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Research paper

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ABSTRACT

Antimicrobial peptides (AMP) comprise a wide range of small molecules with direct antibacterial activity and immunostimulatory role and are proposed as promising substitutes of the antibiotics. Additionally, they also exert a role against other pathogens such as viruses and fungi less evaluated. NK-lysin, a human granulysin orthologue, possess a double function, taking part in the innate immunity as AMP and also as direct effector in the cell-mediated cytotoxic (CMC) response. This molecule is suggested as a pivotal molecule involved in the defence upon nervous necrosis virus (NNV), an epizootic virus provoking serious problems in welfare and health status in Asian and Mediterranean fish destined to human consumption. Having proved that NK-lysin derived peptides (NKLPs) have a direct antiviral activity against NNV *in vitro*, we aimed to evaluate their potential use as a prophylactic treatment for European sea bass (*Dicentrarchus labrax*), one of the most susceptible cultured-fish species. Thus, intramuscular injection of synthetic NKLPs resulted in a very low transcriptional response of some innate and adaptive immune markers. However, the injection of NKLPs ameliorated disease signs and increased fish survival upon challenge with pathogenic NNV. Although NKLPs showed promising results in treatments against NNV, more efforts are needed to understand their mechanisms of action and their applicability to the aquaculture industry.

1. Introduction

Conventionally, search and demonstration of the antiviral potential of synthetic or natural compounds has been relegated to the background in pursuit of bacterial control (Cabello, 2006). In particular, the problematic of the viruses spread in aquaculture has upraised with the intensive fish culture and the constant transport of eggs and larvae between farms all over the world, with no practical solutions in the short-middle term. Antimicrobial peptides (AMP) comprise a large number of different gene-encoded short peptides, generally cationic and amphipathic, with high number of hydrophobic residues recognized to act in a receptor-independent manner, and *a priori* not generating undesirable bacterial resistance as antibiotics do (Rakers et al., 2013). Therefore, though most of the research has focused on the immunomodulatory actions and the antibacterial properties of AMPs, they have a direct lytic activity against a wider spectrum of targets including viruses, fungi, parasites or tumour cells (Bahar and Ren, 2013). Thus, they are considered promising candidates to replace antibiotics but also to be used against other pathogens in both human health and animal production.

NK-lysin, an orthologous to human granulysin, is synthetized by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells and is stored into granules awaiting to be released after proper *stimuli* (Andersson et al., 1996; Peña and Krensky, 1997). Apart from its

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primary role in the cell-mediated cytotoxicity (CMC), NK-lysin and shorter-derived peptides (NKLPs) have also demonstrated to play a role as AMP against bacteria, viruses, fungi, parasites and tumour cells (Jacobs et al., 2003). NK-lysin possesses a saposin-B (SapB) domain, composed by 3 α-helix and 6 well-conserved cysteines that conform 3 disulphide bonds, and from which all the assayed NKLPs showing AMP activity are derived. NK-lysin has been already identified and characterized in several fish species (Acosta et al., 2019; Ding et al., 2019; Han et al., 2019; Liu et al., 2020; Pereiro et al., 2015, 2017; Valero et al., 2020a; Wang et al., 2006, 2018; Zhang et al., 2019; Zhou et al., 2019). Beyond the direct involvement in the CMC response, which has not been properly demonstrated yet, fish NK-lysin caught the attention due to its immunomodulatory and antimicrobial activities upon a significant range of pathogens including virus (Chen et al., 2021; Hirono et al., 2007; Lama et al., 2018; León et al., 2020; Liu et al., 2020; Pereiro et al., 2017; Zhang et al., 2013, 2014; Zhou et al., 2016, 2019). Although the mechanisms by which its antiviral function is developed is still poorly understood, *in vitro* assays demonstrate that NKLPs disrupt the spring viremia of carp virus (SVCV) ability to fix membranes in a dose- and pH-dependent manner, and in turn, its fusion to the cell (Falco et al., 2019). Moreover, several fish-derived NKLPs are shown to decrease the viral replication of nervous necrosis virus (NNV), viral septicaemia haemorrhagic virus (VHSV), infectious pancreatic necrosis virus (IPNV) and SVCV *in vitro* due to direct viral lysis (León et al., 2020). However, the *in vivo* potential antiviral activity of NK-lysin or NKLPs has been slightly evaluated in fish. In the teleost tongue sole (*Cynoglossus semilaevis*) the administration of NK-lysin expressing plasmids or the synthetic NKLP27, derived from the NK-lysin, is able to reduce the viral load throughout tissues upon infection and up-regulates the transcription of immune-related genes at early times though the effective protection was not demonstrated (Zhang et al., 2013, 2014). Recently, synthetic NKLP showed the ability to reduce the mortality of barbel steed (*Hemibarbus labeo*) against *Aeromonas hydrophila* infection as well as to increase the transcription of pro-inflammatory cytokines and the chemotaxis of monocyte/macrophages (Chen et al., 2021).

