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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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(Grimholt et al., 1993; Hashimoto et al., 1990; Loh et al., 2022) have been widely described in several teleost species, the adaptive CMC, at functional level, has been scarcely studied in fish due to the lack of available study models. In recent years, virus-specific killing by adaptive CMC has been clearly demonstrated in very few fish species. Thus, in ginbuna crucian carp (*Carassius auratus langsdorfii*), the MHC class I-restricted CMC has been demonstrated thanks to the use of clonal fish and cell lines derived from them (Somamoto et al., 2002, 2009). In fact, leucocytes from ginbuna crucian carp infected with hematopoietic necrosis virus (CHNV) were able to efficiently kill CHNV-infected cell lines if they are syngeneic, but not when being allogeneic. Moreover, they also demonstrated that CD8α-positive cells are involved in the killing of CHNV-infected cells, in contrast to NK-like cells and monocytes (Somamoto et al., 2013). In the same species, most allo-sensitized CD8α-positive lymphocytes were capable of activating the perforin/granzyme pathway to kill the target cells (Toda et al., 2011a). In rainbow trout (*Oncorhynchus mykiss*), MHC class I-restricted CTL activity has been also demonstrated against infectious hematopoietic necrosis (IHNV)- and viral haemorrhagic septicaemic virus (VHSV)-infected cells (Fischer et al., 2006; Utke et al., 2007). In contrast, in most teleost fish, it is extremely challenging to investigate the CTL activity due to the lack of MHC class I matched effector and target cell systems.

Nodavirus (NNV), or Betanodavirus, is one of the most threatening viruses for marine fish species, being European sea bass (*Dicentrarchus labrax*) and groupers (*Epinephelus* spp.) amongst the most susceptible target species, mainly at larvae and juvenile stages (Chaves-Pozo et al., 2012; Chi et al., 1997). Betanodavirus are small, non-enveloped virus with a genome composed by two positive-sense single-stranded RNA segments (RNA1 and RNA2) and traditionally differentiated into four genotypes (RGNNV, SJNNV, BFNNV and TPNNV) according to the sequence of a variable epitope in the capsid protein (T4 region), coded by the RNA2 (Bandín and Souto, 2020). In addition to traditional genotypes, two reassortant genotypes have also been described and named accordingly with their RNA1/RNA2 composition as RGNNV/SJNNV and SJNNV/RGNNV (Bandín and Souto, 2020). Regarding the innate CMC response against NNV, it has been demonstrated that head-kidney leucocytes (HKLs) from NNV-infected European sea bass showed an increased innate CMC against xenogeneic cells (Chaves-Pozo et al., 2012). In contrast, HKLs from naïve European sea bass failed to activate this activity against several NNV-infected target cell lines, which point out the ability of NNV to escape from the innate CMC as an important factor involved in its high pathogenicity (Chaves-Pozo et al., 2012, 2017). Interestingly, orange-spotted grouper (*Epinephelus coioides*) showed, upon NNV infection, increased levels of circulating $CD8\alpha^{+}$ lymphocytes and adaptive CMC restricted to the MHC I (Chang et al., 2011). Similarly, NNV-infected European sea bass have shown in immune (head-kidney) and NNV-target tissues (brain and retina) a clear increase in various CTL markers, including *cd8a, tcrb, cd28, ctla4* (cytotoxic T-lymphocyte-associated protein 4) and *crtam* (cytotoxic and regulatory T cell molecule) (García-Álvarez et al., 2023; González-Fernandez et al., 2021; Valero et al., 2018) and CMC mediators, such as *gzma, gzmb* and *prf* (Chaves-Pozo et al., 2019b; García-Álvarez et al., 2024; Valero et al., 2018). All these data support the hypothesis about the involvement of CTLs in the adaptive CMC in European sea bass because most of these changes were mainly observed from 15 days of infection onwards, but not earlier. Nevertheless, our knowledge about the adaptive CMC response of European sea bass against NNV is still scarce.

