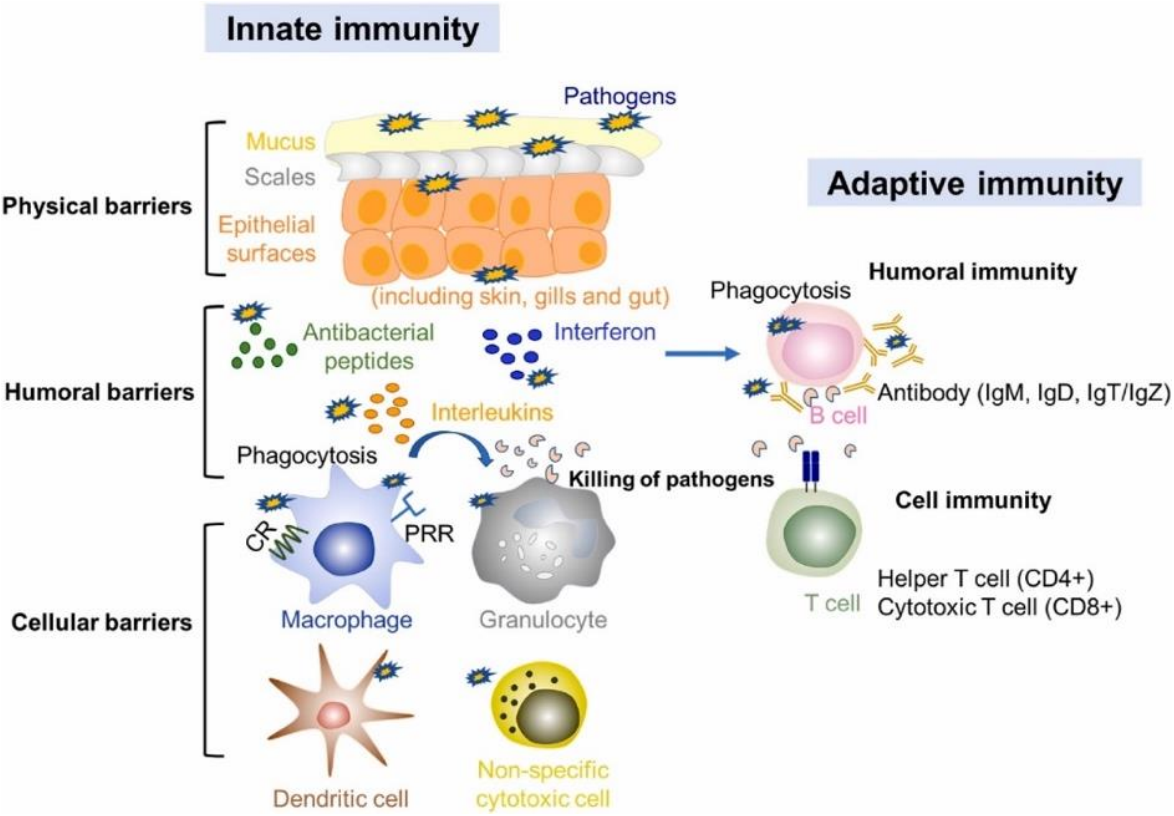

Figure 1. Major components of the innate and adaptive immune system of fish. From: (Wu *et al*., 2022).

Unlike mammals, teleost fish lack lymph nodes and bone marrow but have a primary hematopoietic and immunological organ called the head-kidney (HK) (Fig 2.), which contains macrophages able to form melanomacrophage centers (MMCs) with high antimicrobial activity, similar to mammalian germinal centres (Steinel and Bolnick, 2017; Wolke, 1992). Additionally, HK is crucial for lymphocyte maturation, especially B cells (Deluca *et al*., 1983), and antigen presentation through cells analogous to mammalian dendritic cells (Bassity and Clark, 2012). Fish also have thymus and spleen (Fig. 2), serving as primary and secondary immune organs, respectively. Thymus is involved in T

lymphocyte formation, differentiation and maturation, evidenced by the presence of double negative ($CD8^-CD4^-$) (CD, cluster of differentiation) and double positive ($CD8^+CD4^+$) T lymphocytes, as well as a significant activity of RAG genes (Huttenhuis *et al*., 2005; Picchietti *et al*., 2009). For its part, the spleen is mainly involved in antigen presentation and adaptive immune activation (Arnesen *et al*., 2002).

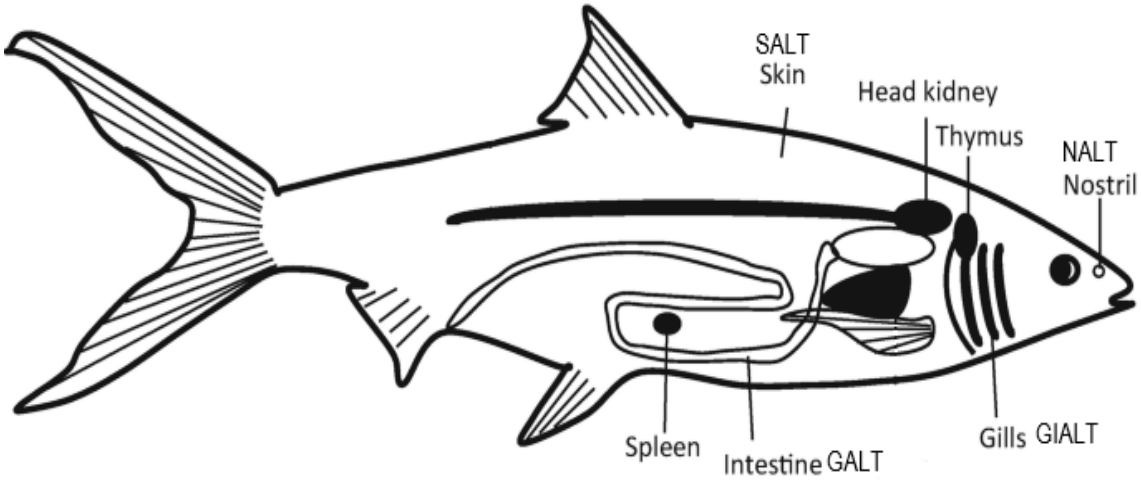

Figure 2. Schematic representation of the main primary lymphoid organs, head-kidney and thymus, and secondary lymphoid organs, spleen and mucosa-associated lymphoid tissues (SALT, GALT, GIALT and NALT). Modified from (Makesh *et al*., 2022).

1.1 Innate immune system of fish

As in mammals, fish innate immunity is characterized by being the first line of defence against the penetration of pathogens, as well as its rapid reaction since this system recognizes common molecular patterns shared by a wide variety of pathogens, known as pathogen-associated molecular patterns (PAMPs), through different types of pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs), nucleotide-oligomerization domain-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I) like receptors (RLRs), C-type lectins and complement components (Zhu *et al*., 2013). The binding of PAMPs to their respective PRRs initiates a molecular cascade that finishes in the production of anti-pathogenic molecules such as antiviral or inflammatory cytokines (Martinon *et al*., 2002; Skjæveland *et al*., 2008).

Innate immunity starts in the surface barriers (skin, intestine and gills) serving as the initial physical barriers against microorganisms. They are called mucosa-associated lymphoid tissues (MALTs) distinguished by their production of a mucus layer, whose

functions are: (i) entrap pathogens, and (ii) house resident leucocytes. Consequently, a significant proportion of innate immune cells stand at the forefront of the immune response in MALTs (Fillatreau *et al*., 2013; Mokhtar *et al*., 2023).

The humoral arm of the innate immunity consists of a diverse range of soluble molecules located in bodily fluids or mucus that limit the growth of pathogens and drive their subsequent eradication and the restoration of homeostasis. These include cytokines, antimicrobial peptides (AMPs), or the complement cascade, among many others. Cytokines are small proteins that facilitate cell communication and are categorized based on their biological roles. Within this family, inflammatory cytokines play a fundamental role in innate immunity, particularly interleukin (IL)-1β, tumor necrosis factor (TNF)-α and interferons (IFNs) (Mokhtar *et al*., 2023). In fish, IL-1β orchestrates the inflammatory response by stimulating leucocytes such as macrophages or T cells (Mokhtar *et al*., 2023; Wang *et al*., 2009) while IFNs constitute the main antiviral response triggering a cascade that culminates with the activation of interferon-stimulated genes (ISGs) and viral clearance (Robertsen, 2006; Verrier *et al*., 2011). By its side, AMPs are small peptides (between 12 and 60 amino acids) with antimicrobial and immunomodulatory functions (Shabir *et al*., 2018). The composition of AMPs, combining positive charges with hydrophobic domains, primes them for engaging with the negatively charged, lipid-dense cytoplasmic membranes of microbial cells resulting in destabilization and ultimately disintegration of these microorganisms (Brown and Hancock, 2006). Fish synthesize a wide variety of AMP families, being piscidins, hepcidins, beta-defensins, cathelicidins and histone-derived peptides the most extensively studied, displaying a broad spectrum of functions, encompassing not only antibacterial activity but also antiviral, antifungal and even antitumor activities (Broekman *et al*., 2011; Chen *et al*., 2009a; Jin *et al*., 2010; Pinzón-Arango *et al*., 2013). Finally, the complement system consists of a large number of soluble and membrane-associated proteins, which are classified into three different routes: classical, lectin and alternative pathways. These routes converge at the formation of C3 convertase, an enzyme complex pivotal in the activation of the complement system. Subsequent steps involve the opsonization of pathogens, which marks them for destruction by phagocytes, the release of chemoattractants that recruit immune cells to sites of infection and the formation of the membrane attack complex, leading to the lysis of the pathogen (Holland and Lambris, 2002).

Regarding the cellular components of the innate immunity, the most studied functions are those mediated by the phagocytes and the cytotoxic cells. Phagocytes are responsible for phagocytosis, respiratory burst and inflammatory responses. In fish, the main phagocytic cells are monocytes/macrophages, granulocytes and dendritic cells, devoted to the recognition of pathogens, their destruction and production of humoral factors to restore the homeostasis (Wu *et al*., 2022). On the other hand, fish non-specific cell-mediated cytotoxicity (CMC) is mediated by non-specific cytotoxic cells (NCCs) and natural killer (NK)-like cells (Mokhtar *et al*., 2023), both responsible for recognizing and killing virus-infected and tumor cells in a spontaneous response without any induction period (Jaso-Friedmann *et al*., 2000; Yoshida *et al*., 1995).

1.2 Adaptive immune system of fish

As previously mentioned, teleost fish represent the earliest vertebrate group to exhibit adaptive immunity (Mitchell and Aspinall, 2008), marking an evolutionary milestone, which is distinguished from innate immunity by its specific recognition of pathogens and the development of immunological memory, ensuring that subsequent infections by the same pathogen elicit a swifter immune response, ultimately fortifying the organism against future encounters (Smith *et al*., 2019).

The main component of the adaptive humoral response is represented primarily by antibodies, also called immunoglobulins (Igs), which are high molecular size glycoproteins (around 70-80 kDa) that can be either soluble and secreted by B lymphocytes or attached to membranes as membrane-bound receptors. Three major Ig isotypes, IgM, IgD and IgT are found in teleost fish, being the last one specific of this group (Mutoloki *et al*., 2014). IgM is the most predominant, found mainly in the serum, and is expressed as a tetramer (Bromage *et al*., 2004). The roles of IgM encompass both innate and adaptive immunity, with the activation of the complement system, clustering of pathogens for easier engulfment, promotion of antibody-dependent cell-mediated cytotoxicity (ADCC) and elimination of pathogens (Boshra *et al*., 2004; Shen *et al*., 2003; Ye *et al*., 2013).

Regarding the adaptive cellular response, B lymphocytes are a fundamental axis in the adaptive system, being the main antigen-presenting cells (APCs), antibody producers and they also possess phagocytic function, the latter in fish (Li *et al*., 2006).

Different subpopulations of B lymphocytes have been described, depending on the type of immunoglobulin secreted (Li *et al*., 2006). Besides B lymphocytes, T lymphocytes constitute the major subpopulation of the lymphoid lineage and are the main cytotoxic executioner arm of the adaptive immune system. This group is characterized by the presence of the TCR in its cellular membrane by the heterodimer TCRα/β, vital for the recognition of the antigenic peptides (Mutoloki *et al*., 2014). Additionally, other T cells expressing the heterodimer TCRγ/δ are preferentially present in mucosal tissues (Nielsen *et al*., 2017). T cells are divided in two subpopulations depending on the presence of the co-receptor CD8 or CD4, which are called cytotoxic T cells (CTLs) and T helper cells (Ths), which match with MHC-I and MHC-II, respectively (Fig. 3) (Squier and Cohen, 1994). Fish, as in mammals, have both types of T cell subpopulation, with the genes encoding *cd8α* and *cd8β*, found to be similar to their mammalian counterparts, as well as two different *cd4* genes, named *cd4-1* and *cd4-2*, and also orthologous genes for *mhcI* and *mhcII* (Yamaguchi *et al*., 2019). Additionally, *mhcI* genes have been documented in fish exhibiting their classical polymorphic sequences and allelic variation (Aoyagi *et al*., 2002). The heavy chain of MHC-I, along with β2-microglobulin, can bind and present small peptides, pointing a similar role in antigen presentation to CTLs as in mammals (Chen *et al*., 2017). In addition, the presence of different Ths groups in fish has been confirmed, such as Th1, Th2 or Th7, due to their expression of cytokines (Nakanishi *et al*., 2015). For the correct functionality of T lymphocytes, especially CTLs, other co-receptors are needed to participate in their activation, cessation, or regulation of their cytotoxic activity, which are described in section 2.2. Additionally, other cell groups participate in the adaptive response. Hence, APCs stand out, whose primary functions lie in displaying antigenic peptides from pathogens bound to MHC-II, enhanced by co-stimulatory proteins such as CD86, CD80 and CD83, and migrate to lymphoid organs where present the antigens to naïve T lymphocytes for its subsequent activation (Tian *et al*., 2022). In fish, as in mammals, the principal APCs are dendritic cells, (DCs), macrophages and B lymphocytes, deeply characterized in zebrafish (*Danio* rerio) and rainbow trout (*Oncorhynchus mykiss*) (Bassity and Clark, 2012; Lewis *et al*., 2014). Nevertheless, in other fish species, other cells such as granulocytes or erythrocytes also act as APCs (Cuesta *et al*., 2006; Iliev *et al*., 2013).