Although viral pandemics wrongly appear to be restricted to humans, there are epizootic viruses severely affecting fish species that are spread all over the world. Moreover, the replication, mutation and reassortment abilities of different viruses have led to diminish the welfare and health of farmed fish and cause impressive economic losses in the aquaculture sector (Lafferty et al., 2015). One of these viruses is NNV (genus *Betanodavirus*), the causative agent of the viral encephalopathy and retinopathy (VER) disease, that affects more than 170 fish species worldwide (Bandín and Souto, 2020). One of the most susceptible ones is the European sea bass (*Dicentrarchus labrax*), which is the highest contributor to the Mediterranean aquaculture in both economic value and production volume (APROMAR, 2019). NNV is lethal mainly to early stages of sea bass development (larvae and early juveniles) reaching in most cases up to 100% of mortality rates at those stages (Breuil et al., 1991). NNV triggers in European sea bass innate (antimicrobial, type-I interferon, CMC or inflammatory responses) and acquired (specific antibodies, B and T cellular responses) immunity at both local (brain and retina) and systemic levels (Buonocore et al., 2017; Chaves-Pozo et al., 2012, 2017, 2019; González-Fernández et al., 2020, 2021; Esteban et al., 2013; Moreno et al., 2018; Novel et al., 2013; Scapigliati et al., 2010; Valero et al., 2015a, 2015b, 2015c, 2015d, 2016, 2020a, 2020b). However, the immune response was inefficient and certainly insufficient to kill and clear the virus. Regarding sea bass NK-lysin, gene expression or protein levels are increased upon NNV infection in several tissues as well as upon leucocyte incubation with T cell mitogens (Valero et al., 2020a).

Taking all this information into account, we have injected European sea bass with different NKLPs and evaluated the transcription of NNV capsid gene and some immune markers and the protection upon NNV challenge. Our results show that though synthetic NKLPs produce little changes in the transcriptomic profile of sea bass, the disease signs were reduced and survival of fish improved, pointing to their potential application as an effective preventive measure in the aquaculture.

2. Material and methods

2.1. Synthetic peptides

Peptides used were previously designed, synthetized and their characteristics resumed elsewhere (León et al., 2020). In brief, synthetic European sea bass NKLPs were based on the complete sequence available in the UniProtKB database (https://www.uniprot.org/; acc. number **A0A218MG56**) and contained the following sequences: NKLP23 (KLLAVCDQIGLLKSLCRKFVKKH), NKLP20.1 (AGKLPGLCWACKWALKKVKK) and NKLP20.2 (CKWALKKVKKVMGPNATAEN). The synthetic NKLP27 (KVKARLIKICNKIGFLKSRCHKFVITH) from tongue sole (acc. number R**4TXU3**) was also used for comparisons. Sequences, properties, helical wheel models, *in silico* predictions and determinations of their antimicrobial activity were already published (León et al., 2020). All the peptides were resuspended in sterile ultrapure water at 10 mg/mL and aliquots stored at −20 °C till use.

2.2. Animals

Healthy juvenile specimens of European sea bass (15.5 ± 0.7 g body weight) were purchased from a hatchery (PREDOMAR S.L., Carboneras, Almería, Spain). The fish were maintained in closed flow-through marine aquaria (28% salinity, 22-26 °C and with a 12 h light: 12 h dark photoperiod) with suitable aeration and filtration systems and were fed daily with a commercial diet (Skretting). The handling of the specimens was always performed in accordance with the Guidelines of the European Union Council (2010/63/UE) and the Bioethical Committees of the University of Murcia (reference REGA ES300305440012 and Permit Number A13150104).

2.3. Fish treatment and sampling

Two-hundred and fifty European sea bass fish specimens were randomly divided into five experimental groups and acclimatised for 15 days. Six additional fish were reserved for later infection control. After light sedation with 40 μg/L of clove oil in sea water, specimens received an intramuscular injection (im) with 100 μL of phosphate buffered saline (PBS) alone (Control) or containing the synthetic peptides NKLP23, NKLP20.1, NKLP20.2 or NKLP27 at a dosage of 15 μg of NKLPs/fish (~1 μg NKLP/g fish). After injection, fish (n = 6 fish/group and time point) were sampled at 3, 24 and 72 h post-injection. Fish were sacrificed by an overdose of clove oil, completely bled, immediately beheaded and weighed. Head-kidney, the main lymphohematopoietic tissue in fish, was removed by dissection, immediately frozen in TRIzol Reagent (Life Technologies) and stored at −80 °C until use.