Therefore, we aimed to study the adaptive CMC response of European sea bass using NNV-infected head-kidney (HK) cells at different times post-infection against mock- or NNV-infected cells from a European sea bass brain derived cell line susceptible to NNV (Chaves-Pozo et al., 2019a). This work will shed light on the adaptive CMC in European sea bass and how it is regulated by NNV infection.

2. Material and methods

2.1. Animals

Healthy adult specimens of European sea bass (*Dicentrarchus labrax*) were bred at the Oceanographic Centre of Murcia (Spanish Institute of Oceanography (COMU-IEO), Mazarrón, Spain) and transported to the Marine Fish Facilities at the University of Murcia. Animals (250 ± 25 g) were kept in marine recirculating aquaculture systems (RAS) (30‰ salinity, 22–25 °C, 12:12 light: dark photoperiod) with suitable aeration and filtration systems and fed *ad libitum* with a commercial pellet diet (Skretting). Specimens were allowed to acclimatize for at least 2 weeks. Procedures were approved by the Bioethical Committee of the University of Murcia (reference REGA ES300305440012 and Permit Number A13170109).

2.2. Nodavirus stocks

Parental NNV (genotype RGNNV, strain 411/96; genotype SJNNV, isolate SJNag97; and genotype TPNNV, isolate TPKag93) and NNV reassortants (genotype RGNNV/SJNNV, isolate 367.2.2005 and genotype SJNNV/RGNNV, isolate 389/I96) were propagated in the E−11 cell line as elsewhere (Iwamoto et al., 2001). NNV stocks were titrated and the mean tissue culture infectious dose ($TCID_{50}$/mL) calculated (Reed and Müench, 1938).

2.3. Nodavirus infections

Two experiments were performed, with duplicate tanks per group in each trial. In all cases, European sea bass were slightly sedated with 40 μg/L of clove oil in sea water and received a single intramuscular injection of 100 μl culture medium or containing 10^6 RGNNV $TCID_{50}$/fish (mock-or NNV-infected groups, respectively). In the first experiment, six fish from each group were sampled at 0, 2, 7, 15 and 30 days post-infection (dpi). In the second experiment, six fish from each group were sampled at 15 dpi to validate and evaluate the specificity of the cytotoxic activity.

2.4. Fish sampling and preparation of effector cells

European sea bass HK cells were isolated from mock- and RGNNV-infected fish and used as effectors to evaluate the cytotoxic activity. Briefly, after bleeding, head-kidney was cut into small fragments and transferred to 10 mL of Leibovitz's L-15 (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco), 2 mM glutamine (Gibco), penicillin (100 IU/mL; Gibco), streptomycin (100 μg/mL; Gibco) and 20 mM HEPES (Gibco). Cell suspensions were obtained by forcing fragments through a nylon mesh (100 μm). HK cells were washed three times in Leibovitz's L-15, counted in a Z2 Coulter Particle Counter (Beckman Coulter) and adjusted to 10^7 cells/mL. Cell viability was determined by the trypan blue exclusion test.

2.5. Preparation of target cells

The nodavirus-susceptible European sea bass brain DLB-1 cell line (Chaves-Pozo et al., 2019a) was used as target cells in the cytotoxicity assays. Exponentially growing DLB-1 cells cultured in Leibovitz's L-15 culture medium supplemented with 2% FBS, glutamine and antibiotics as above were detached by routine trypsinization, seeded at 15,000 cell/well in 96-flat well plates (Nunc) and incubated for 24 h at 25 °C. Afterwards, DLB-1 cell cultures were infected with 10^5 $TCID_{50}$ of RGNNV, SJNNV, TPNNV, SJNNV/RGNNV or RGNNV/SJNNV/mL for 24 h at 25 °C. As controls, uninfected DLB-1 cells were used.