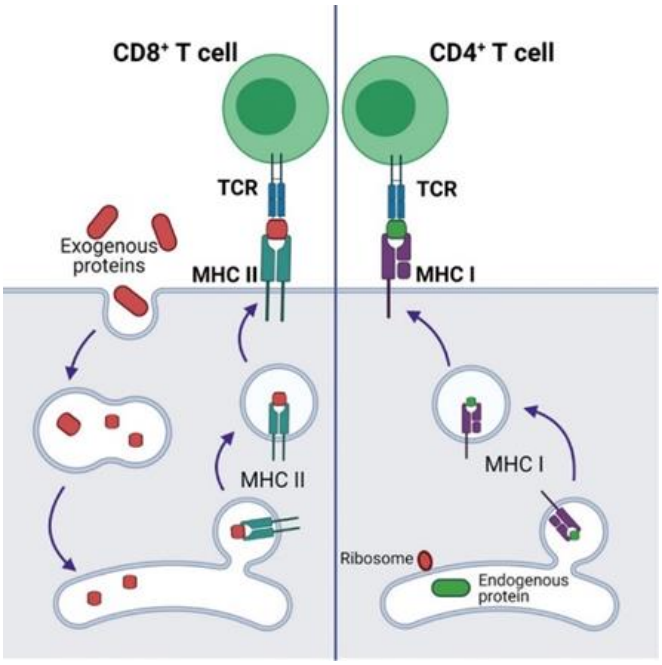

Figure 3. Schematic representation of antigen presentation to $CD8^+$ T cells (left) and $CD4^+$ T cells (right) through MHC-I and MHC-II, respectively. From: (Chasov *et al*., 2021).

2. Cell-mediated cytotoxicity

Numerous studies in fish, similar to mammals, have revealed the existence of numerous cell lineages with the capability to identify and destroy pathogen-infected or tumor cells, thereby neutralizing them. This process, known as CMC, is particularly noteworthy. During the CMC process, cytotoxic effector cells recognize and bind to the target cells, forming an immunological synapse, triggering the activation of different biochemical pathways leading to the death of target cells, either through apoptosis or necrosis, and effectively halting its replication and dissemination (Squier and Cohen, 1994). Therefore, the main types of CMC described in teleost fish, along with the associated cells and regulatory and effector molecules, are detailed in the following sub-sections.

2.1 Innate or non-specific cell-mediated cytotoxicity

The first evidence of the innate cytotoxicity in teleost fish was the discovery of a cell type named as NCC from head-kidney of channel catfish (*Ictalurus punctatus*), where NCCs produced the lysis of human and murine tumor cell lines by direct cell-cell contact in a non-specific manner, as no prior contact with the target cells was necessary (Evans

et al., 1984; Graves *et al*., 1984). These initial experiments, and the activation of NCCs by NKTag, a conserved target antigen found on numerous targets, as human tumor cell lines, led to the hypothesis that these NCCs could be the evolutionary ancestors of mammalian NK cells (Evans *et al*., 1996, 1984). Non-specific CMC carried out by NCCs has been also documented in various teleost fish, such as tilapia (*Oreochromis niloticus*) (Faisal *et al*., 1989), rainbow trout (Greenlee *et al*., 1991) or gilthead seabream (Cuesta *et al*., 2002) among others. However, only in channel catfish, another cytotoxic cell type was discovered in the peripheral blood leucocytes (PBLs), finally named as NK-like cells, which preferentially killed allogeneic cells (Yoshida *et al*., 1995). While NK-like cells are granular and large, as the mammalian NK cells, NCCs are agranular and exhibit pleiomorphic morphology, and also show tissue-based distribution (Evans *et al*., 1987; Shen *et al*., 2004). However, further studies in other fish species found that NCCs might consist of a heterogeneous cell population consisting of lymphocytes, monocyte-macrophages and/or granulocytes. This was followed by the identification of the exclusively non-specific cytotoxic cell receptor (NCCRP)-1 protein on NCC, which recognizes the peptide NKTag, that is shared by the surface of tumor cells (Evans *et al*., 1996; Jaso-Friedmann *et al*., 1997). After that, NCCs and NCCRP-1 have been described in several fish species (Cuesta *et al*., 2005; Greenlee *et al*., 1991; Ishimoto *et al*., 2004; Suzumura *et al*., 1994).

Once the effector cytotoxic cells bind to the target cells, the next steps are similar to mammals, where some well-studied pathways are activated to kill the target cells. These include the participation of two major effector cytotoxic proteins, perforin and granzymes (granule-dependent pathway, PRF/GZM), as well as the Fas/FasL protein system (granule-independent pathway, Fas/FasL) (Hogan *et al*., 1999; Jaso-Friedmann *et al*., 2000; Shen *et al*., 2002), being found all of these genes in several fish species (Athanasopoulou *et al*., 2009; Mao *et al*., 2021; Matsuura *et al*., 2014; Toda *et al*., 2011). NCCs have been found to express granzyme-like serine proteases and *prf* transcripts, indicating that teleost NCCs display this cytotoxic pathway (Praveen *et al*., 2006, 2004). The addition of Ca^{2+} chelators significantly inhibit the cytotoxic activity of NCCs, probably inhibiting the polymerization of perforin and therefore, the formation of the cytotoxic pore in the membrane of target cell, hampering the later entry of granzymes and other proteins inside target cells to induce apoptosis and necrosis cell death (Hogan *et al*., 1999). At the same time, NCCs also possess FasL, which binds to the target cell Fas

receptor during cytotoxic reactions leading to apoptosis through caspase activation in a granule-independent manner (Cuesta *et al*., 2003; Evans *et al*., 2000). Granulysin and NK-lysin are other cytotoxic effector molecules related to the non-specific CMC though their characterization and implications have received little attention (Praveen *et al*., 2006; Valero *et al*., 2020).

On the other hand, fish NK-like cells are also able to exert innate CMC like NCCs, being able to eliminate virus-specific-infected cells through cytoplasmic granules via perforin/granzyme mechanism (Hogan *et al*., 1996; Mutoloki *et al*., 2014; Shen *et al*., 2004; Thorpe *et al*., 2016). Additionally, the cytotoxic activity of NK-like cells from channel catfish can be inhibited using an antibody that binds to the leucocyte-function-associated antigen-1 (LFA-1), an adhesion molecule (Yoshida *et al*., 1995). In mammalian NK cells, different proteins belonging to the killer-cell immunoglobulin-like receptors (KIRs) are distributed among the cellular surface and lead to the activation or inhibition of NK cells, depending on the activation (ITAM) or inhibitory (ITIM) intracellular motifs (Biassoni *et al*., 2001). In fish, some orthologs to KIRs have been found, named as novel immune-type receptors (NITRs) and novel immunoglobulin-like transcripts (NILTs) (Kock and Fischer, 2008; Yoder *et al*., 2010); however, their functional properties are scarcely known nowadays.

2.2 Adaptive or specific cell-mediated cytotoxicity

As commented previously, numerous homologs of *cd8*, *mhcI* and *tcr* genes have been described in fish, suggesting a similar role for CTLs as in mammals (Nakanishi *et al*., 2015; Yamaguchi *et al*., 2019). Nevertheless, with the identification of different T cell markers in fish, correlations between specific CMC reactions and the cell groups that displayed cytotoxicity could be established. Firstly, one study in channel catfish showed that in mixed leucocyte reactions (MLR) the presence of alloantigen-specific cytotoxic cells displaying *tcra* and *tcrb* genes was similar to mammalian CTLs (Stuge *et al*., 2000). Subsequently, in rainbow trout, the use of different monoclonal antibodies (mAB) specific to IgM, granulocytes, thrombocytes and monocytes evidenced that fractions of IgM^- PBLs, expressing *cd8a* genes and exerted cytotoxic activity against allogeneic target cells (Fischer *et al*., 2003). Similarly, in ginbuna crucian carp (*Carassius auratus langsdorfii*), using kidney leucocytes from virus-infected fish, was found that lymphocyte and IgM^-

leucocyte fractions exhibited high levels of CMC activity and expressed large amounts of *cd8a* and *trcb* mRNA levels (Somamoto *et al*., 2006).

The first evidence for the presence of fish CTLs at functional level was carried out in ginbuna crucian carp (Somamoto *et al*., 2000). Thus, it was found that the effector donor cells must be sensitized by allogeneic antigen to exert cytotoxic activity, whereas in non-sensitized fish this was not performed (Somamoto *et al*., 2005). Later on, this alloantigen-specific killing was confirmed to be carried out by $CD8^+$ T cells (Toda *et al*., 2009). Indeed, leucocytes from crucian carp hematopoietic necrosis (CHNV)-infected ginbuna crucian carp were capable of effectively eliminating syngeneic CHNV-infected cell lines, but not allogeneic CHNV-cell lines, being $CD8\alpha^+$ cells the main anti-viral cytotoxic executioners (Somamoto *et al*., 2013). Furthermore, in rainbow trout, activity of CTLs has been demonstrated against viral haemorrhagic septicaemic virus (VHSV)- and infectious hematopoietic necrosis (IHNV)-infected cells (Utke *et al*., 2007). Taking all this into account, it is clear that CTLs execute the cytotoxic activity of fish adaptive immunity. As observed in mammals, through the immunological synapse, fish CTLs must come into direct contact with the cells that display the antigen on their surface bound to MHC-I (MHC-I-restricted recognition), inducing the PRF/GZM and Fas/FasL pathways and leading to either necrosis or apoptosis (Halle *et al*., 2017) (Fig. 4). Furthermore, evidence of MHC-I restriction has also been found in fish, initially observed in ginbuna crucian carp, where cytotoxic responses were virus-specific and occurred only in syngeneic cells and not in allogeneic cells infected with the same virus. Additionally, no cytotoxic activity was observed against syngeneic cells infected with another virus (Somamoto *et al*., 2000). Subsequently, in the same species, it was also observed that *in vitro* generation of virus-specific cytotoxic T cells was only able to lyse those syngeneic cells infected with the same virus, while this was not observed in virus-infected allogeneic cells (Somamoto *et al*., 2009). However, these studies provided indirect evidence of MHC-I-restricted cytotoxicity. Using the rainbow trout line C25 and the RTG-2 cell line of the same species, which shares the same MHC-I alleles, it was shown that PBLs from low dose virus-infected trout killed MHC-I-matched virus-infected RTG-2 cells, correlating with high expression of *cd8a* gene (Utke *et al*., 2007). The main cytotoxic effector molecules of CTLs are perforin, granzymes and FasL in mammals, (Mitchell and Aspinall, 2008). Both pathways have also been implicated in the CTLs activity of fish. For instance, the $TCR\alpha\beta^+$ alloantigen specific leucocytes of catfish, considered the

equivalent of mammalian CTLs, displayed inhibited cytotoxicity with the addition of EGTA (ethyleneglycol-bis(β-aminoethyl) tetraacetic acid) and concanamycin A (CMA), which both are recognized as perforin inhibitors (Zhou *et al*., 2001), as also occurred for ginbuna carp CTLs (Toda *et al*., 2011). In common carp (*Cyprinus carpio L*.), adaptive CMC activity was compromised with the use of calcium chelators, resulting in a poor activity of PRF/GRZ pathway (Companjen *et al*., 2006).

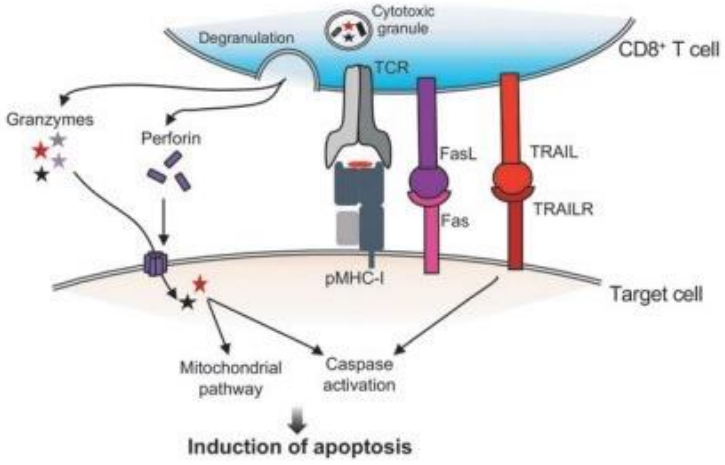

Figure 4. Function of cytotoxic T lymphocytes. Binding between the TCR receptor of $CD8^+$ T cell and MHC-I of target cell (immunological synapse) causes activation of the perforin/granzyme and Fas/FasL pathway, leading to the induction of apoptosis in the target cell. Modified from: (Kedzierska and Koutsakos, 2020).