2.4. Nodavirus infection in vivo

After the sampling at 72 h post-injection, remaining fish (n = 32 fish/group) were infected with NNV (strain It/411/96, genotype RGNNV) as previously described (Chaves-Pozo et al., 2012). For this, fish slightly sedated received a single im injection of 100 μL of culture medium containing $10^{7.75}$ NNV $TCID_{50}$/fish (Aranguren et al., 2002). Six untreated fish were mock-infected with 100 μL of culture medium alone and served as controls. Three days after NNV infection, brain (the main target tissue for NNV replication) and head-kidney (n = 6 fish/group) were removed by dissection, immediately frozen in TRIzol Reagent and stored at −80 °C until use. Disease signs and mortalities were recorded daily for 25 days post-infection (dpi). Four ranks of disease signs were established attending to their severity as follows: 1) changes of the colour of the skin, slower rhythm of swimming and/or slower reaction to external *stimuli* as feeding, 2) alterations in the swimming balance

and/or erratic swimming spasms, 3) continuous erratic swimming and 4) complete incapacity to keep balance, swim and/or move without external *stimuli*. Mortality was presented by Kaplan-Meier survival curves. Relative percent survival (RPS) was determined: RPS = (1 – cumulate mortality in NKLP-treated and NNV-infected group/cumulate mortality in PBS-treated and NNV-infected group).

2.5. Gene expression

Total RNA from TRIzol Reagent-frozen head-kidney or brain tissues was isolated following the manufacturer's instructions. One μg of total RNA from each individual fish was treated with DNAse I to remove genomic DNA, and the first strand of cDNA was synthesized by reverse transcription using the SuperScript IV™ Reverse Transcriptase (Invitrogen) with random hexamers (Invitrogen).

The expression of the genes coding for the proteins of the i) NK-lysin; ii) type-I interferon/antiviral response marker; iii) cellular markers of innate and specific immune response; iv) pro-inflammatory cytokines; v) chemokines and their receptors, and vi) NNV capsid were analysed by real-time PCR and are described in Table 1. PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using PowerUp SYBR Green PCR Core Reagents (Applied Biosystems) as elsewhere (Valero et al., 2020a). Briefly, the reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min 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 L13 alpha (*l13a*) content in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the geometric mean of the endogenous genes *l13a* and *ef1a* Ct values from the target Ct. The primers used are shown in Table 1. A melting curve analysis of the amplified products validated the primers for specificity. Negative controls with no template were always included in the reactions.

2.6. Calculations and statistical analysis

Data are represented as the mean ± standard error of the mean (SEM). Data were analysed by one-way ANOVA ($p \leq 0.05$) followed by Tukey's post-hoc analysis to study the differences between groups. A non-parametric Kruskal–Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. All statistical differences were conducted using IBM SPSS20 software.

3. Results

3.1. Synthetic NKLPs slightly regulate the transcription of cellular immune markers

The injection of European sea bass with the different synthetic NKLPs provoked some alterations in the gene expression of different immune markers at early time-points compared with controls (Fig. 1). Genes coding for *nkl*, *mx, mpo* or *cxcl9* were not significantly altered by the NKLP injection at any time (Fig. 1A,B,F,J). Sea bass NKLP23 significantly up-regulated the gene expression of *ighm* and *csf1r* after 3 h of treatment and of *cxcr3* after 24 h whilst NKLP20.2 induced and increase in the transcript levels of the *tcrb, il1b, il8* and *cxcr3* genes after 24 h, when compared with controls (Fig. 1C,D,E,G,H,I). The comparison of the effect of the different peptides revealed that, at 3 h post-injection, the gene expression of *ighm*, *csf1r* and *cxcr3* was statistically different in specimens treated with NKLP23 compared with the rest of peptides (Fig. 1C,E,I), whilst in the case of the *tcrb*, *il1b* and *il8* genes, NKLP23- and NKLP20.1-treated fish showed significant differences with those receiving NKLP20.2 and NKLP27 (Fig. 1D,G,H). Otherwise, fish injected with NKL20.2 showed the highest transcriptional levels of *tcrb* at 24 h when compared with the rest of the peptides (Fig. 1D). No mortality rates were registered during the NKLP treatment (72 h; data not shown).

3.2. Sea bass NKLPs improve the resistance to NNV

NKLP-treated sea bass juveniles were challenged with NNV after 72 h and disease signs and mortality recorded during 25 dpi (Fig. 2, Supplementary Videos 1–6) as well as the gene expression after 3 dpi (Fig. 3). Regarding the clinical signs, the number of fish with each score were annotated daily and the cumulated number of fish with each score

Table 1
Primer sequences used for gene expression analysis.