2.6. Cell-mediated cytotoxicity assays

Cytotoxic activity of the HK cells was determined by measuring the release of lactate dehydrogenase (LDH) (Korzeniewski and Callewaert, 1983) from killed target cells using the commercial CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's instructions. Briefly, uninfected- or NNV-infected DLB-1 cell cultures were washed twice with culture medium to remove extracellular virus and/or cellular debris. Subsequently, 10^6 HK cells from mock- or RGNNV-infected fish were added to each well (~50 effector cells: 1 target cell) to a final volume of 100 μL. The plate was then centrifuged at 400 g for 1 min to promote cell contact and incubated for 4 h at 25 °C. Thereafter, the plate was centrifuged at 400 g for 5 min and 50 μL of the supernatant from each well was transferred to another 96-flat well plate where LDH release was measured according to the manufacturer indications including a positive control with LDH-standard solution. Targets (DLB-1) or effectors (HK cells) cultured alone were used for measuring the spontaneous release of LDH as controls. The cytotoxic activity was calculated by the following formula:

$$\text{Cytotoxic activity (\%)} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} * 100$$

In addition, cytotoxicity assays performed with HK cells from mock- or RGNNV-infected specimens after 15 dpi were processed for gene expression study. In this case, after incubation of mock- and RGNNV-infected HK cells with the target cells, samples were centrifuged, the supernatant discarded and 200 μL TRIzol® Reagent (Invitrogen) added and stored until being used. Cytotoxicity samples formed by mock-infected HK cells and uninfected-DLB-1 cells alone served for gene expression normalization.

2.7. Gene expression by real-time PCR

Total RNA was isolated from TRIzol® Reagent frozen independent samples ($n = 6$/group) following the manufacturer's instructions. One microgram of total RNA was treated with DNAse I (Promega) to remove genomic DNA. Superscript IV Reverse Transcriptase (Life Technologies) was used to synthesize the first strand cDNA. Real-time qPCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. For each mRNA, gene expression was corrected by the elongation factor 1 alpha (*ef1a*) and ribosomal protein S18 (*rps18*) gene expression in each sample and expressed as either $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ (Pfaffl, 2001). The primers used are shown in the Supplementary Table S1. Prior to the experiment, the specificity of each primer pair was studied using positive and negative samples. A melting curve analysis of the amplified products validated the primer for specificity. Negative controls with no template were always included in the reactions.

2.8. Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Data of the CMC and gene expression were analysed by one- or two-way ANOVA followed by a Tukey comparison of means test. The level of significance was fixed at either 0.1 or 0.05. A Pearson correlation tests were applied to test correlations among gene expression levels and adaptive CMC.

3. Results

The accumulated mortalities after 30 and 15 days of NNV challenge in trials 1 and 2 were of 60 and 57%, respectively.

3.1. CMC against NNV-infected cells increases in infected European sea bass

The cytotoxic activity of HK cells isolated from RGNNV-infected fish against uninfected-, RGNNV- and SJNNV/RGNNV-infected target cells was determined at 0, 2, 7, 15 and 30 dpi (Fig. 1). HK cells from RGNNV-infected European sea bass also showed very low cytotoxic activity against uninfected DLB-1 target cells at all the experimental times (Fig. 1), very similar to that of HK cells from mock-infected fish (data not shown). However, HK cells from RGNNV-infected European sea bass displayed a statistically significant increase in the cytotoxic activity from 7 until 30 dpi against RGNNV- and SJNNV/RGNNV-infected DLB-1 target cells, peaking at 15 dpi for both genotypes (Fig. 1). Two-way ANOVA test revealed that the infection time ($p < 0.001$) and the target cell type ($p < 0.001$) factors, and their interaction ($p < 0.0235$), showed statistical significance.

3.2. CMC is specific to RGNNV and SJNNV/RGNNV genotypes

Firstly, we evaluated if European sea bass specimens and the DLB-1 target cells share some MHC class I alleles, which is mandatory for the adaptive response. Fish used in this study and the one used to generate the DLB-1 cell line come from the same broodstock so might share genetic families. Thus, we searched data from RNA-seq studies carried out in our laboratory (Chaves-Pozo et al., 2019a; unpublished data). We found 11 MHC class Iα coding alleles shared among fish tissues and DLB-1 cells, which are different according to the sequences and phylogenetic tree (Supplementary Fig. 1A). In addition, 9 out 11 were significantly increased in the DLB-1 cells upon infection with RGNNV (Supplementary Fig. 1B).