For the proper function of CTLs, the participation of different membrane receptors that regulate their activity is essential. In mammals, up to five different co-receptors have been identified in T cells, divided in positive costimulatory receptors such as CD28 and inducible costimulatory signal (ICOS), and three negative costimulatory receptors as cytotoxic T-lymphocyte-associated protein 4 (CTLA4), B and T-lymphocyte attenuator (BTLA) and programmed cell death-1 (PD-1) (Bugeon and Dallman, 2000). Nevertheless, only CD28 and CTLA4 have been discovered in teleost fish, with high degree of similarity and conservation, which potentially indicates similar functions as their respective mammalian orthologues (Bernard *et al*., 2006; González-Fernández *et al*., 2021). In contrast, other co-receptors have been discovered, such as T cell immunoglobulin-3 (Tim-3) or T cell immunoglobulin and ITIM domain (TIGIT) (Anderson *et al*., 2016). One of the most representative co-receptor in mammals is CRTAM, which is up-regulated in active CTLs against virus-infected or tumor cells (Patiño-Lopez *et al*., 2006). Unfortunately, there is no information on its presence or

function in fish, which could help to improve the understanding of CTLs. Cytokines also play a pivotal role in the control of CTLs and have been described in several fish species. IL-2, a growth factor for antigen-stimulated T cells that control their clonal expansion, and IL-15, that induces the expansion of CTLs, cytokines have been described in several species. Rainbow trout recombinant IL-2 increased the expression of IFN-γ (Díaz-Rosales *et al*., 2009) while common carp IL-15 induced the gene expression of *cd8α* and *cd8β* (Wang *et al*., 2018). By contrast, mammalian IL-10 is a negative regulator of the T cell response. Nevertheless, in common carp, IL-10 failed to regulate T cells in naïve animals but increased their activity when carps were sensitized (Piazzon *et al*., 2015). The presence of cytotoxic and regulatory protein molecules of fish CTLs is extremely important and deserves considerable attention.

2.3. Antibody-dependent cellular cytotoxicity

A further mechanism of the adaptive cytotoxic action observed in mammals is known as ADCC. This process involves effector cells that possess the Fc membrane receptor, mostly NK-cells or macrophages among others. These effector cells can identify cells that have been marked with antibodies. Such marked target cells typically display pathogenic or tumor-associated antigens on their surface and once these antibody-coated cells are recognized, the effector cells activate the perforin/granzyme pathway to lead target cells death (Gómez Román *et al*., 2013). The initial direct observation of ADCC in fish was made in sharks (*Ginglymostoma cirratum*). Some studies demonstrated that shark adherent leucocytes were able to kill target cells prior to engagement by 19S IgM (McKinney and Flajnik, 1997; Pettey and Mckinney, 1988). Later, studies on channel catfish revealed that a certain group of NK-like cells had the Fc receptor for IgM, leading to the subsequent demonstration of their ability to perform ADCC (Shen *et al*., 2003, 2002). Unfortunately, characterization of this ADCC has been almost ignored in the field of fish immunology.

3. Nodavirus

One of the most threatening infectious viral agents that cause innumerable economic and biological losses in the aquaculture sector is nodavirus, or nervous necrosis virus (NNV), which is the causative agent of the viral encephalopathy and retinopathy

(VER) disease (Bandín and Souto, 2020). Since its detection in the late 1980s, NNV infection has been described in more than 180 teleost fish species, both marine and freshwater, including many farmed fish (Gomez *et al*., 2006; Mori *et al*., 2003; Olveira *et al*., 2009; Parameswaran *et al*., 2008). VER disease has been mainly described in the earliest developmental stages, larvae and juveniles, where mortalities up to 100% can be reached (Parameswaran *et al*., 2008). Furthermore, adult fish that overcome the infection will become asymptomatic carriers of NNV (Gjessing *et al*., 2009). The main symptoms produced, although they depend on the infected species, are classified into four stages depending mainly on the motor behavior of the infected fish. Among the symptoms are abnormal swimming, which begins with slight erratic movements that prevent the animal from following a straight trajectory and continues with spiral or circular movements until the fish stops responding to external stimulation and finally ends up swimming with the ventral part facing the surface (Bandín and Souto, 2020). This progression of VER disease is related to the tropism that NNV shoots for the central nervous system (CNS) since it has been observed in different histopathological analyses that NNV produces necrosis and vacuolation of CNS cells (Kuo *et al*., 2012b).

Another point of special interest is the transmission route of nodavirus, being the horizontal one the most predominant. Horizontal transmission of fish to fish or through water has been reported for several fish species (Glazebrook *et al*., 1990; Péducasse *et al*., 1999; Souto *et al*., 2015b), with the ability to penetrate through the epithelial surface, gills and nasal or oral cavities (Péducasse *et al*., 1999; Souto *et al*., 2018; Tanaka *et al*., 2004). Surprisingly, the presence of NNV has been also observed in feces of fish-eating birds, helping to increase the dissemination and transmission of the virus (Kuo *et al*., 2012b). In addition, vertical transmission capacity has also been reported via the shedding into the gametes and the detection in embryos (Breuil *et al*., 2002; Kuo *et al*., 2012a; Valero *et al*., 2015). Furthermore, in striped jack (*Pseudocaranx dentex*), the release of viral particles from the intestine might result in the infection of eggs (Nguyen *et al*., 1997). Therefore, early detection of virus carriers is vital to avoid cross-infection in aquaculture farms.

3.1 Viral structure

NNV are naked viruses with icosahedral symmetry composed of a single capsid protein. At genomic level, it harbors two single-stranded positive-sense RNA fragments,

RNA1 and RN2, where RNA1 encodes the RNA-dependent RNA polymerase (*rdrp*), which determine the thermal tolerance of NNV (Hata *et al*., 2010), and RNA2 synthesizes the capsid protein (*cp*), which is responsible for host specificity (Fig. 5) (Ito *et al*., 2008; Mori *et al*., 1992). In the viral replication process, a subgenomic fragment, called RNA3, is produced from the RNA1 fragment and encodes two non-structural proteins named B1 and B2 (Iwamoto *et al*., 2005). Although there are few studies that delve into the functions of B1 and B2 proteins, the available data suggest that B1 protein has an anti-necrotic function that increases the viability of the infected cells (Chen *et al*., 2009b) while B2 is expressed early in the infection and seems to participate in the replication and proliferation of virions by inhibiting the RNA interference mechanisms of the host cell (Iwamoto *et al*., 2005).

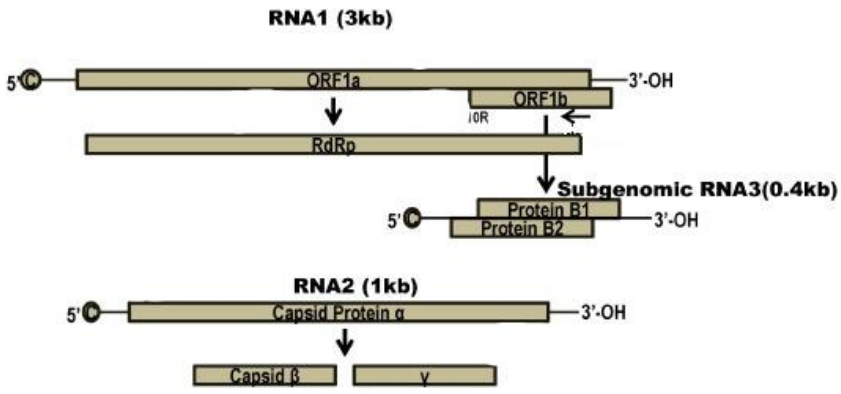

Figure 5. Representation of NNV genome, composed of RNA1 and RN2 coding for the RNA-dependent RNA polymerase *(rdrp)* and capsid protein *(cp)* genes, respectively. Modified from: (Agnihotri *et al*., 2016)

3.2 NNV taxonomy and distribution

The classification of the different NNV genotypes has been conducted by analyzing a variable region present in the RNA2 fragment called T4. Thus, nowadays, NNV is divided into 5 genotypes: red-spotted grouper nervous necrosis virus (RGNNV), striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV) and turbot nodavirus (TNV) (Bandín and Souto, 2020). However, the genomic nature of NNV has led to the discovery in the last decade of two reassortant genotypes derived only from the RGNNV and SJNNV genotypes, named RGNNV/SJNNV and SJNNV/RGNNV, based on which parental genotype the RNA1 and RNA2 fragments belong to, respectively (Olveira *et al*.,

2009; Toffolo *et al*., 2007). Molecular analyses of the RNA fragments from the reassortant genotypes indicated that they show slight differences compared to their parental genotypes, being more pronounced for RdRp than for CP (Olveira *et al*., 2009).

The worldwide geographical distribution of NNV is closely linked to its thermal tolerance, with the highest incidence in European and Asiatic waters. In colder climates, the BFNNV genotype is the most prevalent. Notwithstanding, in the Mediterranean region, where water temperature is higher, the predominant genotype is RGNNV, being also the one with the highest number of susceptible species (Bandín and Souto, 2020). In addition, SJNNV and SJNNV/RGNNV and RGNNV/SJNNV reassortants are also present in the Mediterranean sea (Bandín and Souto, 2020).

3.3 Host immune defence against NNV

In recent decades, research into the immune response against NNV has gained prominence due to the global impact in the aquaculture sector. Although both the innate and adaptive immune systems of fish have been implicated against NNV infection, the former has been more extensively studied. As PRRs are responsible for the detection of viral pathogens, some studies have been conducted proving their relevance in NNV infection. Thus, an *in vitro* study performed with a zebrafish cell line evidenced an increase in the activation of RLRs and TLRs. Furthermore, *rig1* gene knockdown demonstrated the inhibition in the expression of *ifnφ2* and *ifnφ3* genes as well as in the recruitment of monocytes (Chen *et al*., 2015). In contrast, in Atlantic halibut (*Hippoglossus hippoglossus*), NNV infection increased the expression in the eye of TLR7 receptor (Øvergård *et al*., 2012), which recognizes viral single-stranded RNA, a finding also observed in seven-band grouper (*Epinephelus septemfasciatus*) gills along with up-regulation of *tlr3* gene (Krishnan *et al*., 2020). Because of modulation of PRRs, the expression of type I IFN and IFN-related genes is also altered upon NNV infection, especially in the brain, the main target organ for NNV. Some of these include increased mRNA levels of *interferon regulatory factor 3* (*irf3*), *isg15*, *ifn-induced protein 44* (*ifi44*), *ifn-induced proteins with tetratricopeptide repeats (ifit) 5*, *mixovirus (influenza) resistance protein* (*mx)* and type I *ifn* genes (Álvarez-Torres *et al*., 2017; Kim *et al*., 2017; Liu *et al*., 2016; Lu *et al*., 2008; Poisa-Beiro *et al*., 2008). Interestingly, in the orange spotted grouper (*Epinephelus coioides*), overexpression of IFIT1 suppressed the RGNNV replication *in vitro* (Zhang *et al*., 2019). Additionally, pro-inflammatory cytokines are also

modulated and play a key role in NNV resistance. In fact, the long-term expression of *il1β* gene in the brain of infected fish leads to neuronal cell death, aiding nodavirus colonization and increasing disease (Chiang *et al*., 2017). *In vivo* NNV infections have shown increases of *il1β*, *il6* and *tnfα* genes (pro-inflammatory cytokines) in Atlantic halibut, Senegalese sole (*Solea senegalensis*) and zebrafish (Morick *et al*., 2015; Øvergård *et al*., 2012; Souto *et al*., 2024). With regards to the adaptive immune response, many studies demonstrate the generation of specific antibodies after NNV infection or vaccination exhibiting neutralizing effect against NNV, enhancing immunoprotection and survival of fish (Jaramillo *et al*., 2016; Mushiake *et al*., 1992; Pakingking *et al*., 2009; Yamashita *et al*., 2009). In addition, NNV infections induce an increase in a multitude of T-cell markers as *trcβ, cd4, cd8α* or *zeta chain of T cell receptor associated protein kinase (zap)-70* genes (Krasnov *et al*., 2013; Øvergård *et al*., 2013, 2012; Toubanaki *et al*., 2022), which apparently indicate the participation of T lymphocytes (Krasnov *et al*., 2013; Øvergård *et al*., 2013; Tso and Lu, 2018). Furthermore, the use of a mAB specifically able to detect CD4-1 lymphocytes in olive flounder (*Paralichtthys olivaceus*) evidenced the proliferation of this subpopulation during the onset of NNV infection (Jung *et al*., 2020).

When focusing on Mediterranean fish species, RGNNV infection has been extensively researched in two seawater fish, European sea bass and gilthead seabream, due to their varying susceptibility patterns. European sea bass is very susceptible to developing the VER disease, leading to almost 100% mortality rates in the larval and juvenile stages, whereas seabream shows resistance to the RGNNV genotype, predominant in the Mediterranean basin and serving as a carrier of the virus (Castric *et al*., 2001; Chaves-Pozo *et al*., 2012; Thiéry *et al*., 2006). Furthermore, both species hold significant importance within the Mediterranean aquaculture sector, as they are among the most widely farmed and commercialized fish. Deepening into the available research, many investigations indicate that seabream possesses a more robust innate immune response against NNV-infected cells, effectively hindering the replication of the virus. This is evident when comparing both species, the virus-induced up-regulation of many IFN-related genes and immune receptors is markedly more pronounced in seabream than in European sea bass (Chaves-Pozo *et al*., 2012; Poisa-Beiro *et al*., 2009, 2008). Transcriptomic analyses in juvenile seabream and European sea bass reinforce these observations (Lama *et al.,* 2020; Pereiro *et al.,* 2023). Specifically, in seabream, RGNNV

infections trigger a significant upsurge in the expression of various IFN-related genes and immune receptors in the brain and HK. Furthermore, the containment of inflammatory markers in the brain suggests a neuroprotective response (Pereiro *et al*., 2023). Contrariwise, in European sea bass, there is an observable increase in stress response genes, particularly those involved in cortisol synthesis in HK (Lama *et al*., 2020). This fact implies that the high stress induced by NNV may inhibit various immunoprotective pathways, thereby allowing rapid virus replication and spread (Lama *et al*., 2020). Moreover, the great up-regulation of pro-inflammatory cytokines upon NNV infection in European sea bass supports the idea of an exacerbation of brain inflammation whereas in seabream it is resolved at the onset (Poisa-Beiro *et al*., 2008).

