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Short communication

In vitro immunomodulatory activities of peptides derived from *Salmo salar* NK-lysin and cathelicidin in fish cells

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ABSTRACT

Antimicrobial peptides (AMPs) are amphipathic peptides, which play an important role in innate defence. These peptides are gene-encoded and either constitutively expressed and/or upregulated during an infection. NK-lysins are AMPs with a three-dimensional globular structure. They are larger molecules, which comprise 74–78 amino acid residues and six conserved cysteine residues forming three disulphide bonds. Cathelicidins are a family of antimicrobial peptides that act as important components of the innate immune system with a broad spectrum of antimicrobial activity and immunomodulatory properties. Although they are widely studied in mammals, little is known about their immunomodulatory function. In the present study, we identified and characterized for the first time four NK-lysin-like transcripts from Atlantic salmon (*Salmo salar*) based on EST reported sequences. *In vitro*, NK-lysin derived peptides were able to induce the expression of IL-1β and IL-8 in *Salmo salar* head kidney leukocytes. We also tested *Salmo salar* cathelicidin 1 derived peptide in a similar assay, showing its ability to induce the expression of IFN-γ. These results indicate that NK-lysin and cathelicidin 1 derived peptides are able to modulated immune response, suggesting their potential use to enhance immune response in fish.

1. Introduction

Aquaculture has become an important economic sector worldwide, but is faced with an ongoing threat from infectious diseases from a wide range of bacteria, virus, parasites and fungi, that produce a significant impact on the quality and volume of the fish produced throughout the world [1]. Thus, the knowledge of the structure and function of fish immune system lead to more efficient intervention through specific therapeutic agents and vaccines. This strategy is essential for promoting the aquaculture as an economic activity and for reducing the over use of chemicals [2].

Currently, it has been shown that most live animals produce a broad spectrum of antimicrobial agents that act as true natural antibiotics. These are low molecular weight, cationic and amphipathic peptides synthesized by the ribosomal machinery [3,4]. In the last years, hundreds of antimicrobials peptides (AMPs) have been isolated from a wide variety of plants, invertebrates, amphibians, fish and mammals, as well as bacteria and fungi, with a key role in the defenses of multicellular organisms [5]. Many of these peptides show strong antibacterial, antifungal and antiviral effects, as well as immunomodulatory and antitumor properties through different mechanisms. AMPs also have other functions with an impact on the quality, efficacy and direction of immune responses connecting innate and adaptive immunity [6]. The immunomodulatory activity of cationic AMPs is complex and includes anti-infective immune modulation, such as the induction of chemokines and cytokines, pro/anti-inflammatory activity, direct chemotaxis,

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wound healing, angiogenesis, apoptotic and adjuvant activities [7–9].

The mature AMPs, upon contact with cellular membranes, fold into a variety of structurally diverse groups including: α-helical, β-sheet, cysteine rich and rare amino acid specific peptides. In addition, of these four classes of known AMPs, a fifth class of AMPs has been identified which includes the NK-lysins. These peptides have a three-dimensional globular structure, 74–78 amino acid residues and contain six cysteine residues forming three disulfide bonds. NK-lysins are a type of granulysin that was first isolated from pig small intestine [10]. It is present in $CD8^+$, $CD2^+$, and $CD4^+$ cells, and is produced by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells following over stimulation with interleukin (IL)-2. NK-lysins exhibit potent antibacterial activity and it is also lytic against a number of different types of tumor cells, but is not lytic for erythrocytes [10].

In teleosts, NK-lysins were found in *Ictalurus punctatus* [11], *Paralichthys olivaceus* [12], *Cynoglossus semilaevis* [13], *Takifugu rubripes*, *Salmo salar*, *Larimichthys crocea* [14], *Cyprinus carpio* [15], *Oreochromis niloticus* [16] and *Danio rerio* [17]. Despite NK-lysins have been identified in teleosts, the studies on their immunomodulatory and antibacterial activities are still insufficient. Previous studies demonstrated that AMPs play important roles in innate immune responses of salmonids, but no related work studied NK-lysin. Besides, several reports have shown that synthetic peptides derived from the NK-lysin sequences of chicken, fish and mammals possess antimicrobial activity against bacteria or parasites and immunomodulatory activities [12,18–27].