Then, after proving that CMC was increased only against DLB-1 cells infected with RGNNV and SJNNV/RGNNV genotypes (Fig. 1), which share the same capsid protein, we aimed to evaluate whether this activity really is genotype-specific. For this, we used different NNV genotypes since this capsid protein acts as the major immunogenic antigen (Coeurdacier et al., 2003). The results demonstrated that HK cells from RGNNV-infected sea bass were only capable to increase their CMC response against target cells that expressed the same capsid antigen, RGNNV and SJNNV/RGNNV, but not others, RGNNV/SJNNV, SJNNV or TPNNV (Fig. 2). These data suggest that the recognition is in an antigen-specific manner, and probably restricted to the MHC class I.

3.3. Transcription profile points to T cell activation, PRF/GZM and Fas/FasL pathways and apoptosis during the specific CMC

After demonstrating that the adaptive CMC is activated in RGNNV-infected European sea bass HK cells against infected cells, we evaluated the transcription of some relevant genes at the peak of the CMC response, 15 dpi. Data are expressed as fold change in a heatmap with hierarchical clustering (Fig. 3) using the CMC assays from mock-infected sea bass HK cells against uninfected DLB-1 target cells as calibrators. Regarding groups (columns), the clustering analysis yielded two clusters: Cluster 1 comprised the samples where a control was used, either mock-infected fish or uninfected targets, while cluster 2 contained both infected fish and target cell combinations. As regards the genes (rows), these were divided into two clusters, A and B. Cluster A is composed by *fasl prf1.2, nccrp1, gzma and cd28* genes that were highly up-regulated in cluster 2 when compared to cluster 1 levels (Fig. 3). Cluster B is composed by *ctla4, bcl2, il2, bax, casp3, cd8a, tcrb, nkl, grzmb* and three of the genes coding for perforin (*prf1.3, 1.5* and *1.9*). Similarly to the genes of cluster A, the transcription levels of *prf1.9, gzmb, il2 and bax* genes from cluster B were also increased in samples of cluster 2 compared to cluster 1 (Fig. 3). In fact, the lowest levels of *prf1.9* expression were observed in RGNNV-infected HK cells against uninfected DLB-1 target cell samples. Regarding the transcription of *prf1.5,* the RGNNV-infected HK cells against uninfected DLB-1 target cells, ant the samples of cluster

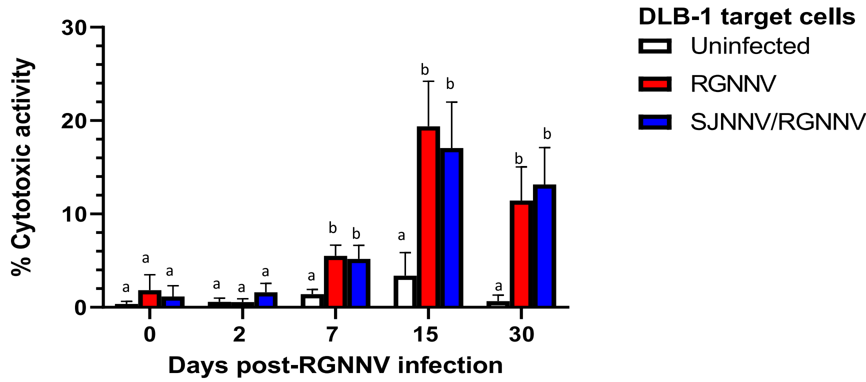

Fig. 1. Cell-mediated cytotoxic activity of RGNNV-infected European sea bass head-kidney effector cells at 0, 2, 7, 15 and 30 days post-infection (dpi) against uninfected-, RGNNV- or SJNNV/RGNNV-infected DLB-1 cells as target cells. Results are expressed as the mean ± SEM ($n = 6$ fish/group). Different letters denote significant differences between the infection time and target cells according to a two-way ANOVA test ($p < 0.05$).