Regarding the innate CMC, head-kidney leucocytes (HKLs) from NNV-infected seabream and European sea bass increased the innate CMC against xenogeneic tumor cells and up-regulated *nccrp-1* gene expression (Chaves-Pozo *et al*., 2012). Regardless, naïve European sea bass HKLs displayed an inability to perform cytotoxic activities against NNV-infected cell lines (Chaves-Pozo *et al*., 2017), including the NNV susceptible DLB-1 cell line derived from sea bass brain (Chaves-Pozo *et al*., 2019a). In stark contrast, naïve seabream HKLs demonstrated enhanced cytotoxic activity against all NNV-infected cell lines. Thus, transcriptomic analysis of sea bass HKLs revealed that most gene expression differences were associated with metabolic processes rather than immune responses (Chaves-Pozo *et al*., 2017). These findings suggest that NNV possesses mechanisms to circumvent the early defence systems in European sea bass, such as the innate CMC, thereby facilitating its replication, resulting in heightened susceptibility and dissemination within this species. In addition, the cellular adaptive immune response is also modulated by NNV infection. Hence, in European sea bass, NNV infection of target organs provoked an increase in several CTL markers, such as *cd28, ctla4, cd8α, cd4* and *tcrβ* (González-Fernández *et al*., 2021; Valero *et al*., 2018), and cytotoxic genes, including *gzma, gzmb* and *prf* (Chaves-Pozo *et al*., 2019b; Valero *et al*., 2018), especially towards the end of the infection, suggesting the generation and involvement of CTLs in the eradication of NNV-infected cells. Similar results have been documented in seabream, where NNV infection led to an increase in *cd8α*, *tcrβ* and *cd4* genes, with an earlier expression pattern than in European sea bass (López-Muñoz *et al*., 2012). Additionally, the use of immunohistochemistry techniques has revealed the

infiltration of IgM^+ cells in the infected brain of seabream, as well as $GZMB^+$ cells (Chaves-Pozo *et al*., 2019b; López-Muñoz *et al*., 2012).

Although seabream is refractory to the RGNNV, multiple unexpected natural outbreaks at seabream culture facilities exhibiting classic signs of VER disease and high mortality rates have been documented in recent years. These outbreaks have been attributed to the reassortant genotype RGNNV/SJNNV (NaveenKumar *et al*., 2017; Toffan *et al*., 2017; Volpe *et al*., 2020). Notably, RGNNV/SJNNV genotype appears to have a heightened affinity and lethality for the very early larval stages of seabream, in comparison to later developmental stages (Toffan *et al*., 2021). On the opposite side, European sea bass do not display symptoms towards RGNNV/SJNNV genotype (Bandín and Souto, 2020). An RNA-seq analysis performed in gilthead seabream larvae infected with RGNNV/SJNNV revealed an increased expression of genes associated with heat-shock proteins, whereas genes tied to the immune response, including IFN and other antiviral pathways, were notably suppressed (Peruzza *et al*., 2021). In contrast, no outbreaks due to the SJNNV/RGNNV genotype in seabream have been detected.

2.OBJECTIVES

The present Doctoral Thesis proposes to delve into the dynamics of cell-mediated cytotoxicity of fish leucocytes against nodavirus. For this purpose, the specific objectives are:

1. Study the pathogenicity of the RGNNV/SJNNV and SJNNV/RGNNV reassortant genotypes in gilthead seabream larvae of different ages and the modulation of their immunogenetic profile.
2. Characterize the cytotoxic and regulatory T cell co-receptor (CRTAM), and its ligand CADM1, in European sea bass and gilthead seabream, and their implication during a NNV infection.
3. Identify the perforin genes in European sea bass and describe their regulation and involvement during a NNV infection.
4. Evaluate the adaptive cell-mediated cytotoxic response of NNV-infected European sea bass leucocytes against NNV-infected cells.

3.EXPERIMENTAL CHAPTERS

CHAPTER I: Effect of NNV reassortants on gilthead seabream larvae of different ages

García-Álvarez, M.Á., Arizcun, M., Chaves-Pozo, E., Cuesta, A., 2022. Profile of innate immunity in gilthead seabream larvae reflects mortality upon betanodavirus reassortant infection and replication. Int. J. Mol. Sci. 23, 1–15. https://doi.org/10.3390/ijms23095092

CHAPTER II: Characterization of the cytotoxic and regulatory T cell coreceptor (CRTAM), and its ligand CDAM1, and their implication in a NNV infection in European sea bass and gilthead seabream

García-Álvarez, M.Á., González-Fernández, C., Esteban, M.Á., Cuesta, A., 2023. Molecular characterization of the cytotoxic and regulatory T cell coreceptor (CRTAM), and its ligand CADM1, in the European seabass and gilthead seabream. Fish Shellfish Immunol. 134, 108569. https://doi.org/10.1016/j.fsi.2023.108569

CHAPTER III: Identification of European sea bass perforins and their probable implication in the adaptive CMC against NNV

García-Álvarez, M.Á., Cervera, L., Valero, Y., González-Fernández, C., Mercado, L., Chaves-Pozo, E., Cuesta, A., 2024. Regulation and distribution of European sea bass perforins point to their role in the adaptive cytotoxic response against NNV. Fish Shellfish Immunol. 144, 109244. https://doi.org/10.1016/j.fsi.2023.109244

CHAPTER IV: Evaluation of the adaptive cell-mediated cytotoxicity in NNV-infected European sea bass

García-Álvarez, M.Á., Chaves-Pozo, E., Cuesta, A., 2024. Cytotoxic activity and gene expression during *in vitro* adaptive cell-mediated cytotoxicity of head-kidney cells from betanodavirus-infected European sea bass. Dev. Comp. Immunol. 152, 105124. https://doi.org/10.1016/j.dci.2023.105124

CHAPTER I: Effect of NNV reassortants on gilthead seabream larvae of different ages

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Profile of Innate Immunity in Gilthead Seabream Larvae Reflects Mortality upon Betanodavirus Reassortant Infection and Replication

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Abstract: Historically, gilthead seabream (*Sparus aurata*) has been considered a fish species resistant to nervous necrosis virus (NNV) disease. Nevertheless, mortality in seabream hatcheries, associated with typical clinical signs of the viral encephalopathy and retinopathy (VER) disease has been confirmed to be caused by RGNNV/SJNNV reassortants. Because of this, seabream larvae at 37 and 86 days post-hatching (dph) were infected by immersion with RGNNV/SJNNV and SJNNV/RGNNV reassortants under laboratory conditions, and mortality, viral replication and immunity were evaluated. Our results show that gilthead seabream larvae, mainly those at 37 dph, are susceptible to infection with both NNV reassortant genotypes, with the highest impact from the RGNNV/SJNNV reassortant. In addition, viral replication occurs at both ages (37 and 86 dph) but the recovery of infective particles was only confirmed in 37 dph larvae,; this value was also highest with the RGNNV/SJNNV reassortant. Larvae immunity, including the expression of antiviral, inflammatory and cell-mediated cytotoxicity genes, was affected by NNV infection. Levels of the natural killer lysin (Nkl) peptide were increased in SJNNV/RGNNV-infected larvae of 37 dph, though hepcidin was not. Our results demonstrate that the seabream larvae are susceptible to both NNV reassortants, though mainly to RGNNV/SJNNV, in an age-dependent manner.

Keywords: nodavirus; reassortants; virus; gilthead seabream; larvae; immunity

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1. Introduction

Marine aquaculture is a continuously expanding sector, which must deal with numerous pathogens that cause economic losses. Among these pathogens, nervous necrosis virus (NNV) is one of the most threatening viruses, affecting to more than 170 marine teleost fish species, and being the causative agent of the viral encephalopathy and retinopathy (VER) disease. NNV is a 25–30 nm icosahedral virus, non-enveloped, whose genome consists of two molecules of singled-stranded positive-sense RNA, called RNA1 and RNA2 [1]. The RNA1 segment codifies for the RNA-dependent RNA polymerase (RdRP) whereas the RNA2 segment encodes the capsid protein (CP). Additionally, a third RNA segment, RNA3, is synthesized during viral replication due to the cleavage of RNA1, producing B1 and B2 proteins, the latter being needed for the repression of cellular RNA interference in infected cells [2,3]. Traditionally, NNV has been classified into four genotypes, based on a variable region of the RNA2 segment named T4: striped jack nervous necrosis virus (SJNNV), red-spotted grouper nervous necrosis virus (RGNNV), barfin flounder nervous necrosis virus (BFNNV) and tiger puffer nervous necrosis virus (TPNNV). Nonetheless, an additional genotype has been accepted, named as turbot nodavirus (TNV) [4]. However, recent studies have indicated the existence of two natural genotype reassortants: the first one,

CHAPTER II: Characterization of the cytotoxic and regulatory T cell coreceptor (CRTAM), and its ligand CDAM1, and their implication in a NNV infection in European sea bass and gilthead seabream

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Full length article

Molecular characterization of the cytotoxic and regulatory T cell coreceptor (CRTAM), and its ligand CADM1, in the European seabass and gilthead seabream

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ABSTRACT

T cell activation is a multifaceted process that depends on the activation of the T cell receptor (TCR). However, other coreceptors are also strictly necessary to provide co-signals and modulate the immune response. However, to date, most of these coreceptors are unknown in fish or their information is very limited. Therefore, in this work, we have identified the cytotoxic and regulatory T cell molecule, CRTAM, and its ligand, the cell adhesion molecule 1, CADM1, in European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*); and evaluated their transcriptional levels. Both putative proteins showed the canonical architecture observed in mammals, where CRTAM exhibited two immunoglobulin domains and CADM1, both the a and b forms, exhibited three of these domains. In addition, phylogeny and synteny analyses showed their conservation throughout vertebrate evolution. We found constitutive expression of all three genes, with *crtam* and *cadm1a* being predominant in immune tissues such as spleen, thymus and head-kidney (HK), while *cadm1b* expression was more limited to the brain. *In vitro*, only the T cell mitogen phytohemagglutinin (PHA) up-regulated the transcription of *crtam* and *cadm1a* in HK leucocytes. Nodavirus (NNV) infection elicited an up-regulation of *crtam* and *cadm1a* in brain and HK, appearing earlier in seabream than in seabass, which could explain the resistance of seabream to the development of nodavirus disease. In addition, they are up-regulated during the innate cell-mediated cytotoxic response in seabream but not in seabass. Altogether, our data seem to indicate that CRTAM is more related to the innate cytotoxicity in seabream and more in the specific and T cell-mediated cytotoxicity in seabass. Our results highlight the importance of CRTAM and CADM1 as important molecules in the activation of T lymphocytes in seabass and seabream, but further studies are needed.

1. Introduction

The presence of an adaptative immune system is unique to vertebrates, first appearing in jawed fish almost 500 million years ago, suggesting a clear evolutionary advantage due to its high specificity and immunological memory capacity against numerous pathogens. Among its components, T lymphocytes are one of the most important, with two main populations: cytotoxic (CTL) and helper (Th) T lymphocytes, the latter with different subpopulations. Participation of T lymphocytes in the mammalian immune response requires their activation based on the binding of the T cell receptor (TCR) with the antigen-presenting cell (APC) through a pathogen-derived peptide bound to its major histocompatibility complex (MHC) proteins. However, it is also necessary the participation of several coreceptors on the T cell membrane for proper activation, regulation and cessation of activity, with CD8 and CD4 being the classical coreceptors of CTL and Th populations, respectively [1]. In mammals, numerous coreceptors that induce the activation or inhibition of T cells have been characterized [2]. CRTAM (cytotoxic and regulatory T cell molecule or Class-I MHC-restricted T Cell-associated molecule) is an important coreceptor belonging to the immunoglobulin (Ig) superfamily due to the existence of two, V- and C1-type, Ig-like domains in the extracellular region of the protein, whose expression is mostly restricted to MHC-class I-activated cells such as NK (natural killer) cells and $CD8^+$ T lymphocytes [3], but also in cytotoxic

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CHAPTER III: Identification of European sea bass perforins and their probable implication in the adaptive CMC against NNV

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Full length article

Regulation and distribution of European sea bass perforins point to their role in the adaptive cytotoxic response against NNV

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ABSTRACT

Cell-mediated cytotoxicity is a complex immune mechanism that involves the release of several killing molecules, being perforin (PRF) one of the most important effector players. Perforin is synthesized by T lymphocytes and natural killer cells in mammals and responsible for the formation of pores on the target cell membrane during the killing process. Although perforin has been extensively studied in higher vertebrates, this knowledge is very limited in fish. Therefore, in this study we have identified four *prf* genes in European sea bass (*Dicentrarchus labrax*) and evaluated their mRNA levels. All sea bass *prf* genes showed the typical and conserved domains of its human orthologue and were closely clustered by the phylogenetic analysis. In addition, all genes showed constitutive and ubiquitous tissular expression, being *prf1.9* gene the most highly expressed in immune tissues. Subsequently, *in vitro* stimulation of head-kidney (HK) cells with phytohemagglutinin, a T-cell activator, showed an increase of all *prf* gene levels, except for *prf1.3* gene. European sea bass HK cells increased the transcription of *prf1.2* and *prf1.9* during the innate cell-mediated cytotoxic activity against xenogeneic target cells. In addition, sea bass infected with nodavirus (NNV) showed a similar expression pattern of all *prf* in HK and brain at 15 days post-infection, except for *prf1.3* gene and in the gonad. Finally, the use of a polyclonal antibody against PRF1.9 showed an increase of positive cells in HK, brain and gonad from NNV-infected fish. Taken together, the data seem to indicate that all *prf* genes, except *prf1.3,* appear to be involved in the European sea bass immunity, and probably in the cell-mediated cytotoxic response, with PRF1.9 playing the most important role against nodavirus. The involvement of the PRFs and the CMC activity in the vertical transmission success of the virus is also discussed.