	Protein name	Gene name	Accession number	Sequence (5' – 3')	
NK-lysin	NK-lysin	*nkl*	KY801205	F	GAAGAAACACCTCGGGGAAT
				R	GCAGGTCCAACATCTCCTTC
Antiviral response marker	Mx Interferon-induced GTP-binding protein Mx	*mx*	AM228977 HQ237501 AY424961	F	GAAGAAGGGCTACATGATCGTC
				R	CCGTCATTGTAGAGAGTGTGGA
Cellular markers of specific immune response	Immunoglobulin mu heavy chain	*ighm*	FN908858	F	AGGACAGGACTGCTGCTGTT
				R	CACCTGCTGTCTGCTGTTGT
	T cell receptor beta chain	*tcrb*	FN687461	F	GACGGACGAAGCTGCCCA
				R	TGGCAGCCTGTGTGATCTTCA
Cellular markers of innate immune response	Macrophage colony-stimulating factor 1 receptor 1	*csf1r*	KM225787	F	TTTCGGAAAGGTTGTTGAGG
				R	TCTCATCTGAATGGGCACTG
	Myeloid-specific peroxidase	*mpo*	CX660745	F	GAAGAGTGGGGCCTTTGTTT
				R	CTGGGCCTCAGTGAAGACTC
Pro-inflammatory cytokines	Interleukin 1 beta	*il1b*	AJ269472	F	CAGGACTCCGGTTTGAACAT
				R	GTCCATTCAAAAGGGGACAA
Chemokines and receptors	Interleukin 8	*ill8*	AM490063	F	GTCTGAGAAGCCTGGGAGTG
				R	GCAATGGGAGTTAGCAGGAA
	CXC chemokine receptor 3	*cxcr3*	ENSDLAT00005001752	F	ATCCTGTACGCCTTTGTGGG
				R	GTCGGCAGACTCAGACCAAA
	CXC chemokine ligand 9	*cxcl9*	DLAgn_00012980	F	TCTGTCAGCTCGCCTTTCTG
				R	TTCGTACTTGGACACGCACA
Viral genome	NNV capsid protein	*cp*	D38636	F	CAACTGACAACGATCACACCTTC
				R	CAATCGAACACTCCAGCGACA
Housekeeping	60S Ribosomal Protein L13A	*l13a*	DT044539	F	GCGAAGGCATCAACATCTCC
				R	AGACGCACAATCTTGAGAGCAG
	Elongation factor 1 alpha	*ef1a*	FM019753	F	CGTTGGCTTCAACATCAAGA
				R	GAAGTTGTCTGCTCCCTTGG