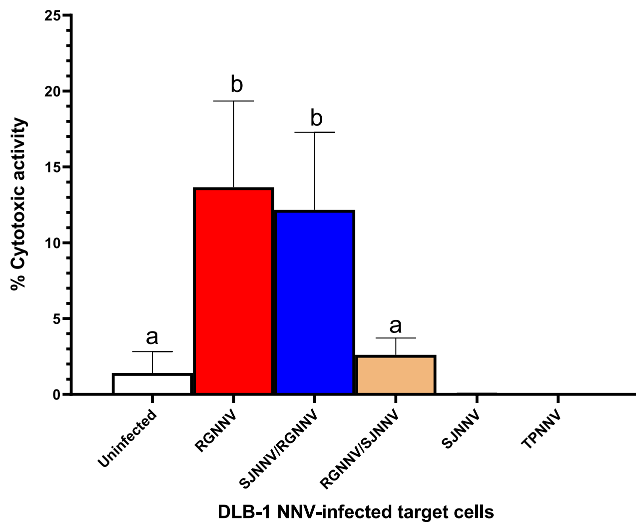

Fig. 2. Cell-mediated cytotoxic activity of RGNNV-infected European sea bass head-kidney effector cells at day 15 of infection against DLB-1 cells infected with different NNV strains as target cells. Results are expressed as the mean ± SEM ($n = 6$ fish/group). Different letters denote significant differences between uninfected- and NNV-infected target cells according to one-way ANOVA and Tukey's comparison of means tests ($p < 0.05$).

2, showed significantly lower levels than mock-infected HK cells against infected DBL-1 target cells. In contrast, *tcrb* and *nkl* gene expressions increased in the mock-infected fish against RGNNV-infected target cells (Fig. 3). In summary, during the adaptive CMC against RGNNV the transcription of T cell activation markers (*il2* and *cd28*), CMC mediators (*prf1.2, prf1.9*, *gzma*, *gzmb*, *fasl*) and apoptosis (*bax*) was significantly increased.

In order to support the potential implication of apoptosis during the adaptive CMC, two of the master regulators, the antiapoptotic gene *bcl2* and the proapoptotic gene *bax* were analysed. Transcription of *bcl2* was unaltered during the CMC whilst *bax* was significantly increased. Therefore, the ratio of *bax/bcl2* was significantly increased during the CMC using RGNNV-infected cells as effectors (Fig. 4) and positively correlated with the adaptive CMC (Pearson coefficient = 0.562; $P < 0.0001$).