1. Introduction

Perforin (PRF) is one of the main death effectors released, together with granzymes, during the cell-mediated cytotoxicity (CMC) immune response. PRF is mainly synthesized by cytotoxic $CD8^+$ T lymphocytes (CTL) and natural killer (NK) cells and stored in their cytolytic granules [1,2]. In humans and mice, perforin is produced from a single-copy gene called *prf1*, which codes for a glycoprotein of about 70 kDa that belongs to the membrane attack complex/perforin (MACPF) superfamily. The mature protein consists of a MACPF domain at the N-terminal end, a Ca^{2+} binding (C2) domain and an intermediate epidermal growth factor-like (EGF) domain [2,3]. Perforin is mainly involved in eliminating viruses-infected or tumour cells [4–7], although there is evidence about its role in clearing bacteria-infected cells [8]. The mechanism of action of perforin has been exhaustively studied. Briefly, inactivated perforin remains inside the cytotoxic granules of killer lymphocytes together with other molecules as granzymes. Once the recognition of the killer and the target cells takes place and the immunological synapse proceeds, the exocytosis of the lytic granules causes the release of their contents [6]. In this scenario, the high level of Ca^{2+} and the neutral pH

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CHAPTER IV: Evaluation of the adaptive cell-mediated cytotoxicity in NNV-infected European sea bass

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Cytotoxic activity and gene expression during *in vitro* adaptive cell-mediated cytotoxicity of head-kidney cells from betanodavirus-infected European sea bass

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ABSTRACT

Cell-mediated cytotoxicity (CMC) is essential in eradicating virus-infected cells, involving $CD8^+$ T lymphocytes (CTLs) and natural killer (NK) cells, through the activation of different pathways. This immune response is well-studied in mammals but scarcely in teleost fish. Our aim was to investigate the adaptive CMC using head-kidney (HK) cells from European sea bass infected at different times with nodavirus (NNV), as effector cells, and the European sea bass brain cell line (DLB-1) infected with different NNV genotypes, as target cells. Results showed low and unaltered innate cytotoxic activity through the infection time. However, adaptive CMC against RGNNV and SJNNV/RGNNV-infected target cells increased from 7 to 30 days post-infection, peaking at 15 days, demonstrating the specificity of the cytotoxic activity and suggesting the involvement of CTLs. At transcriptomic level, we observed up-regulation of genes related to T cell activation, perforin/granzyme and Fas/FasL effector pathways as well as apoptotic cell death. Further studies are necessary to understand the adaptive role of European sea bass CTLs in the elimination of NNV-infected cells.

1. Introduction

Cell-mediated cytotoxicity (CMC) is a pivotal immunological process in mammals devoted to the elimination of virus-infected and tumour cells (Golstein and Griffiths, 2018; Russell and Ley, 2002). Natural killer (NK) cells and cytotoxic $CD8^+$ T lymphocytes (CTLs) are key effector leucocytes of the innate and adaptive CMC response, respectively (Andersen et al., 2006; Smyth et al., 2005). During the CMC process, NKs and/or CTLs directly interact with the altered cells (targets) and induce their death through a series of coordinated steps, which encompasses the recognition and engagement of antigens presented on the surface of target cells by effector receptors. For CTLs, the response is initiated by the specific binding of the T cell receptor (TCR), and its co-receptor CD8, with the major histocompatibility complex (MHC) class I of the target cells presenting the viral or anormal peptides (Cole et al., 2007). Subsequently, effectors activate and deliver a cascade of cytotoxic mediators that culminate in the efficient destruction of the target cells (Halle et al., 2017; Squier and John Cohen, 1994). Overall, cytotoxic effectors mainly use the perforin/granzyme (PRF/GZM) or the Fas/FasL pathways, granule- and Ca^{++}-dependent or -independent respectively, leading to the target cell death by either apoptosis or necrosis (Halle et al., 2017).

CMC is also present in fish, with both innate and adaptive arms, though slightly studied. Regarding the innate CMC, two types of NK homologues have been discovered, known as non-specific cytotoxic cells (NCC) and NK-like cells (Nakanishi et al., 2015). Although studies on these cell types are limited, they have been univocally linked to innate cytotoxic functions as they clearly show the ability to kill xenogeneic, allogeneic and virus-infected cells by using the PRF/GZM and/or Fas/-FasL pathways (Bishop et al., 2000; Hogan et al., 1996; Jaso-Friedmann et al., 2000; Yoshinaga et al., 1994). Focusing on the study model, the European sea bass (*Dicentrarchus labrax*), previous studies have identified the effector leucocytes, demonstrated that target cells suffer morphological features proper of necrotic and apoptotic cell death or determined the innate CMC (Cammarata et al., 2000; Chaves-Pozo et al., 2012, 2017; Meloni et al., 2006; Meseguer et al., 1996; Mulero et al., 1994). Although homologous sequences for TCR (Hordvik et al., 1996; Nam et al., 2003; Wermenstam and Pilström, 2001), CD8 (Buonocore et al., 2006; Somamoto et al., 2005; Xu et al., 2011), and MHC class I

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4.GENERAL DISCUSSION

Viruses are considered one of the most lethal pathogens in fish, as they possess numerous mechanisms to evade the immune response. Among the different immune mechanisms, CMC plays a key role in controlling virus evasion. Different immune cells from the innate and adaptive systems can exert cytotoxic activity; however, CTLs stand out for their specificity. Therefore, this Doctoral Thesis has focused on the characterization of some aspects of the CMC response of European sea bass and gilthead seabream leucocytes against different NNV genotypes.

Thus, in **Chapter I**, we proposed to investigate the interaction of the naturally occurring reassortant NNV genotypes, RGNNV/SJNNV and SJNNV/RGNNV, in seabream larvae of different ages to study how infection occurs over time and the involvement of the seabream immune response. Regarding *in vivo* infection by immersion, both genotypes were able to induce mortality in 37 and 86 days post-hatching (dph) larvae, with the highest mortality rates observed in the 37 dph larvae infected with the RGNNV/SJNNV genotype. These findings were correlated with the replication of viral genes and the recovery of infected particles, again observing a higher viral titer in 37 dph larvae, which could explain the high pathogenicity of RGNNV/SJNNV, in agreement with other studies (Toffan *et al*., 2021; Volpe *et al*., 2020). For the first time, in this study we also demonstrated the larval susceptibility to the SJNNV/RGNNV genotype, also with an age-dependency, probably due to changes in RNA1 and RNA2 fragments of the reassortant genotypes, which increase their pathogenicity against seabream (Souto *et al*., 2015a). Principal component analysis (PCA) and hierarchical clustering of the transcriptional profile of immune genes divided the infected larvae into different expression patterns, correlating with the mortality data, viral replication and recovery. Apparently, immunity decreased along with the infection with the RGNNV/SJNNV genotype in 37 dph larvae, explaining their higher susceptibility. Initially, in the 37 dph larvae infected with the RGNNV/SJNNV genotype, there was an increase in the antiviral *mx* gene, which later decreased, as seen in a previous study (Peruzza *et al*., 2021) and an increase in the inflammatory *il1b* gene, explaining the susceptibility and high mortality of larvae at this age, possibly due to over-inflammation, as seen in other species, causing necrosis (Chiang *et al*., 2017). As the infection progresses, most genes decrease, especially those related to cytotoxicity, supporting the idea that specimens are not capable of mounting a defence throughout the infection. In 86 dph larvae, where we observed less mortalities, there was an increase in antiviral defence

genes against the RGNNV/SJNNV genotype, while the pro-inflammatory *il1b* gene decreased. In contrast, for the SJNNV/RGNNV genotype, the effect was the opposite, with an increasing tendency until the end of the infection. Thus, in larvae infected with the SJNNV/RGNNV genotype, although cytotoxic genes like *gzma* and *gzmb* are initially decreased, they later increase, explaining the lower mortality and virus replication, since these two molecules are involved in the control of NNV (Chaves-Pozo *et al*., 2019b). This fact explains the high survival upon infection with this genotype, possibly helped by the production of NKL peptide, which might combat NNV (Valero *et al*., 2020). In 86 dph larvae, the SJNNV/RGNNV genotype did not modulate any immune gene.

Chapter II consisted of the characterization of CRTAM and CADM1 molecules in both species. In humans, CRTAM is primarily expressed in $CD8^+$ T lymphocytes, indicating that they are in an active state. However, to accomplish this purpose, CRTAM needs to bind with its ligand, CADM1. Bioinformatic analysis revealed a single version of *crtam* gene and two *cadm1* genes, *cadm1a* and *cadm1b*, possibly arising from a genomic duplication, common in fish (Taylor *et al*., 2001). At the protein level, alignment with their human orthologs demonstrated the conservation of two Ig domains in CRTAM and three Ig domains in CADM1, which are vital for the formation of the CRTAM/CADM1 complex (Galibert *et al*., 2005). Genetic analysis of basal expression indicated that *crtam* gene of both species was highly expressed in immune-related tissues such as the spleen, thymus or HK, similar to mammals (Kennedy *et al*., 2000). However, the sea bass brain showed a significant expression, suggesting that *crtam* could play an important role in neural interactions as described in mammals (Patiño-Lopez *et al*., 2006). For *cadm1a*, differences were found between the two species. In sea bass, interestingly, there was higher expression of *cadm1a* in the brain but also immune organs such as the thymus or spleen. In mammals, this gene has a more widespread expression, as it is a protein involved in cell-cell adhesion. However, it is also present in $CD8^+$ dendritic cells, subpopulation capable of establishing contact with T lymphocytes (Galibert *et al*., 2005). In fish, the presence of this same subpopulation has been confirmed (Granja *et al*., 2015), therefore, the correlation in the expression of *crtam* and *cadm1a* in sea bass could be due to its existence, although it requires further study. In seabream, as in sea bass, the highest expression of *cadm1a* occurs in the brain, where *cadm1* participates as a synaptic adhesion molecule as described in the CNS of mammals (Biederer *et al*., 2002). Finally, *cadm1b* in both species was mainly expressed in the skin and brain, which suggests that

it might have a specialized function in cell-cell junctions. Regarding the regulation of their expression in HKLs, only *crtam* showed an increase in sea bass with T mitogens, indicating that T lymphocytes may be the cellular population that mostly expresses *crtam*, as in mammals (Patiño-Lopez *et al*., 2006). This expression pattern also coincides with other co-receptors such as *cd4*, *ctla4*, and *cd28* (González-Fernández *et al*., 2021). On the other hand, in seabream, the expression of *crtam* was not affected by any stimulus, and interestingly, *crtam* decreased with the use of lipopolysaccharide (LPS). Regarding *cadm1a* expression, an increase upon T mitogens used has been observed in sea bass and seabream, supporting the idea of the existence of a population like dendritic cells. In the innate cytotoxicity assay, where NCCs are the main executors, only an increase in *crtam/cadm1* genes was produced in seabream against the NNV-infected cell line. This observation, along with the fact that HKLs from seabream exhibit a higher innate CMC than HKLs from sea bass (Chaves-Pozo *et al*., 2017), seems to indicate that while in sea bass the main cells producing *crtam* might be T cells, in seabream, they might be NCCs. Based on previous data, we also aimed to study the modulation of the three genes in an *in vivo* NNV infection. Curiously, in sea bass the expression of *crtam* in the brain followed a similar pattern to that observed for *gzma* and *cd8* (Chaves-Pozo *et al*., 2019b; Verrier *et al*., 2011), with low expression at the onset of the infection that later increases, suggesting that CMC activity is not able to act efficiently at an early stage of infection, which causes high susceptibility of this organ to NNV. However, this trend seems to reverse later, as indicated by the increase in this gene expression at day 15 in the HK, indicative of the possible generation of CTLs. On the other hand, in seabream, we observed a different pattern that may explain its resistance to this pathogen. It appears that there was a mobilization of immune cells from the HK to the brain, as well as higher production of *cadm1a* in the brain and HK, coupled with the increase in both genes in the CMC assay. All this data may indicate a more effective innate CMC response than in sea bass. The data on the expression of *crtam* and *cadm1a* seem to support the hypothesis of our group where, although sea bass is not capable of mounting an initial cytotoxic response to halt the virus, it can arm an adaptive CMC response at the later stages of the infection in the survivors, whereas in gilthead seabream, this does not occur because the innate CMC response is sufficient.