On the other hand, cathelicidins have been described in several salmonid fish [28–31]. The members of the cathelicidin family have a highly divergent antimicrobial C-terminal region and a conserved N-terminal cathelin-like pro-region which allows their classification [32]. Sequence analyses revealed the existence of 2 different cathelicidins in most salmonidae, includind *Salmo salar* [31]. Differently from their well-studied mammalian counterparts, little is known about the cathelicidin functions in fish. It is know that the synthetic C-terminal domain of rainbow trout rtCATH-1 and rtCATH-2 displayed antibacterial activity against Gram-positive or Gram-negative bacteria and low cytotoxic effects on host cells [28,33]. Additionally, purified cod-CATH showed antifungal activity [34] and some synthetic CATH-derived peptides increased phagocytic uptake by bacterial-stimulated head kidney lymphocytes and intracellular killing of live *E. coli* [35]. Besides, Atlantic salmon cathelicidin 1 and 2 (asCATH1 and asCATH2) stimulated the transcription of the chemokine interleukin-8 in peripheral blood leukocytes (PBL) [36].

In the present study we identified four NK-lysin-like transcripts from Atlantic salmon (*Salmo salar*) based on EST reported sequences and demonstrated that some of peptides derived from *S. salar* NK-lysin were able to induce the expression of IL-1β and IL-8 in head kidney leukocytes (HKL) cells. Besides, we showed that a peptide derived from *S. salar* cathelicidin-1 induced the expression of IFN-γ in HKL cells.

2. Materials and methods

2.1. Database searching for NK-lysin ESTs

Salmo salar EST databases in GenBank (http://www.ncbi.nlm.nih.gov/) were searched for putative NK-lysin sequences using *Salmo salar* predicted NK-lysin sequence (GenBank accession nos.: NM_001141110.1). Homologous EST sequences were retrieved and translated into protein sequences, which were then used as bait sequences to analyze the databases again. Default algorithm parameters were used, but in some cases the expected threshold was changed to 50, in order to score sequences with low homology. In this way, additional three putative NK-lysin ESTs for *Salmo salar* (GenBank accession nos.: EG932844.1; GenBank accession nos.: EG810337.1; GenBank accession nos.: EG819316.1) were obtained. Multiple alignments were performed using the protein sequences translated from the EST sequences and the fish NK-lysin published.

2.2. Sequence analysis

Sequence analysis was done using BLASTX (http://www.ncbi.nlm.nih.gov/blast/blastx) or CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw). Phylogenetic tree of NK-lysin of *Salmo salar* and other species was constructed using the Neighbor-joining (N-J) method in the MEGA 7.0 software, with 10000 bootstrap replicates. Tertiary structure prediction was performed using the I-Tasser server (http://zhang.bioinformatics.ku.edu/I-TASSER/about.html) [37–39]. The mature peptide for all NK-lysin like peptide genes was predicted using SignalP v3.0 (http://www.cbs.dtu.dk/services/SignalP/) and CLUSTALW. The molecular weights of each peptide were calculated using the Compute pI/Mw from ExPASy (http://web.expasy.org/compute_pi/).

2.3. Peptides synthesis

The peptides derived from *Salmo salar* NK-lysin, NK1 (TLKQKLLSVCDKVGFLKSMCKGLMKKH) and NK2 (EIKQKLLSYCGKLPLVKSTCEDLVKKH), and the derived peptide from *Salmo salar* Cathelicidin-1 (RRSQARKCSRGNGGKIGSIRCRGGGTRL) (referred in the literature as asCATH-1 [28]; GenBank accession number BG935125) were chemically synthesized by GenScript Company (https://www.genscript.com/). The peptides were purified by high-performance liquid chromatography to 90% of purity. Lyophilized peptides were stored at −20 °C and dissolved in PBS (pH 7.5) before use.

2.4. Isolation of head-kidney leukocytes

HKLs were isolated from *S. salar* following the method previously described [40]. Briefly, specimens were sacrificed by overexposure to benzocaine (20%) and the head kidney was removed aseptically and homogenized through a 70 μm nylon mesh using Leibovitz medium (L-15, Gibco, USA) supplemented with penicillin (100 IU/ml, Gibco, USA), streptomycin (100 μg/ml), heparin (2%) and 2% fetal bovine serum (FBS, Hyclone, USA). The resulting cell suspension was placed onto Percoll gradients with a density of 51%/34%, and then was centrifuged at 800 g for 40 min at 15 °C. The fraction corresponding to the leukocytes was collected and washed twice, and centrifuged at 800 g for 5 min at 15 °C in L-15 medium supplemented with 10% FBS. Viable cell concentration was determined by Trypan blue exclusion method, and the cells were resuspended in L-15 medium supplemented with 10% FBS. Cells were seeded into 24-well culture plates at a concentration of 10^6 cells/well.