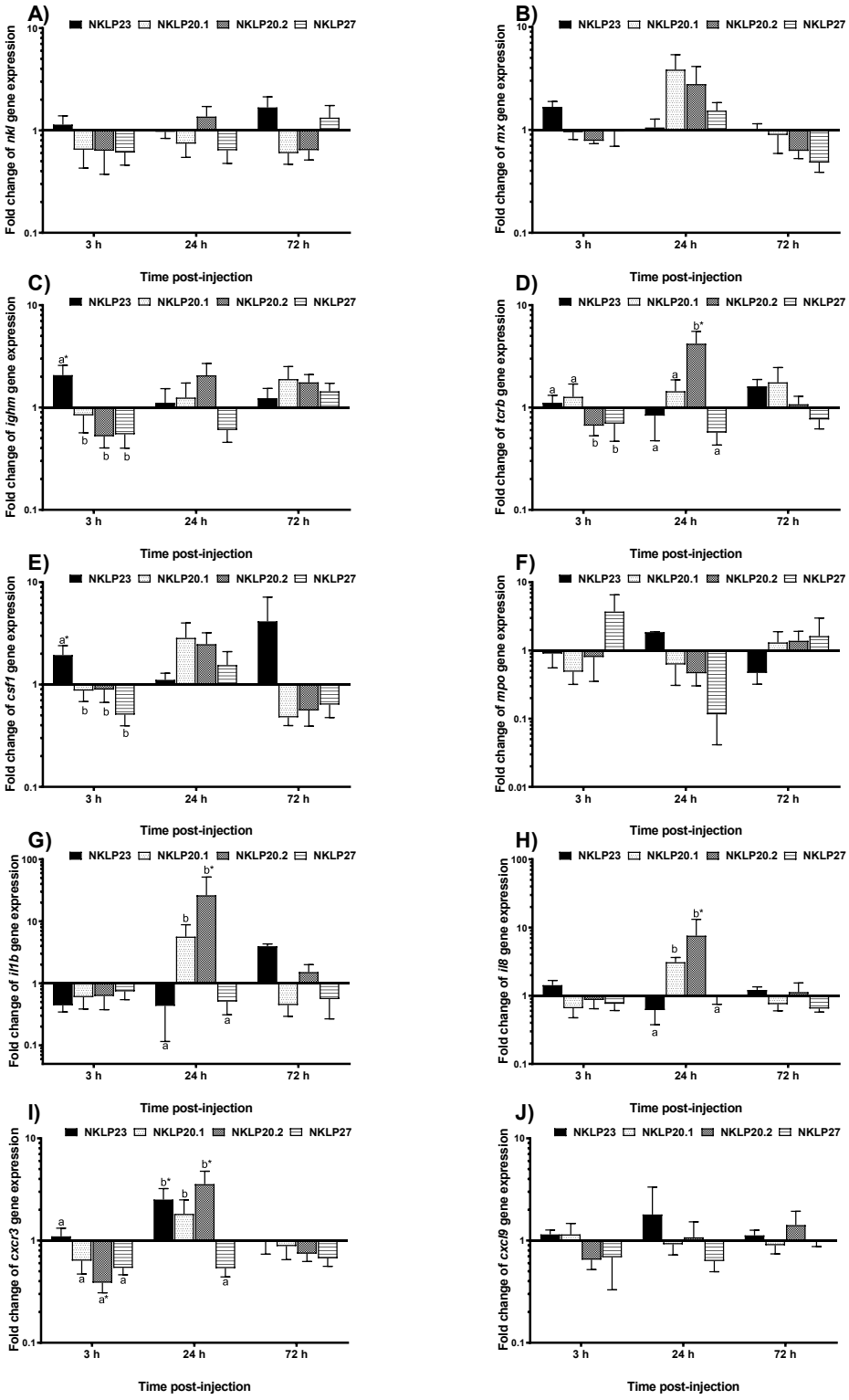

Fig. 1. Synthetic NK-lysin peptides (NKLPs) produce little changes in the transcription of immune-related markers in European sea bass juveniles. European sea bass juveniles were intramuscularly injected with phosphate buffer (PBS-Control) or with 1 μg of synthetic NKLP23, NKLP20.1, NKLP20.2 or NKLP27/g fish and sampled 3, 24 or 72 h post-injection to evaluate the transcription of *nkl* (A), *mx* (B), *ighm* (C), *tcrb* (D), *csf1* (E), *mpo* (F), *il1b* (G), *il8* (H), *cxcr3* (I) and *cxcl9* (J) in the head-kidney by real-time PCR. Data represent the mean fold change of the relative gene expression ± SEM (n = 6/group and time). Statistical differences between groups were analysed by ANOVA ($p < 0.05$) followed by Tukey's post-hoc test. Asterisks denote differences with respect to the PBS-Control while different letters do for differences among NKLP treatments.

during the 25 days challenge presented. First, mock-infected fish displayed no clinical signs (Supplementary Video 1). In the PBS-Control group, and those in the group pre-treated with the sole NKLP27, NNV-challenged fish started to show the most typical signs (alterations in the swimming balance and/or erratic swimming) of the disease around 5–7 dpi and many of them reached scores of 3 or 4 before they died; but score 1 was never observed (Fig. 2A; Supplementary Videos 2 and 6). By contrast, all the fish treated with sea bass-derived NKLPs showed lighter signs upon NNV infection. Thus, fish treated with NKLP20.2 only showed score 1, followed by NKLP20.1 and NKLP23 groups, where scarce fish displayed scores of 1–3 or 1 to 4, respectively (Fig. 2A, Supplementary Videos 3–5).

Besides, the survival curves (Fig. 2B) and the RPS (Fig. 2C) were calculated and presented. PBS-injected fish challenged with NNV showed a cumulated mortality of 41.7%. However, fish treated with synthetic sea bass-derived NKLP23, NKLP20.1, NKLP20.2 showed