Given the suspected role of CTLs in sea bass during the NNV infection, in **Chapter III,** we aimed to further investigate the underlying mechanism by studying

perforin, a pivotal cytotoxic executor protein in mammalian CMC (Law *et al*., 2010). First, the *in silico* study identified four *prf* genes within the sea bass genome, named *prf1,2, prf1.3, prf1,5* and *prf1.9*, which probably emerged from a genomic duplication event (Taylor *et al*., 2001). Compared with the mammalian *prf1* orthologue, all predicted sea bass perforins evidenced the canonical MACPF and C2 domains, suggesting potential functional similarity. Indeed, all sea bass *prf* genes showed a high grade of conservation compared to other fish perforin, macrophage-expressed gene 1 (MPEG) and C6-C9 complement proteins, which belong to the same family (Dangelo *et al*., 2012). However, within the perforin clade, all sea bass PRFs did not group in the same cluster, which might point to a principle of subfunctionalization. This idea is also supported by their basal expression, where only *prf1.9* appears to be expressed in the thymus or spleen, exactly as the mammalian *prf1* gene (Mitchell and Aspinall, 2008). However, the rest of *prf* genes showed a wider distribution, as in zebrafish or rainbow trout (Athanasopoulou *et al*., 2009; Varela *et al*., 2016). Interestingly, all genes were up-regulated in HKLs stimulated with PHA in line with the expression patterns of other T lymphocyte markers in sea bass (Chaves-Pozo *et al*., 2019b) except *prf1.3*. The synthesis of different *prf* genes showing unequal basal expressions could be explained by the presence of different immune cells distributed throughout the fish body, since not only T cells express this gene, as can be documented in NCCs of tilapia or in a rainbow trout cell line formed mainly by monocytes/macrophages (Ordás *et al*., 2011; Toda *et al*., 2011). Unexpectedly, exposure to LPS, a primary virulence factor in gram-negative bacteria, and *V. anguilllarum* reduced the expression of *prf1.9*, pointing to a possible strategy employed by bacteria to circumvent the immune response, as seen in the decreased respiratory burst activity of sea bass HKLs exposed to *V. anguillarum* (Sepulcre *et al*., 2007). By contrast, in other fish, LPS treatment causes an increase in *prf* genes (Li *et al*., 2018; Varela *et al*., 2016). The *in vivo* NNV infection supported our hypothesis regarding the involvement of CTLs in NNV-infected sea bass, as there was a significant increase in all *prf* genes in HK and brain after 15 dpi, but not earlier, except for *prf1.3*. In contrast, in the gonad, only *prf1.9* was increased initially and then disappeared. These findings suggest a clear stimulation of CTLs towards the end of the infection and their subsequent mobilization to the affected organs to combat the infection. In addition, this was later demonstrated using a specific polyclonal antibody against PRF1.9, revealing that PRF1.9^{+} cells increased in HK, brain and gonad at 15 dpi. Furthermore, the expression levels of most *prf* genes were significantly and positively correlated with *gzma* and *gzmb* genes, pointing out the

activation of perforin/granzyme pathway which is characteristic of CTLs for the elimination of NNV-infected cells in sea bass, as demonstrated in other species (Companjen *et al*., 2006). However, in the gonad, even though the presence of PRF1.9^{+} cells was observed, they did not orchestrate an effective response and nodavirus can colonize the gonads and be transmitted vertically (Breuil *et al*., 2002; Valero *et al*., 2015). Probably because of *prf1.9* gene expression is not kept high through infection time and decreases in the gonad at the end of the infection. Therefore, the presence of immune cells producing perforin after 15 dpi is not efficient in eliminating the virus in this tissue. Moreover, during the innate CMC assay, there was a significant decrease of *prf1.2* and *prf1.9* in response to NNV-infected cells, supporting our previous data that the virus can evade the immune response early in the infection and spread to different tissues including the gonad. Subsequently, once individuals have overcome the initial phase of infection, they are able to initiate a specific CMC response through CTLs and the overexpression of *prf1.2, prf1.5*, and *prf1.9* in the HK and brain, but not in gonad.

Based on the previous data of **Chapter II** and **Chapter III**, suggesting the potential role of sea bass CTLs against NNV infection, **Chapter IV** delves into unravelling this hypothesis at a functional level. Therefore, in this study we employed leucocytes from RGNNV-infected sea bass as effector cells, and RGNNV- and SJNNV/RGNNV-infected DLB-1 sea bass cell line as target cells, since both genotypes share the same capsid protein, the immunogenic part of the virus (Coeurdacier *et al*., 2003). This study revealed that HKLs from mock-infected sea bass lacked cytotoxic activity, aligning with prior findings (Chaves-Pozo *et al*., 2017). Nevertheless, this scenario changed with HKLs from RGNNV-infected sea bass, exhibiting significant CMC activity peaking at 15 dpi on both NNV-infected target cells. The repetition of the experiment infecting target cells with other NNV genotypes with distinct viral capsids demonstrated that this CMC activity was restricted to those cells infected with NNV presenting the RGNNV RNA2, and therefore was specific. Furthermore, this specificity was probably MHC-I-restricted, based on the numerous MHC-I alleles shared by both target and effector cells, as they come from the same broodstock, supporting the idea that observed specific CMC was carried out mainly by CTLs, as hypothesized in previous chapters. To date, the involvement of specific CTLs against NNV-infected cells has been demonstrated only in orange spotted grouper (Chang *et al*., 2011). Further analyses underlying this mechanism involved assessing genes linked to CTLs and CMC activity

are still mandatory. On the one hand, leucocytes from mock-infected sea bass underwent slight activation of T cells, as evidenced by high expression of *tcrβ* and *nkl* genes, the last one also linked to T cell and NNV infection in sea bass (Valero *et al*., 2020); however, it was not enough to kill NNV-infected cells. On the other hand, in experimental groups playing the specific CMC, CTLs participation was also suggested by the increased levels of *cd28* and *il2* genes. A previous study evidenced the role of *cd28* in the activation of T cells in sea bass (González-Fernández *et al*., 2021). In addition, in mammals, the binding of CD28 to its ligand leads to the production of IL2, a growth factor for T cells (Esensten *et al*., 2016), as reported previously in sea bass (Buonocore *et al*., 2020). Behind the specific CMC observed, the perforin/granzyme and Fas/FasL pathways seemed to be the pivotal mechanisms, as several genes of both routes, such as *gzma, gzmb, prf1.2* and *prf1.9* were up-regulated. As discussed in Chapter 3, perforin/granzyme pathway is activated under the participation of CTLs in the CMC response (Companjen *et al*., 2006; Toda *et al*., 2011). Nevertheless, the increase in *fasl* was in line with a previous study where NNV infection in pacific cod (*Gadus macrocephalus*) might increase apoptosis through the Fas/FasL pathway (Mao *et al*., 2021). Finally, the increase in the *bax/bcl2* ratio, an indicator of apoptosis (Raisova *et al*., 2001), was also high in both groups, consistent with the activation of apoptosis, probably through the Fas/FasL. Thus, although NCCs are also capable of producing perforin, granzyme or even FasL (Jaso-Friedmann *et al*., 2000), our data demonstrate that the main effector cells of CMC were CTLs, due to their specificity to detection against the RGNNV capsid, although the increased expression of the *nccrp1* gene suggests a possible participation of NCCs in the process.

5.CONCLUSIONS

1. Gilthead seabream larvae are susceptible to infection by RGNNV/SJNNV and SJNNV/RGNNV reassortants with age-dependency.
2. RGNNV/SJNNV reassortant is more pathogenic for seabream larvae than SJNN/RGNNV, with a higher rate of viral replication and a decrease in the immune response along the infection.
3. European sea bass and gilthead seabream possess one *crtam* and two *cadm1* genes, showing constitutive expression, with *crtam* and *cadm1a* showing highest transcription in lymphoid tissues.
4. In both fish species, *crtam* and *cadm1a* are modulated by NNV infection.
5. In European sea bass, transcription and regulation of *crtam* seems to be related to T lymphocytes.
6. European sea bass exhibits four *prf* genes, with conserved domains and characteristics, being *prf1.9* the most highly expressed in lymphoid tissues.
7. All *prf* genes, except *prf1.3,* seem to be mainly expressed in T lymphocytes and increase upon NNV infection, simultaneously with the increase in PRF1.9^{+} cells in HK, brain and gonad, indicating a possible role in the adaptive CMC against NNV infection.
8. Leucocytes from RGNNV-infected European sea bass exert CMC activity against NNV-infected target cells in a specific manner, demonstrating the adaptive CMC response.
9. Perforin/granzyme and Fas/FasL pathways are activated in leucocytes, leading to apoptosis in target cells death, during this adaptive CMC response.
10. This Doctoral Thesis demonstrates that the CMC mediated by CTLs is fundamental in the antiviral response against nodavirus.

6.RESUMEN EN CASTELLANO

6.1 Introducción

El reciente incremento en la demanda de la producción acuícola ha provocado numerosos problemas en este sector, muchos de ellos relacionados con la salud y el bienestar de las especies cultivadas, como puede ser el alto estrés que sufren los animales debido a un aumento en la densidad del cultivo, provocando la dispersión y propagación de patógenos emergentes que generan grandes mortalidades y pérdidas económicas en el sector (Walker and Winton, 2010). Los peces son el primer grupo animal en presentar un sistema inmune innato y adaptativo, al igual que en mamíferos; sin embargo, presentan diferencias a nivel tisular, celular y molecular (Mitchell and Aspinall, 2008). Dentro del sistema inmune innato, destaca el componente humoral, con la presencia de citoquinas y otras moléculas efectoras como la interleucina (IL)-1β, factor de necrosis tumoral (TNF)-α, interferones (IFNs), péptidos antimicrobianos (AMPs) y las proteínas del complemento, cuya función principal es la movilización de leucocitos hasta el lugar de la infección, la coordinación de los mecanismos moleculares inmunes y la eliminación directa de los patógenos (Holland and Lambris, 2002; Mokhtar *et al*., 2023; Verrier *et al*., 2011). En la respuesta celular, destacan las células fagocíticas, como los monocitos/macrófagos, granulocitos y células dendríticas, y las células citotóxicas innatas, compuestas principalmente por las células citotóxicas no específicas (NCCs) y células NK-*like*, capaces de reconocer y eliminar células infectadas por virus y células tumorales de manera espontánea y sin un periodo de inducción previo (Jaso-Friedmann *et al*., 2000; Yoshida *et al*., 1995). Dentro del sistema adaptativo, el componente humoral está formado por las inmunoglobulinas, caracterizadas por promover la activación del sistema del complemento, la opsonización de patógenos para facilitar la fagocitosis y promover la citotoxicidad celular dependiente de anticuerpos (Boshra *et al*., 2004; Mutoloki *et al*., 2014). Respecto a la inmunidad celular, los peces teleósteos presentan células presentadoras de antígenos, linfocitos B y linfocitos T. El primer grupo está compuesto por células dendríticas, macrófagos, linfocitos B, e incluso granulocitos y eritrocitos y tienen como función la presentación de antígenos mediante el complejo mayor de histocompatibilidad (MHC)-II a los linfocitos T (Tian *et al*., 2022). Por otro lado, los linfocitos B son las células encargadas de sintetizar las inmunoglobulinas M, D y T, siendo la IgM la más importante (Mutoloki *et al.,* 2014). Por último, los linfocitos T constituyen la mayor subpoblación de la estirpe linfoide y son las principales células citotóxicas del sistema inmune adaptativo. Están caracterizados por la presencia del receptor de células T (TCR), así como de los correceptores CD8 y CD4, que dividen a

este grupo en linfocitos T citotóxicos (CTLs) y linfocitos T colaboradores, portadores del MHC-I y MHC-II, respectivamente (Mutoloki *et al*., 2014; Squier and Cohen, 1994).