2.5. Cytokine induction assay by qRT-PCR

S. salar head kidney leukocytes were resuspended in L-15 medium with 10% FBS and cultured at 18 °C in the absence or presence of 50 μM of the SsNK-lysin-1 and SsNK-lysin-2 derived peptides (NK1 and NK2, respectively) or *Salmo salar* Cathelicidin-1 derived peptide (CAT). The dose of peptides is not cytotoxic for these cells (data not showed). Four hours after stimulation, cells were harvested and total RNA was extracted using TRIzol Reagent (Invitrogen, USA), as the manufacturer's instructions. The concentration and purity of the RNA were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). Real-time PCR was performed using Brilliant II SYBR® Green QRT-PCR Master Mix, 1-Step (Agilet, USA) and AriaMx Real-Time PCR System (Agilet, USA). The results were analyzed as $2^{-\Delta\Delta CT}$ relative quantification. The comparative threshold cycles values were normalized for EF-1 mRNA. The primers are listed in Table 1.

2.6. Statistical analysis

Statistical analysis was carried out using GRAPHPAD PRISM version 6.00 (GraphPad Software, San Diego, CA).

Table 1
Primer sequences.

Gene	Accession number	Primer	Nucleotide sequence (5 → 3′)
IFN-α	NM001123570	Forward	TGGGAGGAGATATCACAAAGC
		Reverse	TCCCAGGTGACAGATTTCAT
IL-1β	AY617117	Forward	GCTGGAGAGTGCTGTGGAAGA
		Reverse	TGCTTCCCTCCTGCTCGTAG
IL-8	HM162835.1	Forward	ATTGAGACAGAAAGCAGACG
		Reverse	CGCTGACATCCAGACAAATCT
IFN-γ	FJ263446.1	Forward	CCGTACACCGATTGAGGACT
		Reverse	GCGGCATTACTCCATCCTAA
EF1-α	AF321836	Forward	GTGACACCGAAACTAAGCGAC
		Reverse	TGTAGATCAGATGGCCGGTG

3. Results

3.1. Sequences analysis

In this study, four putative novel NK-lysin-like peptides from *Salmo salar* were identified based on EST database. The four transcripts identified were named SsNK-lysin-1 (GenBank accession nos.: NM_001141110.1), Ss-NK-lysin-2 (GenBank accession nos.: EG932844.1), SsNK-lysin-3 (GenBank accession nos.: EG810337.1) and SsNK-lysin-4 (GenBank accession nos.: EG819316.1), The open reading frames (ORF) and domains of NK-lysin-like peptides from *Salmo salar* were deduced from an amino acid sequences alignment with related reported sequences using the CLUSTALW function and by prediction of conserved domains (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

The SsNK-lysin 1 ORF consisted of 384 bp and encoded a protein of 127 amino acid residues. The theoretical molecular mass of the mature SsNK-lysin 1 is 312041.04 Da. The SsNK-lysin-2 ORF consisted of 390 bp and encoded a protein of 129 amino acid residues. The theoretical molecular mass of the mature SsNK-lysin 2 is 12228.38 Da. The SsNK-lysin-3 ORF consisted of 390 bp and encoded a protein of 129 amino acid residues. The theoretical molecular mass of the mature SsNK-lysin 3 is 12208.42 Da. The SsNK-lysin-4 ORF consisted of 411 bp and encoded a protein of 136 amino acid residues. The theoretical molecular mass of the mature SsNK-lysin 4 is 12992.35 Da. The first 22 amino acids in each sequence were identified as the signal peptide. Sequence analysis showed that all SsNK-lysins contain a surfactant-associated protein B (saposin B) domain that is located at aa 44–117 in SsNK-lysin 1, aa 47–119 in SsNK-lysin-2, aa 47–119 for SsNK-lysin-3 and aa 54–116 for SsNK-lysin-4 (Fig. 1A).

3.2. Homology and phylogenetic analysis

Multiple alignments revealed six cysteine residues in the four SsNK-lysins that are highly conserved among compared species (Fig. 2). Nevertheless, the four SsNK-lysins contain at least an additional cysteine residue conserved among them and SsNK-lysin-1 has eight cysteine residues.

Using I-Tasser, the putative 3D structures of the four peptides were modelled. TM-scores of the first model of each peptide were 0.56 ± 0.15 for SsNK-lysin-1, 0.44 ± 0.14 for SsNK-lysin-2, 0.55 ± 0.15 for SsNK-lysin-3 and 0.53 ± 0.15 for SsNK-lysin-4 indicating a model of correct topology. For the four peptides models, the C-score was approximately in the range of −5 to 2 (−1.25, −2.3, −1.37 and −1.5, respectively), with an estimated RMSD of 6.6 ± 4.0 Å for SsNK-lysin-1, 9.5 ± 4.6 Å for SsNK-lysin-2, 6.9 ± 4.1 Å for SsNK-lysin-3 and 7.4 ± 4.2 Å for SsNK-lysin-4. The hypothetical 3D-structures of these peptides revealed the characteristic four/five-helical-bundle structure observed in the family of saposin-like proteins (Fig. 1B).