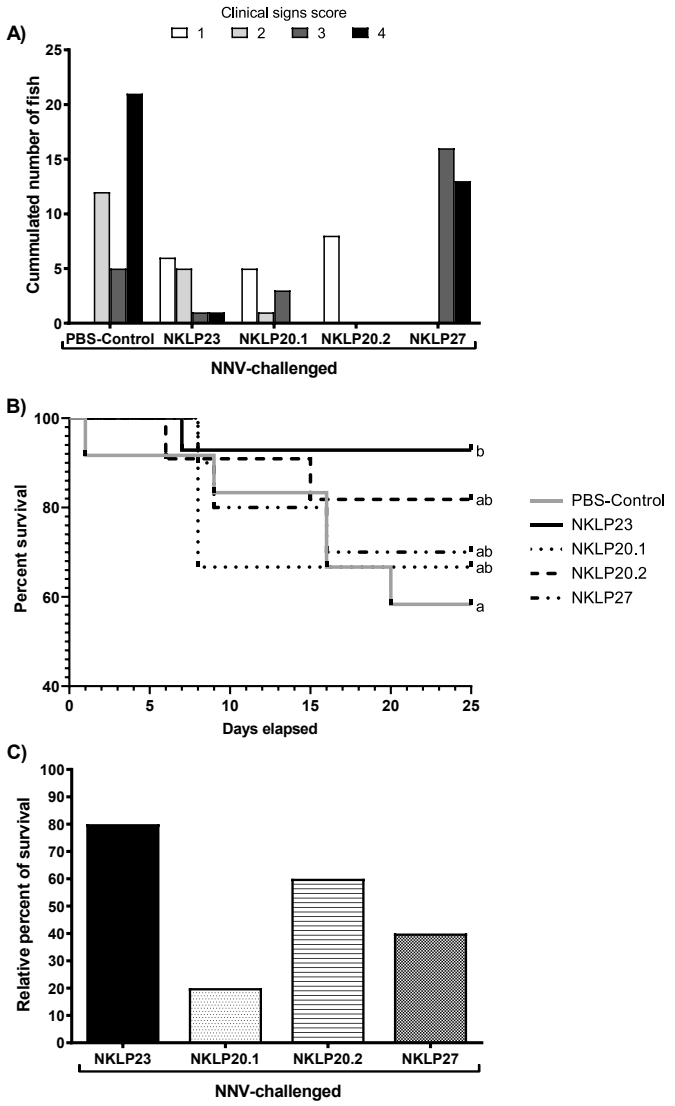

Fig. 2. Synthetic NK-lysin peptides (NKLPs) reduce the disease signs and mortality of European sea bass upon nervous necrosis virus (NNV) infection. European sea bass juveniles were intramuscularly injected with phosphate buffer (PBS-Control) or with 1 μg of synthetic NKLP23, NKLP20.1, NKLP20.2 or NKLP27/g fish and after 72 h challenged by an intramuscular injection with $10^{7.75}$ NNV $TCID_{50}$/fish. (A) Clinical signs were daily observed and the cumulated number of fish showing VER disease signs presented attending to their severity: 1, changes of the colour of the skin, slower rhythm of swimming and/or slower reaction to external *stimuli* as feeding; 2, alterations in the swimming balance and/or erratic swimming spasms; 3, continuous erratic swimming; and 4, complete incapacity to keep balance, swim and/or move without external *stimuli*. (B) Kaplan-Meier survival curves showing the proportion of European sea bass survivors upon NNV infection. Different letters denote significant differences among groups according to the Log-rank (Mantel-Cox) test ($p < 0.05$). (C) Relative percent survival in NKLPs-treated European sea bass after NNV infection.

partial protection of 80, 20 and 60% upon NNV challenge, respectively though only in those treated with the NKLP23 the RPS reached significance respect to the control (Fig. 2C). Sole NKLP27-treated fish showed a RPS of 40%.

Finally, we also evaluated the transcriptional level in the head-kidney and the brain upon NNV challenge (Fig. 3A and B). First, NNV capsid gene was detected by qPCR in all the NNV-challenged fish with no statistical differences between treatments (Fig. 3B). NNV was able to significantly induce the transcription of the antiviral *mx* gene in both the

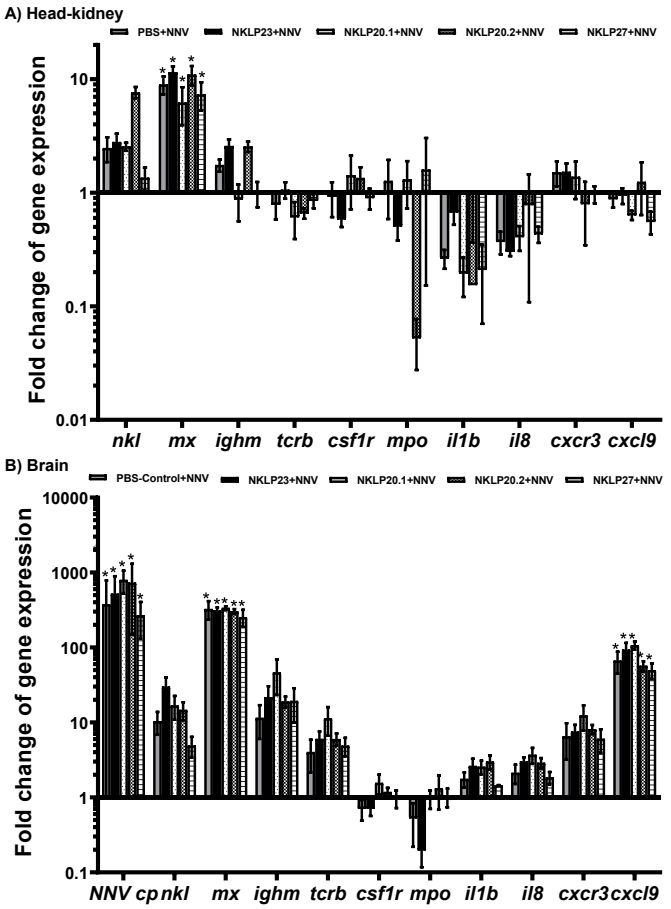

Fig. 3. Synthetic NK-lysin peptides (NKLPs) fail to modulate the NNV-induced transcription of immune-related genes in European sea bass. European sea bass juveniles were intramuscularly injected with phosphate buffer (PBS-Control) or with 1 μg of synthetic NKLP23, NKLP20.1, NKLP20.2 or NKLP27/g fish g and after 72 h challenged by an intramuscular injection with $10^{7.75}$ NNV $TCID_{50}$/fish. A group of resting fish were mock-infected and served as controls. After 3 days of infection, head-kidney (A) and brain (B) tissues were sampled to evaluate the transcription of NNV *cp*, *nkl*, *mx*, *ighm*, *tcrb*, *csf1*, *mpo*, *il1b*, *il8*, *cxcr3* and *cxcl9* by real-time PCR. Data represent the mean fold change of the relative gene expression ± SEM (n = 6/group and time) respect to the mock-infected group. Statistical differences between groups were analysed by ANOVA ($p < 0.05$) followed by Tukey's post-hoc test. Asterisks denote differences with respect to the mock-infected control.

head-kidney and brain, and of *cxcl9* in the brain, in control specimens. However, pre-treatment of sea bass juveniles with NKLPs failed to produce any additional change in the *mx* or *cxcl9* transcription (Fig. 3A and B). Transcription of *nkl*, *ighm*, *tcrb*, *il1b*, *il8* or *cxcr3* in the brain was always increased upon NNV infection but never reached significant values (Fig. 3A and B).