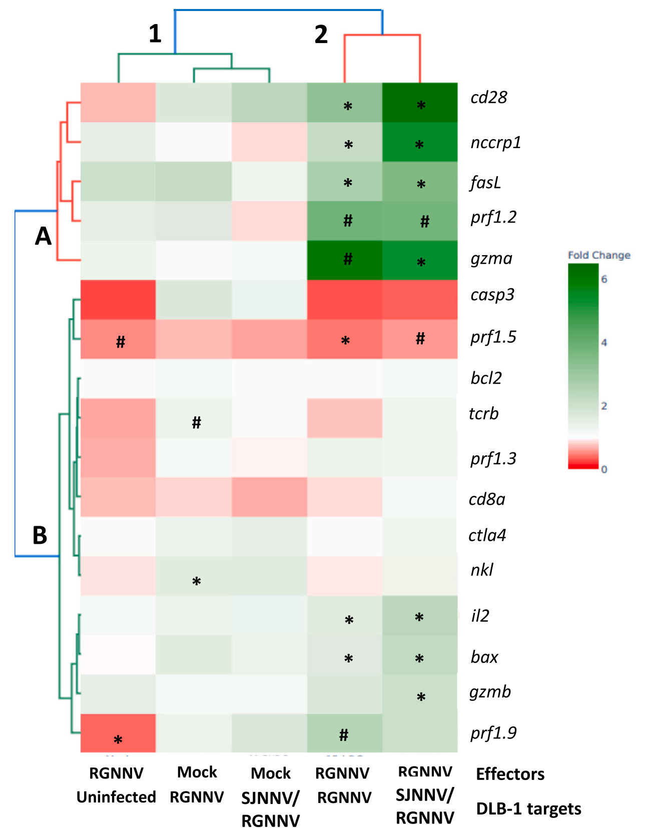

Fig. 3. Heatmap and two-way hierarchical clustering analysis (Euclidean method) of immune related-genes expression in cell-mediated cytotoxic assays between mock- and RGNNV-infected sea bass head-kidney cells (effectors) after 15 days of infection against uninfected- or NNV-infected DLB-1 target cells. Data are shown as mean of fold change ($n = 6$ fish/group) respect to the mock effectors against uninfected target cells. Asterisk and # symbols denote significant differences at $p < 0.05$ or $p < 0.1$ respectively, according to one-way ANOVA and Tukey's comparison of means tests.

4. Discussion

As in mammals, the existence of a CMC carried out by different effector cells has been evidenced in fish, even in a MHC class I-restricted manner. In this study, for the first time, we have evaluated the adaptive

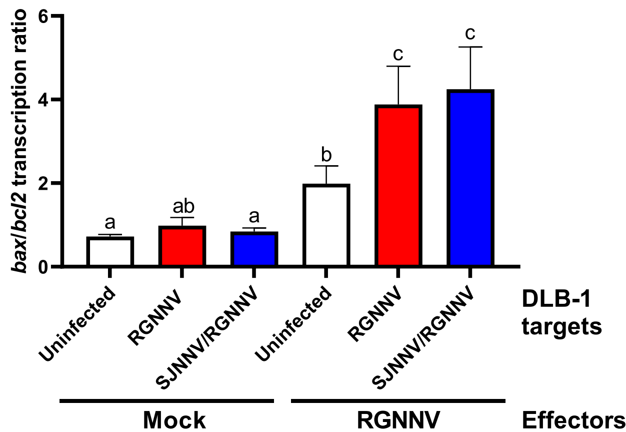

Fig. 4. Transcriptional ratio between the pro-apoptotic *bax* and the anti-apoptotic *bcl2* genes (*bax/bcl2*) in cell-mediated cytotoxic assays between mock- and RGNNV-infected European sea bass head-kidney cells (effectors) after 15 days of infection against uninfected- or NNV-infected DLB-1 target cells. Results are expressed as the mean ± SEM ($n = 6$ fish/group). Different letters denote significant differences between groups according to one-way ANOVA and Tukey's comparison of means tests ($p < 0.05$).

CMC in RGNNV-infected European sea bass specimens using as target cells the NNV-susceptible DLB-1 cell line (Chaves-Pozo et al., 2019a) infected with different genotypes of NNV. Previous studies have demonstrated that leucocytes from different teleost species were capable of exert innate CMC against virus-infected cells via NCC or NK-like cells (Hogan et al., 1996; Moody et al., 1985). In fact, European sea bass leucocytes from naïve (Cammarata et al., 2000; Meloni et al., 2006; Meseguer et al., 1996; Mulero et al., 1994) or NNV-infected specimens (Chaves-Pozo et al., 2012) were able to kill xenogeneic tumour cells thanks to the engagement of NCCs and the up-regulation of the *nccrp1* gene. However, leucocytes derived from naïve sea bass showed the same innate killing efficiency against NNV-infected target cell lines, including the DLB-1, than against the mock-ones (Chaves-Pozo et al., 2017). We found similar results in this study, where leucocytes from mock-infected sea bass did not increase innate CMC against NNV-infected targets.