The percent of identity of SsNK-lysin peptides with other species ranged from 15% to 53%. The observed phylogenetic relationship implies that SsNK-Lysin peptides were grouped with other fish NK-lysins and separate from those of their avian and mammalian counterparts (Fig. 3). Concerning to *Salmo salar* Nk-lysins, SsNK-lysin-2 and SsNK-lysin-3 grouped together. Alignment of the four protein SsNK-lysins sequences revealed percent identities between 68% and 89% and the SsNK-lysin-2 and SsNK-lysin 3 have the highest percent identity with 89.15%.

3.3. Designing and sequences analysis of synthetic Salmo salar NK-lysin and cathelicidin derived peptides

The length of the NK-lysin proteins makes they difficult for chemical synthesis or biosynthesis. Therefore, short peptides derived from NK-lysin with immunomodulatory activities have been studied. Two small peptides, NK1 and NK2, derived from SsNK-lysin-1 and SsNK-lysin-2 were synthesized based on sequences alignment between NK-lysin sequences identified in *Salmo salar* and NKLP27, a peptide of 27 amino acids derived from *Cynoglossus semilaevis* NK-lysin. NK1 and NK2 are composed of 27 residues that form the H2 and H3 α-helices of the SapB domain of SsNK-lysin-1 and SsNK-lysin-2, respectively (Fig. 1B). Sequence alignment showed that NK1 and NK2 shares 55.6% identities between them and 40.7% and 33.3% identities with NKLP27, respectively. On the other hand, a 28 amino acids peptide derived from as-CATH-1 (CAT) was synthesized.

3.4. Effects of NK-lysin and cathelicidin derived peptides on cytokines expression in head kidney leucocytes

After peptide synthesis, they were evaluated *in vitro* to assess whether these shorter peptides modulate the immune response in the *Salmo salar* HKL. To this aim, the induction of IL-1β, IL-8, IFN-γ and IFN-α were analyzed. As results, NK-lysin derived peptides (NK1 and NK2) induced the expression of IL-1β and IL-8 after 4 h of stimulation with 50 μM of the peptides ($p < 0.001$), while cathelicidin derived peptide induced the expression of IFN-γ ($p < 0.001$) (Fig. 4). None of the peptides significantly stimulated IFN-α expression after 4 h of stimulation.

4. Discussion

In this study, four NK-lysin-coding transcripts were identified in Atlantic salmon. These transcripts have a signal peptide, indicating that they are secretory protein. The conserved Saposin domain was also detected in the four NK-lysins, as well as the six conserved cysteine residues. In addition, the four SsNK-lysins contain additional cysteine residues conserved among them, similar to what happened in mammals and specifically SsNK-lysin-1 has eight cysteine residues. The same number and the positioning of cysteine residues in the saposin family proteins strongly suggests a common structural organization among these proteins and also indicates that a favorable tertiary structure combining amphipathic helices with a disulfide-bond compact folding is conserved. It has been hypothesized that the conserved arrangement of disulfide-linked amphipathic helices provides a common structural framework which support a wide range of biological functions [41].

It's important to mention that *Salmo salar*, as in *Danio rerio* [17], *Ictalurus punctatus* [11] and *Cyprinus carpio* [15], presented more than one copy of NK-lysin. However, other teleost and higher vertebrates seem to possess only one Nk-lysin/granulysin [12,13]. This diversity could indicate a specialization of the different proteins into different functions. The multiple sequences alignment and phylogenetic analyses suggest that the proteins reported herein belong to the NK-lysin family.

Peptides are remarkable biomolecules with a large diversity of important functions *in vivo*. They are small molecules (2–50 amino acids) relatively easier to obtain in comparison with larger protein molecules; therefore, they are attractive agents and targets for the development of therapies and diagnostics. However, as the size of the peptide increases,