4. Discussion

Although AMPs have been widely proposed as an alternative to reduce the use of antibiotics, due to their antibacterial properties, their application against other pathogens is very limited, being this even more evident in the case of fish and aquaculture. In this sense, the use of AMP to prevent or treat viral diseases is a reliable and promising field of research. Thus, fish AMPs have shown very interesting immunomodulatory effect on individuals and antiviral activity *in vitro* but their function against viral diseases *in vivo* has been slightly and preliminarily evaluated (Valero et al., 2020c). Among the potential fish AMPs, NK-lysin derived peptides are remarkable examples that, apart from the

immunomodulatory role, have shown direct or indirect antibacterial, antiviral and antiparasitic activities (Chen et al., 2021; Hirono et al., 2007; Lama et al., 2018; León et al., 2020; Pereiro et al., 2017; Valero et al., 2020a, 2020b; Zhang et al., 2013, 2014). However, the scarce studies evaluating the antiviral activity of fish NK-lysin *in vivo* were limited to quantify the viral load in fish tissues upon infection but the protection was not confirmed (Zhang et al., 2013, 2014). Thus, in this work, we selected different synthetic NKLPs derived from European sea bass (NKLP23, NKLP20.1 and NKLP20.2) or from tongue sole (NKLP27), all of them previously designed, synthetized and their direct antibacterial and antiviral activity probed *in vitro* (León et al., 2020; Zhang et al., 2014). Since all of the NKLPs described inhibited the NNV replication (León et al., 2020; Zhang et al., 2014), and European sea bass is one of the most susceptible fish species to NNV, we aimed to evaluate whether the administration of NKLPs to sea bass was able to reduce the disease and mortality upon a NNV challenge. This might represent a potential preventive candidate against NNV disease in aquaculture.

Firstly, we administered the synthetic NKLPs intramuscularly to juvenile European sea bass and evaluated the transcription of immune-relevant genes in the head-kidney, the fish equivalent to the mammalian bone marrow. After the administration of NKLPs, the transcriptional levels of *nkl* remained stable and steady in all treatments suggesting that the exogenous supply does not interfere in the NKL synthesis pathway, maintaining then the homeostasis in the routes in which NK-lysin is involved. Similarly, we could observe that all the NKLPs failed to alter the transcription of the main antiviral marker, *mx*. In tongue sole overexpression of NK-lysin throughout expression plasmids, or administration with synthetic NKLP27, produced an early up-regulation in the transcription of interleukin 1 beta (*il1b*), *il8*, chemokines (*cck1* and *cxce1*), Toll-like receptor 9 (*tlr9*), myeloid differentiation primary response 88 (*myd88*), interferon-stimulated gene 15 (*isg15*), *cd28* or major histocompatibility complex I alpha (*mhc1a*) in either head-kidney or spleen, indicating the activation of the type-I interferon pathway, chemotaxis, T-cell activation and inflammation (Zhang et al., 2013, 2014). Similarly, barbel steed NKLP also up-regulates the pro-inflammatory cytokines *il1b* and tumour necrosis factor alpha (*tnfa*) both *in vitro* and *in vivo* as well as chemoattract to monocyte/macrophages (Chen et al., 2021). In our hands, only sea bass injected with NKLP23 or NKLP20.2 showed early and transitory up-regulation in the transcription of *ighm*, *csf1r, tcrb*, *il1b*, *il8* or *cxcr3*. This suggests that NKLPs might improve, for the first time in fish, the adaptive immunity by increasing B and T cell biology, as well as the macrophage functions. By one side, NKLP23 up-regulates *ighm* transcription suggesting their involvement in the sea bass antibody-mediated immunity and pointing to its applicability as vaccine adjuvant, as has also been reported for other fish AMPs (Valero et al., 2020c). In addition, NKLP23 also induces the increase of the *csf1r* transcription pointing to the activation and polarization of macrophages though we failed to detect any inflammatory response in contrast to previous studies (Acosta et al., 2019; Chen et al., 2021; Torraca et al., 2015; Zhang et al., 2013, 2014). Interestingly, NKLP23 up-regulated the transcription of *cxcr3*, which is mainly expressed by NK and CD8 cells (Hosking and Lane, 2010; Kohli et al., 2021), suggesting the activation of the sea bass cytotoxic cells. In agreement with this idea, it has been reported that the injection of Epinecidin-1 to mice triggered the increment of IgM/IgG serum levels concomitantly with the increment of genes coding for pro-inflammatory cytokines (Lee et al., 2012). By the other side, NKLP20.2 promoted the up-regulation of the pro-inflammatory *il1b* and *il8* cytokines as well as the *tcrb* and *cxcr3* genes expression indicating inflammation and the activation of the NK and T cell biology, which might also improve the adaptive immunity. However, whether this involves cytotoxic or helper T cells was not elucidated herein. As suggested by our data, fish NK-lysin could be linked to the CMC since increased *nkl* transcription is parallel to increased CMC response (Chaves-Pozo et al., 2012; Huang et al., 2018; Lama et al., 2018; Valero et al., 2020b). Additionally, sole specimens treated with plasmids coding for the NK-lysin resulted in the up-regulated transcription of *cd28* (Zhang et al., 2013), a T-cell co-stimulatory receptor, supporting the activation of both cytotoxic and helper T lymphocytes. Under the light of these results, different sea bass-derived NKLPs seem to promote the inflammation, the CMC response and the adaptive immunity although more exhaustive experiments must be carried to ascertain their immunomodulatory mode of action.