Regarding the adaptive CMC, surprisingly, HK cells from infected fish increased this activity against RGNNV and SJNNV/RGNNV-infected target cells from 7 to 30 dpi, whereas mock-infected HK cells did not. To study if this activity was virus-specific, we repeated the same experiment using HK cells at the peak of the response, 15 dpi, but against DLB-1 target cells infected with the same virus or with RGNNV/SJNNV, SJNNV or TPNNV genotypes. Strikingly, the CMC was only increased against RGNNV and SJNNV/RGNNV-infected target cells, what strongly suggests that this CMC is specific and carried out by CTLs. This hypothesis is supported by the modulation *in vivo* of numerous CTL-related genes upon a NNV infection from 7 to 15 dpi and not earlier (García-Álvarez et al., 2023; González-Fernandez et al., 2021; Valero et al., 2020). The increase of circulating CTLs as well as the adaptive cytotoxic activity upon a NNV infection was also demonstrated in orange-spotted grouper (Chang et al., 2011). Virus-specific CTL activity has been also confirmed in rainbow trout (Utke et al., 2007, 2008) and ginbuna crucian carp (Somamoto et al., 2000, 2006, 2009, 2013; Tajimi et al., 2019). Furthermore, in our model of study, we demonstrated that both, effector and target cells, share numerous MHC class I alleles, which suggests that the adaptive CMC response observed might also be MHC class I-restricted. However, further studies are mandatory to clearly establish the relationship between adaptive CMC and MHC class I alleles in a NNV infection in European sea bass.

At gene level, HK cells from mock-infected fish specimens against RGNNV- or SJNNV/RGNNV-infected targets underwent little or non-transcriptional variation. In fact, the increased levels of *trcb* and *nkl* in mock-infected HK cells responding against RGNNV-infected cells suggest a slight activation of T lymphocytes upon the first contact with RGNNV-infected target cells. However, the displayed CMC was not sufficient to kill them probably due to the lack of a previous contact with the antigen. Interestingly, *nkl* gene in European sea bass is mainly expressed by T lymphocytes and is modulated upon an *in vivo* infection with NNV (Valero et al., 2020). The opposite appears to occur in the HK cells from infected fish against target cells alone, where there was a decrease in *prf1.9* and *prf1.5*, a fact that keeps in line with previous data in our group (García-Álvarez et al., 2024). Also, an RNA-seq study carried out during the innate CMC using naïve HKLs of European sea bass against RGNNV-infected DLB-1 cells, evidenced that most of the up-regulated genes in NNV-infected target cells were related to metabolism and very few to immunity (Chaves-Pozo et al., 2017). As we mentioned previously, our hypothesis posits a significant role of CTLs in the observed cytotoxic activity, which is supported by the substantial up-regulation of *il2* and *cd28* (indicators of T cell activation) and the lack of modulation of *ctla4* (inhibitory co-receptor) genes. In the case of sea bass, the expression of *cd28* is not only associated with T cells but also plays a role in the immune response against NNV infection, as it is involved in the activation of T cells (González-Fernandez et al., 2021). Furthermore, the interaction of CD28 to its ligand leads to an increase in the cytokine IL-2, a well-known T cells growth factor (Esensten et al., 2016). This phenomenon is also observed in our study, where, akin to humans, the involvement of IL-2 in the development of T cells has been documented (Buonocore et al., 2020). Regarding the underlying mechanism activated by European sea bass CTLs, during the specific CMC displayed by HK cells from infected fish against RGNNV- and SJNNV/RGNNV-infected target cells, the increase of the *fasl, prf1.9, prf 1.2, gzmb* and *gzma* gene transcription could evidence the activation of the perforin/granzyme and Fas/FasL pathways characteristic of CTLs. In European sea bass, the up-regulation of *gzma* and *prf1.9* gene expressions suggested a major role of these genes in the fish CMC against virus-infected cells both *in vivo* and *in vitro* (Chaves-Pozo et al., 2019a, 2019b; Valero et al., 2018), agreeing what was observed in this study. However, *prf1.2* was also up-regulated in the HK of RGNNV-infected European sea bass at 15 dpi (García-Álvarez et al., 2024). Strikingly, our data also show that the mRNA level of *prf1.5* decreased in HK cells from RGNNV-infected specimens against infected- and uninfected-target cells. This could be explained by a regulatory feedback induced by the increased production of other *prf* genes such as *prf1.2* and *prf1.9,* as also observed in zebrafish with *prf1.9b* (Varela et al., 2016). It is worthy to note that *gzmb* transcription levels increased in the HK of NNV infected sea bass after 7 and 15 dpi, as well as GzmB$^+$ cells also increase in NNV-infected gilthead seabream (Chaves-Pozo et al., 2019b). In that sense, our data also showed an increase of *gzmb* transcription levels in HK cells from infected European sea bass responding against SJNNV/RGNNV-infected target cells, supporting the idea that *gzmb* plays an important role against NNV. In fact, the participation of the perforin/granzyme pathway, which is a granule- and calcium dependent process, in fish adaptive CMC has also been evidenced in other fish species. For example, the use of calcium chelators had a negative impact in the adaptive CMC in common carp (*Cyprinus carpio* L.) (Companjen et al., 2006). In ginbuna crucian carp, the use of perforin inhibitors, such as concanamycin A, cause the suppression of CD8$^+$ lymphocyte activity in a dose-dependent manners, probably due to perforin depolymerization (Toda et al., 2011a). Additionally, the use of EGTA caused a significant decrease in the CMC, a fact that was reversed with the addition of Ca^{2+}. In this species, the use of carbobenzyloxy-Ile-Glu-Thr-Asp-fluoromethyl ketone (Z-IETD-FMK), that blocks the activity of granzyme B, caused a significant but not complete decrease in the adaptive CMC, suggesting the participation of other molecules in the cytotoxic pathway (Toda et al., 2011b). The levels of *gzma* were also increased in leucocytes of rainbow trout and RTS-11 cell line against VHSV (Ordás et al., 2011) and in Atlantic salmon (*Salmo*