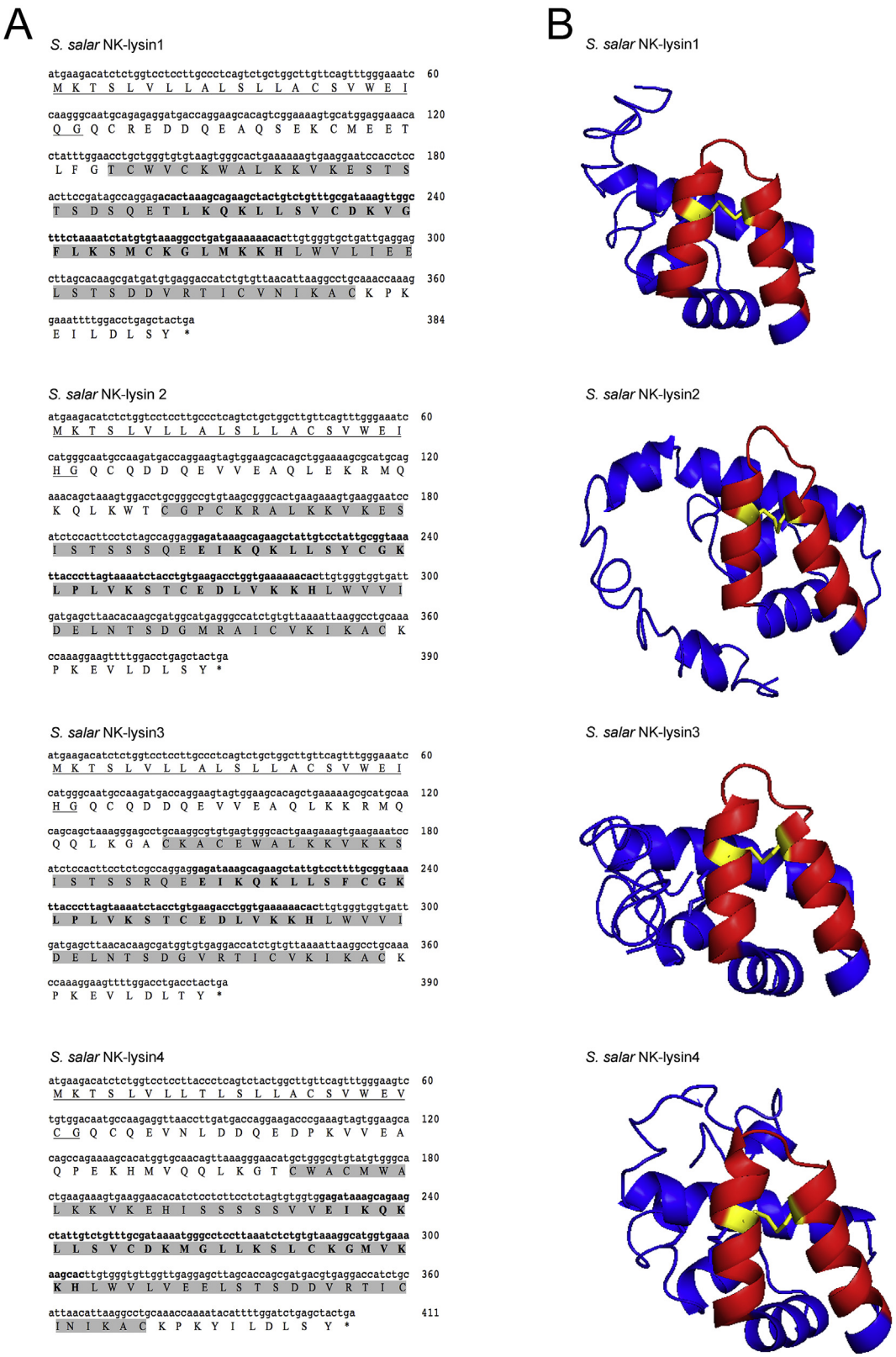

Fig. 1. (A) Nucleotide and amino acid sequence of *Salmo salar* Nk-lysins open reading frames. The predicted signal peptides are underlined, whereas the SapB domains are shaded in the amino acid sequences. (B) Predicted tertiary structure of *Salmo salar* Nk-lysins using I-TASSER server.

it becomes more expensive to obtain, mainly by chemical synthesis. Therefore, it is important to establish a minimum size required for these molecules to retain their biological activity.

Previously an *in vivo* study showed that administration of tongue sole with NKLP27 before bacterial and viral infections significantly reduced pathogen dissemination and replication in tissues. That study also revealed that fish which received NKLP27 exhibited upregulated expression of the immune genes including those that are known to be