Upon an *in vivo* infection with NNV, our results reflect a noticeable decrease of sea bass clinical signs concomitantly with the increment of survival, ranging from 20 to 80%. In this sense, all sea bass-derived NKLP-treated fish showed lower signs than control- or NKLP27-treated ones upon NNV challenge, though this was not completely related to the protection. For example, NKLP27-treated fish displayed more severe disease signs (2–4 rank) than those treated with the sea bass-derived NKLPs, but the protection was higher than in fish treated with the NKLP20.1. However, our results showed no descent viral load in the brain of NKLPs-treated fish compared with controls, pointing to a major immunomodulatory role of these peptides upon NNV infection. In the only available study, NKLP27-treated sole specimens resulted in decreased viral load upon megalocytivirus challenge though the disease signs and protection were not evaluated (Zhang et al., 2014). Previous *in vitro* study also showed that both NKLP23 and NKLP27 showed the same direct anti-NNV activity, which was lower than the antiviral activity of NKLP20.1 and NKLP20.2 (León et al., 2020). All these data point to the complexity and uncertainty of the mechanisms of NKLP response (León et al., 2020). For example, despite that several amino acids such as lysin are related to a direct antiviral activity (Butorov, 2015), the difference in the composition of NKLPs used in this work is mainly based in leucine and alanine residues (Supplementary Table S1). In fact, NKLP20.1 and NKLP20.2 are richer in alanine than NKLP23 or NKLP27. Alanine-rich peptides have shown to possess a potent antiviral activity in mammals or even potentiate it (Bogen et al., 2005; Migliolo et al., 2012); however, deeper studies should be needed to clarify this issue. Thus, NKLP23 and NKLP20.2 show the highest RPS value and were the only producing significant up-regulation of the immune-related genes related to B, T and NK lymphocytes and macrophages. Very interestingly, the transcription of the *cxcl9* chemokine is significantly up-regulated by NNV challenge though the *cxcr3* was increased but not significant. *Cxcl9* is known to be expressed by virus-infected and tumour cells and recruits *cxcr3*-expressing cells (mainly NK and CD8 cells) (Hosking and Lane, 2010; Kohli et al., 2021). Therefore, our data suggest that NKLP23 and NKLP20.2 induce the proliferation of cytotoxic cells in the head-kidney and their recruitment to the infection site, the brain, where the *cxcl9* is greatly up-regulated upon NNV challenge. Unfortunately, no differences in challenged fish with respect to the NKLPs are evidenced. Curiously, NKLP27 failed to produce sea bass immunomodulation whilst it greatly up-regulated the transcription of important immune-genes in sole (Zhang et al., 2014). This implies that fish immunostimulation, or at least the genes evaluated herein, is not strictly necessary to control the NNV infection and suggests a multifactorial mechanism. In fact, sea bass treated with the NKLPs and then challenged with NNV failed to increase the transcription of immune-related genes compared to NNV-challenged fish. This is reasonable since it has been already mentioned that NNV infection induces the sea bass immunity, but this is not enough to clear the virus.

5. Conclusions

To conclude, synthetic NKLPs corresponding to different NK-lysin regions, diverse amino acid composition and structure, produce little immunomodulation of European sea bass involving markers of B and T lymphocytes and macrophages as well as inflammatory cytokines and chemokines. Furthermore, pre-treatment of European sea bass with synthetic NKLPs resulted in decreased disease signs and mortality upon NNV challenge. Although the mechanisms by which these peptides are

able to decrease VER progression and mortality are still unknown, our results point to the use of NKLPs as potential preventive agents against NNV in aquaculture.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2021.105104.

Author's contributions

YV and CG-F performed the infections and analysed the data. FG and CC designed and synthetized the peptides. YV wrote the manuscript draft. AC revised the manuscript. AC and RL conceived the study and obtained the funds. All the authors have read and approved the submitted version of the manuscript.

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