salar) infected with infectious pancreatic necrotic virus (IPNV) (Munang'andu et al., 2013). Additionally, the increment of *fasl* gene suggested the activation of the granule-independent cytotoxic activity against NNV, in line with previous observations in the pacific cod (*Gadus macrocephalus*) where NNV could trigger apoptosis through the Fas/FasL pathway (Mao et al., 2021). Therefore, our data showing increased ratios of *bax/bcl2* indicate the proapoptotic tendency in the adaptive CMC samples in the groups of cluster 2 and show good correlation with CMC, suggesting the induction of apoptosis by specific CTLs. These data, together with the activation of different perforins, granzymes and *fasl* genes reinforce the idea of the activation of both pathways for the clearance of NNV-infected cells, leading to, at least, apoptosis target cell death, as observed in a previous study (Chaves-Pozo et al., 2019a). CMC response from infected fish specimens against RGNNV and SJNNV/RGNNV-infected target cells also resulted in a significant up-regulation of *nccrp1* gene. The role of NCCs against viral infection has been confirmed in crucian carp, where a small fraction of leucocytes could lyse syngeneic cells infected with IPNV (Somamoto et al., 2000), in gilthead seabream (*Sparus aurata*), where innate immune response against VHSV had been described (Esteban et al., 2008), or in European sea bass against NNV (Chaves-Pozo et al., 2012, 2017). Despite the fact that NCCs are also capable of producing cytotoxic molecules such as perforin, granzyme or even FasL (Jaso-Friedmann et al., 2000), our CMC data demonstrated that the main effector cells of such activity are CTLs due to the high specificity displayed against target cells expressing the RGNNV capsid. Anyway, the increase in *nccrp1* expression levels also point to a role of NCCs in the elimination of infected cells. A further characterization of the relation between this receptor and the adaptive CMC is mandatory to ascertain the regulation and interplay of innate and adaptive CMC in fish.

5. Conclusions

To conclude, our study confirms that the CMC in RGNNV-infected European sea bass HK cells increases over infection, peaking at 15 dpi. This cytotoxic activity is specific against DLB-1 target cells infected with different NNV genotypes displaying the RGNNV capsid protein, as expected for adaptive CMC response. At transcriptional level, genes related to T cell activation, the perforin/granzyme and Fas/FasL pathways and apoptosis cell death are up-regulated during this adaptive CMC response. Altogether, our data clearly suggest that the CMC observed is mediated by CTLs. However, further and deeper studies are needed to clarify the nature of this CMC, the leucocytes involved, and the mechanisms elicited for the elimination of NNV-infected cells.

CRediT authorship contribution statement

Miguel A. García-Álvarez: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Elena Chaves-Pozo:** Writing – review & editing, Supervision, Funding acquisition. **Alberto Cuesta:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2023.105124.

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