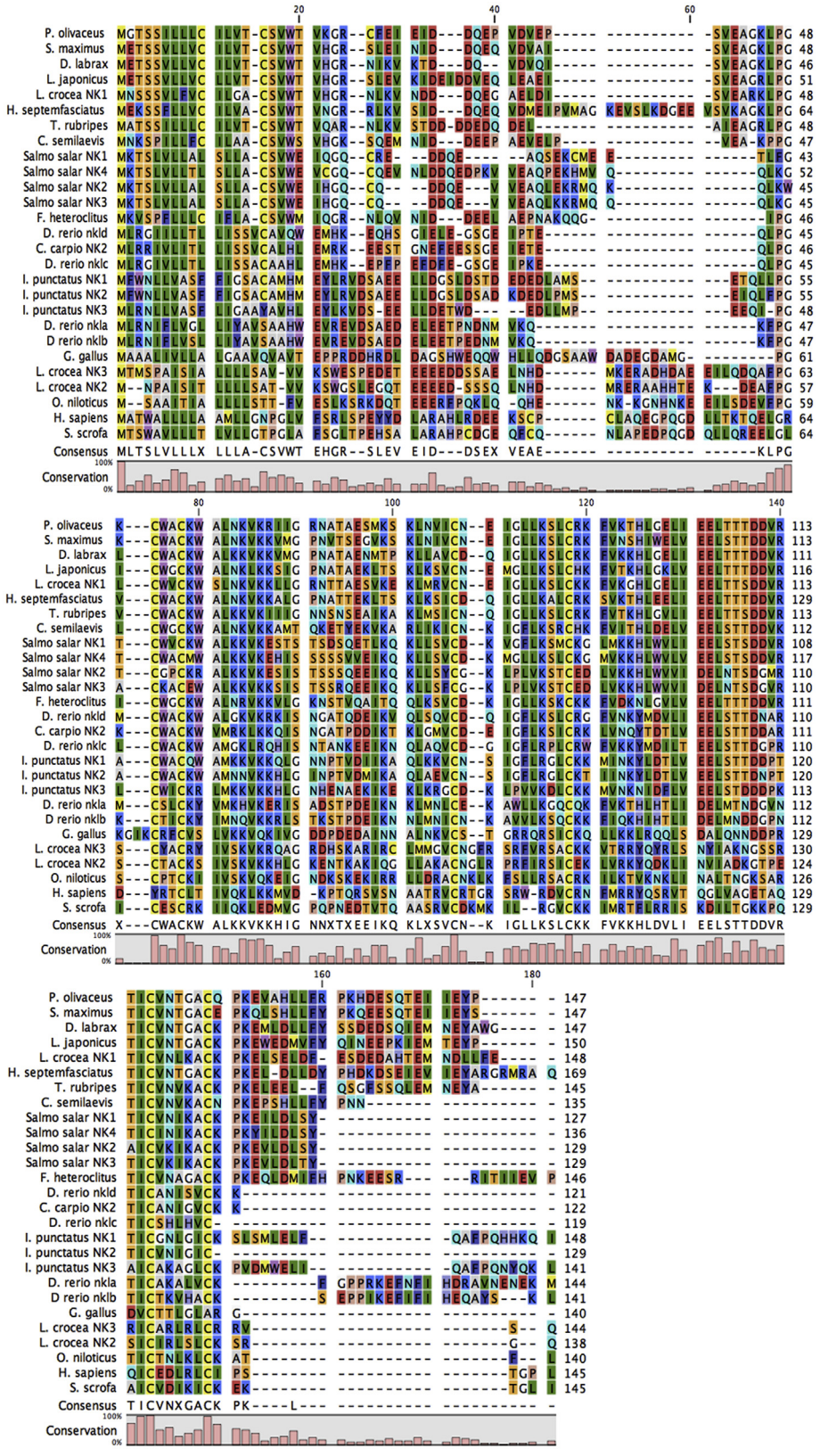

Fig. 2. Multiple amino acid alignment comparing the *Salmo salar* Nk-lysins with other vertebrates. Bars represent the conservation percentage between the amino acids sequences. NCBI GenBank accession numbers of sequences used are listed in Supplementary Data Table S1.

involved in antibacterial and antiviral defense [18]. In the present work we designed two small peptides, NK1 and NK2, derived from SsNK-lysin-1 and SsNK-lysin-2 based on sequences alignment between NK-lysin sequences identified in *Salmo salar* and NKLP27. NK1 and NK2 are composed of 27 residues that form the H2 and H3 α-helices of the SapB domain of SsNK-lysin-1 and SsNK-lysin-2, respectively. On the other hand, several truncated variants of mammal cathelicidins have been evaluated, and it have been demonstrated that they display