Uno de los principales mecanismos para eliminar células infectadas por virus o células tumorales es la citotoxicidad mediada por células (CMC), descrito en peces teleósteos al igual que en mamíferos, pero encontrando diferencias en los tipos celulares efectores (Squier and Cohen, 1994). Dentro de la inmunidad innata de peces, las principales células citotóxicas no específicas son las NCCs y células NK-*like*, capaces de eliminar células tumorales y células infectadas por virus, como se ha observado en la tilapia y en el pez gato americano (Faisal *et al*., 1989; Jaso-Friedmann *et al*., 2000), usando diferentes vías citotóxicas como la vía de la perforina/granzima y Fas/FasL, que desencadenan la muerte celular por necrosis y apoptosis de la célula diana (Hogan *et al*., 1999; Jaso-Friedmann *et al*., 2000). Por otra parte, en la inmunidad adaptativa destacan los CTLs como principales células citotóxicas. Las primeras evidencias se obtuvieron en el pez gato americano, donde células que mostraron expresar los genes *tcra* y *tcrb* presentaron actividad citotóxica específica contra células alogénicas (Stuge *et al*., 2000). La presencia de CTLs en peces a nivel funcional se demostró en primer lugar en la carpa plateada japonesa, mediante la presencia de linfocitos T $CD8^+$ específicos contra células alogénicas (Somamoto *et al*., 2000; Toda *et al*., 2009), en donde se requería de una inducción previa con el antígeno para ejercer la actividad citotóxica (Somamoto *et al*., 2005). De hecho, en esta misma especie los linfocitos T $CD8^+$ fueron capaces de eliminar a células singénicas infectadas por CHNV (Somamoto *et al*., 2013). Una característica fundamental para llevar a cabo esta actividad es que tanto la célula efectora como la diana compartan el mismo alelo de MHC-I, hecho demostrado en peces, en donde la respuesta citotóxica fue específica para un mismo virus en células singénicas infectadas y no en células alogénicas infectadas con el mismo virus o en células singénicas infectadas por otro virus diferente (Somamoto *et al*., 2000). En trucha arcoíris, se demostró la citotoxicidad restringida al MHC-I usando individuos y una línea celular que compartían el mismo alelo de MHC-I, estando a su vez correlacionado con un aumento en la expresión del gen *cd8a* (Utke *et al*., 2007). Las principales vías efectoras de los CTLs para la eliminación de células infectadas por virus en peces son las rutas de la perforina/granzimas y del Fas/FasL, habiéndose demostrado en numerosas especies de peces, mediante el aumento de expresión de estos genes y la aplicación de inhibidores de la perforina que provocan una disminución significativa de la actividad citotóxica

(Companjen *et al*., 2006; Ordás *et al*., 2011; Zhou *et al*., 2001). Un aspecto clave para la correcta modulación de la actividad citotóxica es la presencia y participación de correceptores en la membrana de los linfocitos T, que pueden aumentar dicha actividad, como los receptores CD28 o ICOS, o disminuirla, como los receptores CTLA-4, BTLA o PD-1 (Bugeon and Dallman, 2000). CD28 y CTLA-4 se han identificado en peces, con funciones similares a las de mamíferos (González-Fernández *et al*., 2021), así como al receptor Tim-3 y TIGIT (Anderson *et al*., 2016). Sin embargo, la información respecto a la presencia y caracterización de otros correceptores importantes en mamíferos, como CRTAM, con funciones vitales para la CMC tales como la activación de linfocitos T frente a células infectadas por virus (Patiño-Lopez *et al*., 2006), es escasa en peces, siendo importante una mayor profundización de estos estudios. Las citoquinas también juegan un papel importante regulando la actividad CMC. Por ejemplo, la IL-15 de la carpa común aumentó la expresión de los genes *cd8α* y *cd8β* mientras que la IL-10 incrementó la actividad de los linfocitos T de individuos sensibilizados (Piazzon *et al*., 2015; Wang *et al*., 2018).

Uno de los patógenos virales más letales en la región Mediterránea es nodavirus o el virus de la necrosis nerviosa (NNV), afectando sobre todo al cultivo de lubina (*Dicentrarchus labrax*) y dorada (*Sparus aurata*). Ambas especies son susceptibles a la infección, sin embargo, sólo la lubina es capaz de desarrollar la enfermedad generada por NNV y denominada encefalopatía y retinopatía viral, causando tasas de mortalidad cercanas al 100%, mientras que la dorada es asintomática para la mayoría de las cepas virales (Castric *et al*., 2001; Parameswaran *et al*., 2008). El NNV presenta cinco genotipos parentales, llamados RGNNV, SJNNV, TPNNV, BFNNV, TNV y dos genotipos recombinantes, RGNNV/SJNNV y SJNNV/RGNNV, predominando estos últimos junto con el genotipo RGNNV y SJNNV en aguas del Mediterráneo (Bandín and Souto, 2020). Varios estudios apuntan a que la dorada es capaz de montar una línea defensiva más robusta frente a las células infectadas por NNV, impidiendo su replicación, evidenciado por la mayor regulación positiva que presentan muchos genes relacionados con la ruta del IFN en dorada que en lubina (Chaves-Pozo *et al*., 2012; Poisa-Beiro *et al*., 2009, 2008). Un análisis transcriptómico demostró que en individuos juveniles de dorada infectados con RGNNV, aumentó la expresión de genes relacionados con la ruta antiviral del IFN y receptores inmunes en riñón cefálico y cerebro, mientras que en lubina, se produjo un aumento de genes relacionados con el estrés, lo que podría estar inhibiendo su respuesta

inmune, facilitando la replicación viral y el desarrollo de la enfermedad (Lama *et al*., 2020; Pereiro *et al*., 2023). Respecto a la relación entre la infección por NNV y la actividad citotóxica en ambas especies, los leucocitos de riñón cefálico (HKLs) de lubina y dorada infectadas por RGNNV aumentaron dicha actividad frente a células xenogénicas (Chaves-Pozo *et al*., 2012). Además, leucocitos de dorada sin infectar mostraron activación de dicha actividad frente a diferentes líneas celulares infectadas por NNV, mientras que los leucocitos de lubina sin infectar no aumentaron dicha actividad (Chaves-Pozo *et al*., 2017). El análisis transcriptómico de estas muestras en lubina demostró que la mayoría de los genes expresados diferencialmente estaban relacionados con el metabolismo y pocos con la inmunidad, lo que parece indicar que NNV posee mecanismos capaces de evadir la CMC innata. Sin embargo, numerosos estudios han puesto de manifiesto un aumento en la expresión de genes marcadores de CTLs, tales como *cd28, ctla4, cd8α, tcrβ* (González-Fernández *et al*., 2021), y genes citotóxicos como *gzma, gzmb* y *prf* (Chaves-Pozo *et al*., 2019b; Valero *et al*., 2018) tras la infección de lubina con NNV. Estos hechos, sumado a que dicho aumento de expresión ocurre en etapas tardías de la infección, parecen sugerir la generación y participación de dichos CTLs. En dorada se han encontrado resultados similares, con aumentos de los genes *cd8α, tcrβ* y *cd4* (López-Muñoz *et al*., 2012), así como un aumento de células productoras de GZMB e IgM en el cerebro de individuos infectados (Chaves-Pozo *et al*., 2019b; López-Muñoz *et al*., 2012). Sorprendentemente, en los últimos años han aparecido infecciones por NNV en piscifactorías de dorada en estado larvario, provocando mortalidades cercanas al 100%, habiéndose demostrado posteriormente la implicación del genotipo RGNNV/SJNNV, que parece tener una gran afinidad y letalidad en dicha etapa de desarrollo en dorada (NaveenKumar *et al*., 2017; Toffan *et al*., 2017; Volpe *et al*., 2020). Un estudio de RNA-seq reveló que dicha infección por RGNNV/SJNNV provocó una disminución de numerosos genes inmunes relacionados con las vías antivirales, mientras que aumentaron genes relacionados con el estrés (Peruzza *et al*., 2021). Por el contrario, no hay información respecto al potencial efecto patogénico del recombinante SJNNV/RGNNV en esta especie.

6.2 Objetivos

La presente Tesis Doctoral tiene por objetivo general profundizar en el estudio de la citotoxicidad mediada por leucocitos de lubina contra nodavirus. Para ello, los objetivos específicos fueron:

1. Estudiar la patogenicidad de los genotipos recombinantes RGNNV/SJNNV y SJNNV/RGNNV en larvas de doradas de diferente edad y la modulación de sus perfiles inmunogénicos.
2. Caracterizar la molécula reguladora de los linfocitos T citotóxicos (CRTAM) y su ligando, la molécula de adhesión celular 1 (CADM1), en lubina y dorada y evaluar su implicación durante una infección por NNV.
3. Identificar los genes de perforina en lubina y describir su regulación y participación durante una infección por NNV.
4. Evaluar la actividad citotóxica adaptativa de leucocitos de lubinas infectadas por NNV contra células diana infectadas por NNV.

6.3 Principales resultados y discusión

Los virus son considerados uno de los patógenos más letales en peces, ya que poseen numerosos mecanismos para evadir la respuesta inmune y posteriormente colonizar al animal. Entre los diferentes mecanismos inmunitarios, la CMC juega un papel clave en el control de la evasión viral. Diferentes células del sistema innato y adaptativo pueden ejercer actividad citotóxica; sin embargo, los CTLs destacan por su especificidad. Por lo tanto, esta Tesis Doctoral se ha centrado en la caracterización de algunos aspectos de la respuesta CMC de los leucocitos de lubina y dorada contra diferentes genotipos de NNV.

En el **Capítulo I**, nos propusimos investigar la interacción de los genotipos recombinantes naturales de NNV, denominados RGNNV/SJNNV y SJNNV/RGNNV, en larvas de dorada de diferentes edades para estudiar cómo transcurre la infección a lo largo del tiempo y la implicación del sistema inmune. En la infección *in vivo* llevada a cabo por inmersión, ambos genotipos fueron capaces de inducir mortalidad en las larvas de 37 y 86 días después de la eclosión (dph), alcanzando tasas de mortalidad más altas en las larvas de 37 dph infectadas por el genotipo RGNNV/SJNNV. Estos hallazgos se correlacionaron con la mayor replicación de los genes virales, la recuperación de partículas infectivas y el título viral obtenidos en ese mismo grupo, lo que podría explicar la alta patogenicidad de RGNNV/SJNNV. Un patrón similar se observó en otros estudios (Toffan *et al*., 2021; Volpe *et al*., 2020). Por primera vez, en este estudio se demostró una susceptibilidad de las larvas de dorada al genotipo SJNNV/RGNNV, probablemente debido a cambios moleculares en el fragmento RNA1 y RNA2 de los genotipos recombinantes, que incrementan su patogenicidad contra dorada (Souto *et al*., 2015a). Ambos genotipos demostraron una dependencia con la edad de las larvas. El análisis de correlación PCA y el agrupamiento jerárquico dividieron a los grupos con diferentes patrones de expresión, correlacionándolos con los datos de mortalidad, replicación viral y recuperación de partículas virales y mostrando una disminución en la expresión de genes inmunitarios durante la infección por RGNNV/SJNNV en las larvas de 37 dph que explica su mayor susceptibilidad. Sin embargo, para el genotipo SJNNV/RGNNV el efecto es el contrario, con un aumento de la expresión de los genes inmunitarios al final de la infección, que se correlaciona con su mayor tasa de supervivencia. Destacando la producción del péptido antimicrobiano NKL, que podría combatir a NNV y favorecer la supervivencia (Valero *et al*., 2020). Inicialmente, en el grupo de larvas de 37 dph

infectadas por el genotipo RGNNV/SJNNV, hubo un aumento de la expresión del gen antiviral *mx*, que luego disminuyó, como se observó en un estudio previo (Peruzza *et al*., 2021), y un aumento en la del gen proinflamatorio *il1b*, ocasionando posiblemente una inflamación no controlada que acaba cursando en necrosis y provocando mortalidades altas, como se ha observado en otras especies (Chiang *et al*., 2017). A medida que progresa la infección, la mayoría de los genes estudiados disminuyen, especialmente aquellos relacionados con la citotoxicidad, apoyando la idea de que el animal no es capaz de montar una defensa inmunitaria a lo largo de la infección con el genotipo RGNNV/SJNNV. Sin embargo, en las larvas infectadas por el genotipo SJNNV/RGNNV, aunque los genes citotóxicos *gzma* y *gzmb* disminuyen inicialmente, luego aumentan posteriormente, explicando la menor mortalidad y replicación viral observadas, pues estas dos moléculas están involucradas en el control de NNV (Chaves-Pozo *et al*., 2019b). En las larvas de 86 dph, donde observamos menor mortalidad, hubo un aumento en los genes de defensa antiviral contra el genotipo RGNNV/SJNNV, mientras que el gen *il1b* disminuyó, controlando la inflamación y probablemente ayudando en la defensa. En esta edad, el genotipo SJNNV/RGNNV no modula ningún gen inmunitario.

El **Capítulo II** consistió en la caracterización de las moléculas CRTAM y CADM1 en dorada y lubina. En humanos, CRTAM se expresa principalmente en linfocitos T $CD8^+$ e indica que están activados. Sin embargo, para conseguirlo, CRTAM necesita unirse a su ligando, CADM1. El análisis bioinformático reveló una única versión del gen *crtam* y dos genes *cadm1,* denominados *cadm1a* y *cadm1b,* posiblemente derivados de una duplicación genómica, muy común en peces (Taylor *et al*., 2001). A nivel proteico, el alineamiento con sus ortólogos humanos demostró la conservación de dos dominios Ig en CRTAM y tres en CADM1, vitales para la formación del complejo CRTAM/CADM1 (Galibert *et al*., 2005). El análisis de la expresión basal indicó que el gen *crtam* de ambas especies se expresaba en gran medida en tejidos inmunes como el bazo, el timo o el riñón cefálico, de forma similar a lo que ocurre en mamíferos (Kennedy *et al*., 2000). Sin embargo, *crtam* mostró una expresión significativa en el cerebro de lubina, pudiendo también desempeñar un papel importante en la interacción neuronal, como se ha descrito en mamíferos (Patiño-Lopez *et al*., 2006). Para *cadm1a*, se encontraron diferencias entre las dos especies. En la lubina, curiosamente, hubo una mayor expresión de *cadm1a* en el timo o el bazo. En mamíferos, este gen tiene una expresión más distribuida, ya que es una proteína involucrada en la adhesión célula-célula. No obstante, también está presente en

células dendríticas $CD8^+$, subpoblación capaz de establecer contacto con los linfocitos T (Galibert *et al*., 2005). En peces, se ha confirmado la presencia de esta misma subpoblación (Granja *et al*., 2015), por lo que la correlación en la expresión de *crtam* y *cadm1a* en tejidos inmunes de lubina podría deberse a la existencia de este tipo celular, aunque esta afirmación requiere más estudios. En la dorada, al igual que en la lubina, la mayor expresión de *cadm1a* ocurre en el cerebro, donde *cadm1* participa como una molécula de adhesión sináptica como se ha descrito en el SNC de mamíferos (Biederer *et al*., 2002). Finalmente, *cadm1b* en ambas especies se expresó principalmente en la piel y el cerebro, lo que parece indicar que podría tener una función especializada en las uniones célula-célula. En cuanto a la regulación de su expresión en HKLs, sólo *crtam* mostró un aumento en la lubina con mitógenos de linfocitos T, lo que indica que los linfocitos T pueden ser la población celular de los tejidos inmunes que expresa principalmente *crtam*, al igual que en mamíferos (Kennedy *et al.,* 2000). Este patrón de expresión también coincide con otros correceptores como *cd4, ctla4 o cd28* (González-Fernández *et al*., 2021). Asimismo, en esta especie, *cadm1a* aumentó con el uso de estos mitógenos, lo que respalda la idea de la existencia de las células dendríticas $CD8^+$. Por otro lado, en dorada, la expresión no se vio afectada por ningún estímulo, y curiosamente, *crtam* disminuyó con el uso de lipopolisacárido (LPS). Posteriormente, en el ensayo de citotoxicidad innata, donde las NCCs son las principales ejecutoras, sólo se produjo un aumento en los genes *crtam/cadm1* en los HKLs de dorada contra la línea celular infectada por NNV. Analizando estos datos, junto con el hecho de que los HKLs de la dorada muestran una citotoxicidad innata más alta que los de lubina (Chaves-Pozo *et al*., 2017), podríamos hipotetizar que mientras que en lubina las células principales que producen *crtam* podrían ser los linfocitos T, en dorada podrían ser las NCCs. Basándonos en datos previos, también nos propusimos estudiar la modulación de los tres genes en una infección *in vivo* por NNV. Curiosamente, en lubina, la expresión de *crtam* siguió un patrón similar al observado en *gzma* y *cd8* (Chaves-Pozo *et al*., 2019b; Verrier *et al*., 2011), con una baja expresión al inicio de la infección que luego aumenta, lo que sugiere que la actividad CMC no puede actuar eficientemente en una etapa temprana en el cerebro, lo que causa la alta susceptibilidad de esta especie a NNV. Sin embargo, esta tendencia parece revertirse más tarde en los supervivientes, como indica el aumento de este gen en el día 15 en riñón cefálico, sugiriendo la posible generación de CTLs. Por otro lado, en la dorada, observamos un patrón diferente que puede explicar su resistencia a este patógeno.