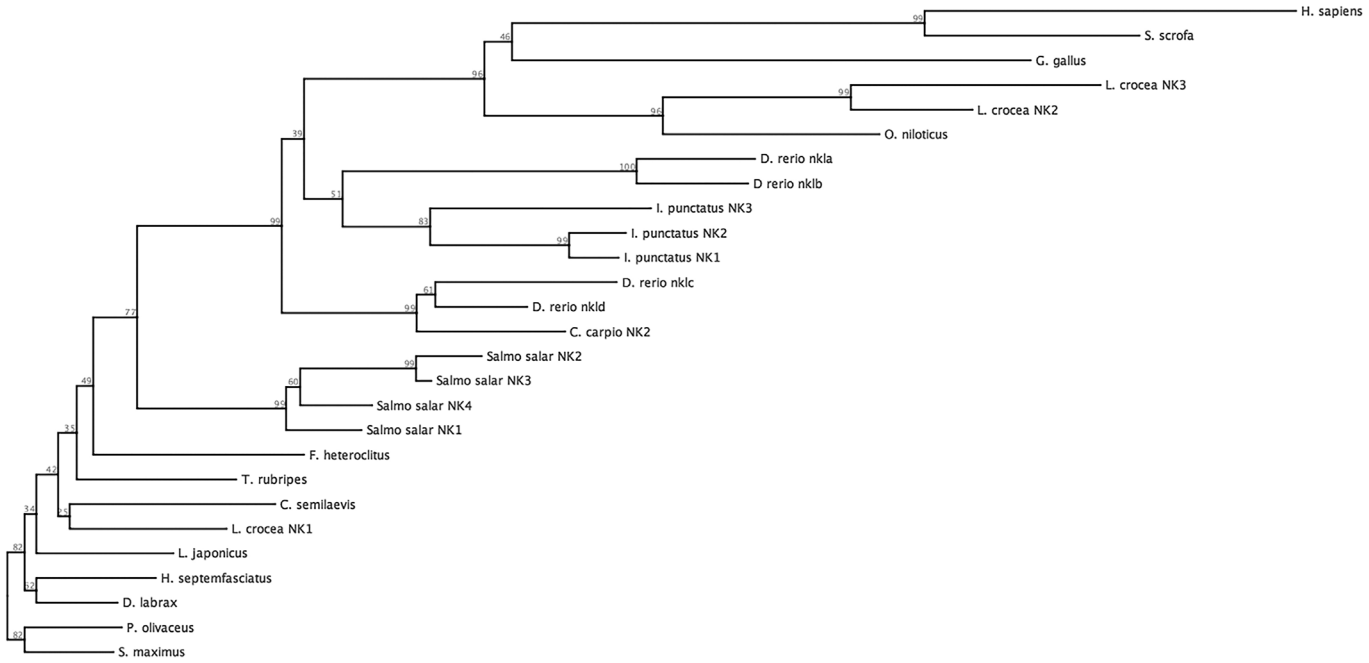

Fig. 3. Phylogenetic relationship between the *Salmo salar* Nk-lysins with other vertebrates. Scale bar indicates that this tree was constructed by the Neighbor-Joining method and bootstrap analysis. The number at each node indicates the percentage of bootstrapping after 1000 replications. NCBI GenBank accession numbers of sequences used are listed in Supplementary Data Table S1.

antimicrobial and immunomodulatory activities [42–48]. Based on these results we also designed a shorter 28 amino acids peptide derived from *Salmo salar* Cathelicidin-1.

Some mammalian studies provide evidence that some AMPs do not simply kill microbes but indirectly confer protection by modulating the immune system [49–51]. Based on these studies we evaluated the expression of IL-1β, IL-8, IFN-α and IFN-γ induced by NK1, NK2 and CAT in *Salmo salar* head kidney leucocytes. We demonstrated that both NK1