Los datos parecen indicar que hay una movilización de células inmunes de riñón cefálico al cerebro, así como una mayor producción de *cadm1a* en el cerebro y riñón cefálico en etapas tempranas de la infección, lo que, junto con el aumento de ambos genes en el ensayo de CMC, indica la existencia de una respuesta CMC innata más eficaz que en la lubina. Los datos sobre la expresión de *crtam* y *cadm1a* parecen respaldar la hipótesis de nuestro grupo donde, aunque la lubina no es capaz de montar una respuesta citotóxica inicial para detener el virus, puede armar una respuesta CMC adaptativa en las etapas posteriores de la infección en los supervivientes, mientras que en la dorada esto no es necesario porque la respuesta CMC innata es suficiente para controlar la infección.

Debido a la sospecha de la implicación de los CTLs de lubina durante una infección por NNV, en el **Capítulo III**, nos propusimos estudiar la perforina, una proteína ejecutora citotóxica crucial en la CMC de mamíferos (Law *et al*., 2010). En primer lugar, el estudio *in silico* evidenció la presencia de cuatro genes *prf* dentro del genoma de la lubina, llamados *prf1.2, prf1.3, prf1.5* y *prf1.9*, que probablemente surgieron por un evento de duplicación genómica (Taylor *et al*., 2001). En comparación con el ortólogo *prf1* de mamíferos, todas las perforinas predichas en la lubina mostraron los dominios MACPF y C2 canónicos, lo que sugiere una posible similitud funcional. De hecho, todas las PRF de lubina mostraron un alto grado de conservación en comparación con otras proteínas de perforina de peces y las proteínas de complemento C6-C9 y MPEG1, que pertenecen a la misma familia de proteínas (Dangelo *et al*., 2012). Sin embargo, dentro del clado de perforina, todos los genes *prf* de la lubina no se agruparon en el mismo clúster, lo que podría apuntar a un principio de subfuncionalización. Esta idea también fue respaldada por su expresión basal, donde solo *prf1.9* parece expresarse en el timo o el bazo, exactamente como el gen *prf1* de mamíferos (Mitchell and Aspinall, 2008). Sin embargo, el resto de los genes *prf* mostraron una expresión más homogéneamente distribuida por diferentes tejidos, como en el pez cebra (*Danio rerio*) o en la trucha arcoíris (*Oncorhynchus mykiss*) (Athanasopoulou *et al*., 2009; Varela *et al*., 2016). Interesantemente, la expresión de todos los genes, a excepción de *prf1.3*, se reguló positivamente en HKLs estimulados con fitohemaglutinina (PHA), siguiendo el patrón de expresión de otros marcadores de linfocitos T (Chaves-Pozo *et al*., 2019b). La expresión basal de los diferentes genes *prf* podría explicarse por la presencia de diferentes células inmunes distribuidas por todo el cuerpo del pez, ya que no solo los linfocitos T expresan este gen, como se puede documentar en las NCCs de tilapia o en una línea celular de

monocitos/macrófagos de trucha arcoíris (Toda *et al*., 2011; Yang *et al*., 2013). Inesperadamente, la exposición al LPS, un factor de virulencia primario en bacterias gramnegativas, y a *V. anguillarum*, redujo la expresión de *prf1.9,* lo que apunta a una posible estrategia empleada por las bacterias para evadir la respuesta inmune, como se observó HKLs de la lubina expuestos a *V. anguillarum*, los cuales disminuyen la producción de reactivos libres de oxígeno (Sepulcre *et al*., 2007), y puede facilitar la evasión de las bacterias del sistema inmune. Por el contrario, en otras especies de peces, el tratamiento con LPS causa un aumento de expresión de los genes *prf* (Li *et al*., 2018; Varela *et al*., 2016). La infección *in vivo* por NNV apoyó nuestra hipótesis sobre la participación de los CTLs en la respuesta de lubina frente NNV, ya que hubo un aumento significativo en todos los genes *prf,* a excepción de *prf.13,* en HK y cerebro, después de 15 dpi y no antes. En contraposición, la expresión de *prf1.9* se incrementó en la gónada al inicio de la infección para luego disminuir. Estos hallazgos sugieren una clara estimulación de los CTLs hacia el final de la infección y su posterior movilización a los órganos afectados para combatir la infección. Además, esto se demostró posteriormente usando un anticuerpo policlonal específico contra PRF1.9, con el cual se observó un aumento de células PRF1.9^{+} en riñón cefálico, cerebro y gónada a los 15 dpi. Teniendo en cuenta que la expresión de la mayoría de los genes *prf* se correlacionó positivamente con los genes *gzma* y *gzmb*, podemos señalar que la vía perforina/granzima característica de los CTLs se está activando en lubina para la eliminación de las células infectadas por NNV en la lubina, como se ha demostrado en otras especies (Companjen *et al*., 2006). Sin embargo, en la gónada, la presencia de células PRF1.9^{+}, no es suficiente para eliminar el virus de este órgano ya que se ha demostrado que, en lubinas, nodavirus puede colonizar las gónadas y transmitirse verticalmente (Breuil *et al*., 2002; Valero *et al*., 2015). Posiblemente porque la expresión de *prf1*.9 en la gónada no se mantiene en el tiempo y desciende al final de la infección mientras que el número de células que producen perforina no aumenta hasta los 15 dpi. Además, en el ensayo de CMC innata, hubo una disminución significativa de *prf1.2* y *prf1.9* en respuesta a células infectadas por NNV, respaldando nuestros datos anteriores de que el virus puede evadirse al principio de la infección y propagarse a diferentes tejidos, incluida la gónada. Posteriormente, una vez que los individuos han superado la fase inicial de la infección, son capaces de iniciar una respuesta CMC específica a través de los CTLs y la sobreexpresión de *prf1.2*, *prf1.5* y *prf1.9* en riñón cefálico y cerebro, pero no en gónada.

Basándonos en los datos anteriores de los **Capítulos II** y **III**, que sugieren la implicación de los CTLs de lubina en respuesta a la infección por NNV, el **Capítulo IV** se adentra en desentrañar esta hipótesis. Por lo tanto, en este estudio empleamos HKLs de lubinas infectadas por RGNNV como células efectoras y células de la línea celular DLB-1 de lubina infectadas por RGNNV y SJNNV/RGNNV como células diana, ya que ambos genotipos comparten la misma proteína de la cápside, la parte inmunogénica del virus (Coeurdacier *et al*., 2003). Este estudio reveló que los HKLs de lubina no infectadas mostraban una actividad citotóxica muy baja, en línea con hallazgos anteriores (Chaves-Pozo *et al*., 2017). Sin embargo, este escenario cambió con los HKLs de lubina infectadas por RGNNV, que exhibieron un aumento de la actividad CMC significativa, alcanzando su máxima actividad a los 15 días post-infección. La repetición del experimento infectando a las células diana con otros genotipos de NNV con cápsides virales distintas demostró que esta actividad CMC estaba restringida a aquellas células infectadas con el virus NNV que presentaba el RNA2 de RGNNV, y por lo tanto era específica. Además, esta especificidad probablemente estuvo restringida por el MHC-I, basado en los numerosos alelos de MHC-I compartidos por las células diana y efectoras, ya que provienen del mismo stock parental, lo que respalda la idea de que la CMC específica observada fue llevada a cabo principalmente por CTLs, como se hipotetizó en capítulos anteriores. Hasta la fecha, la participación de CTLs específicos contra células infectadas por NNV sólo se ha demostrado en mero (Chang *et al*., 2011). Un análisis más detallado de este mecanismo implicó evaluar genes relacionados con los CTLs y la CMC. Por un lado, los HKLs de lubina no infectadas experimentaron una ligera activación de las células T, como lo evidencia la alta expresión de los genes *tcrβ* y *nkl*, este último también vinculado a células T e infección por NNV en lubina (Valero *et al*., 2020); sin embargo, no fue suficiente para matar a las células diana infectadas por NNV. Por otro lado, en los grupos que mostraron CMC específica, la participación de los CTLs se sugirió por la gran expresión observada de los genes *cd28* e *il2*. Estudios previos evidenciaron el papel de *cd28* en la activación de las células T en la lubina (González-Fernández *et al*., 2021). Además, en mamíferos, la unión de CD28 a su ligando conduce a la producción de IL2, un factor de crecimiento para las células T (Esensten *et al*., 2016), como se ha descrito también en lubina (Buonocore *et al*., 2020). Durante esta CMC específica observada, las vías perforina/granzima y Fas/FasL parecían ser los mecanismos principales, ya que varios genes de ambas vías, como *gzma, gzmb, prf1.2* y *prf1.9*, presentaron un aumento significativo en su expresión génica. Como se discutió en el **Capítulo III**, la vía

perforina/granzima se activa durante la participación de los CTLs en la respuesta CMC (Companjen *et al*., 2006; Toda *et al*., 2011). Además, en los grupos experimentales que mostraron CMC específica, el aumento de *fasl* estaba en línea con un estudio anterior en donde se sugirió que la infección por NNV en el bacalao del Pacífico (*Gadus macrocephalus*) aumenta la apoptosis a través de la vía Fas/FasL (Mao *et al*., 2021). Finalmente, la relación *bax/bcl2*, un indicador de apoptosis (Raisova *et al*., 2001), también fue alta en dichos grupos, consistente con la activación de la apoptosis. Por lo tanto, aunque las NCCs también son capaces de producir perforina, granzima o incluso FasL (Jaso-Friedmann *et al*., 2000), nuestros datos demuestran que las células efectoras principales de dicha actividad fueron los CTLs, debido a su especificidad para detectar la cápside de RGNNV, aunque la expresión aumentada del gen *nccrp1,* también sugiere una posible participación de las NCCs en el proceso.

6.4 Conclusiones

1. Las larvas de dorada son susceptibles a la infección por los genotipos recombinantes RGNNV/SJNNV y SJNNV/RGNNV de manera dependiente de la edad.
2. El genotipo RGNNV/SJNNV presenta mayor patogenicidad que SJNNV/RGNNV, con una tasa de replicación viral más alta, resultando en una atenuación de la respuesta inmune a lo largo de la infección.
3. Lubina y dorada poseen un gen *crtam* y dos genes *cadm1*, con expresión constitutiva, aunque *crtam* y *cadm1a* se expresan principalmente en órganos inmunitarios.
4. La expresión de los genes *crtam* y *cadm1a* está modulada durante una infección por NNV tanto en dorada como en lubina.
5. En lubina, la expresión y regulación del gen *crtam* sugieren un papel importante en linfocitos T y en dorada en células NCCs o NK-*like*.
6. La lubina presenta cuatro genes *prf*, siendo *prf1.9* el gen más expresado en órganos inmunitarios.
7. Todos los genes *prf*, excepto *prf1.3*, parecen expresarse en linfocitos T y su expresión aumenta durante la infección por NNV, simultáneamente con el aumento del número de células PRF1.9^{+} en riñón cefálico, cerebro y gónada, indicando un posible papel en la citotoxicidad adaptativa frente a NNV.
8. Leucocitos de riñón cefálico de lubinas infectadas por RGNNV ejercen actividad citotóxica específica contra células diana infectadas por NNV de manera específica, demostrando una respuesta CMC específica.
9. Durante esta citotoxicidad específica, las rutas de perforina/granzimas y Fas/FasL son activadas en los leucocitos, desencadenando la muerte celular de las células diana por apoptosis.
10. Los resultados obtenidos demuestran que la citotoxicidad específica llevada a cabo por CTLs es un mecanismo esencial en la respuesta antiviral contra nodavirus.

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