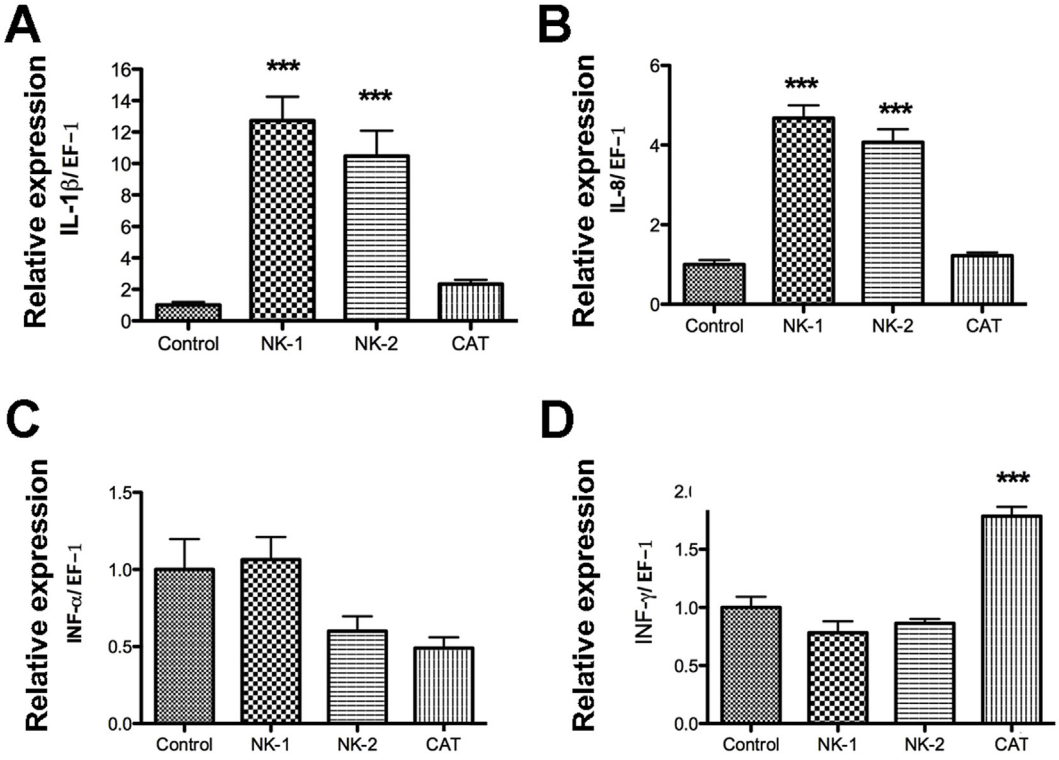

Fig. 4. Relative expression of IL-1β (A), IL-8 (B), IFN-α (C) and IFN-γ (D) induced by *Salmo salar* NK-lysin derived peptides (NK-1 and NK-2) and *Salmo salar* cathelicidin derived peptide in head kidney leucocytes. Cells were stimulated with 50 μM of the SsNK-lysin-1 and SsNK-lysin-2 derived peptides (NK1 and NK2, respectively) or *Salmo salar* Cathelicidin-1 derived peptide (CAT) for 4 h. Expression levels were analyzed by Real-time PCR. The expression of the mRNA was analyzed as $2^{-\Delta\Delta CT}$ relative quantification. The comparative threshold cycles values were normalized for EF-1. Data were expressed the means ± S.D. of three independent experiments, each analyzed in triplicate, and analyzed by ANOVA followed by Dunnett's multiple comparisons test (***$p < 0.001$).

and NK2 induced expression of the chemokine IL-8 and IL-1β but not IFN-α and IFN-γ after 4 h of administration using 50 μM of peptide. The specific stimulation of these pro-inflammatory cytokines supports the argument that NK-lysin derived peptides might act as a signal for the recruitment of immune cells to the infection site and promote the inflammation in order to resolve the infection. Accordingly, a previous study showed that fish which received NKLP27 exhibited upregulated expression of the IL-8 and IL-1β [18]. On the contrary, CAT induced the expression of the IFN-γ after 4 h of stimulation with the dose assayed but not IL-8, IL-1β and IFN-α gene expression. Cathelicidins have been associated with the ability to stimulate IL-8 expression [36,52–55] in humans and Atlantic salmon. The central region of the cathelicidins mature peptide is characterized by five tandem repeats based on the (R/P)(P/L)GGGS motif, and it is flanked by 16 residues at the N-terminal and C-terminal, respectively [56]. Recently, trout cathelicidins (CAMPs) and their truncated variants were evaluated by their capacity to stimulate IL-8 in PBLs *in vitro*. The authors demonstrated that trout CAMPs variants exhibited different abilities to stimulate IL-8 expression in PBLs but this ability decreased with the gradual deletion of the repetitive motif in the central region [56]. The peptide derived from as-CATH-1 evaluated in the present work lacks one repetitive motif in the central region and this could be the possible cause that this peptide does not induce the expression of IL-8 in *Salmo salar* HKLs. Further developmental work will require the assessment of different doses and time points for each peptide and also the study of the peptide effect over different immune cell populations.

The IFNs have important roles in both innate and adaptive immune responses. Specifically, IFN-γ enhances antigen presentation [57–60], the phagocytic and nitric oxide responses of phagocytes [57,61–63]. Fish IFN-γ induces gene expression of many ISGs that also respond to type I IFNs, suggesting cross-activation of the innate antiviral responses elicited by type I and II IFNs. Furthermore, IFN-γ enhances host surveillance against viruses and some bacteria by up-regulation of pattern recognition receptors such as TLR9 [64] and also modulates cytokine and chemokine expression [61,62]. To our knowledge, this is the first report demonstrating upregulation of IFN-γ expression induced by a peptide derived from cathelicidin.

In summary, we identified four NK-lysin-like transcripts from Atlantic salmon (*Salmo salar*) based on EST reported sequence. Afterward, two NK-lysin derived peptides and a 28 aa peptide derived from *Salmo salar* cathelicidin-1 were synthesized and characterized. As a result, these peptides showed immunomodulatory activities in *Salmo salar* head kidney leucocytes. Rational design and functional screening of additional NK-lysin and Cathelicidin derived peptides will allow the identification of analogs with improved safety and therapeutic/prophylactic potential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.03.034.

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