Developmental and Comparative Immunology 117 (2021) 103988

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

Full Length Article

Full recombinant flagellin B from *Vibrio anguillarum* (rFLA) and its recombinant D1 domain (rND1) promote a pro-inflammatory state and improve vaccination against *P. salmonis* in Atlantic salmon (*S. salar*)

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ARTICLE INFO

Keywords:
Adjuvant
Flagellin
ND1 domain
Cytokines
Salmo salar

ABSTRACT

Flagellin is the major component of the flagellum, and a ligand for Toll-like receptor 5. As reported, recombinant flagellin (rFLA) from *Vibrio anguillarum* and its D1 domain (rND1) are able to promote *in vitro* an upregulation of pro-inflammatory genes in gilthead seabream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*) macrophages. This study evaluated the *in vitro* and *in vivo* stimulatory/adjuvant effect for rFLA and rND1 during *P. salmonis* vaccination in Atlantic salmon (*Salmo salar*). We demonstrated that rFLA and rND1 are molecules able to generate an acute upregulation of pro-inflammatory cytokines (IL-1β, IL-8, IL-12β), allowing the expression of genes associated with T-cell activation (IL-2, CD4, CD8β), and differentiation (IFNγ, IL-4/13, T-bet, Eomes, GATA3), in a differential manner, tissue/time dependent way. Altogether, our results suggest that rFLA and rND1 are valid candidates to be used as an immuno-stimulant or adjuvants with existing vaccines in farmed salmon.

1. Introduction

Vaccination is one of the most effective prophylactic strategies against several pathogens in vertebrates, and it is used by the industry to control pathogens in species with commercial value. In aquaculture, vaccines are important tools for the prevention and control of fish diseases; they are routinely used and have been recommended to reduce the use of antibiotics (Adams, 2019; Ma et al., 2019). It was reported that vaccines are not usually able to confer protection for a sufficiently long time, and the use of adjuvants or immunostimulants in combination would be necessary to increase the duration of protection of the vaccine (Tafalla et al., 2013).

Flagellin is a pathogen associated molecular pattern (PAMP), which is recognized by the pattern recognition receptor (PRRs) called toll-like receptor 5 (TLR5) (Jiao et al., 2010). The recognition of PAMPs by TLRs allows the innate immune system to distinguish between the target molecules present in different classes of pathogens and coordinate the immune response, triggering the differential induction of cytokines, co-stimulatory molecules, and the repertoire of TLRs expressed in different cells depending on the type of PAMP and the types of cells stimulated (Kumar et al., 2009). Flagellin is the main protein component of the flagellum in Gram-positive and gram-negative bacteria and is one of the most powerful PAMPs, being able to activate a broad range of cell types within the innate and adaptative immune system to promote cytokine production (Hynes et al., 2011; Tafalla et al., 2013; Lu et al., 2016; Jiang et al., 2017).

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https://doi.org/10.1016/j.dci.2020.103988
Received 8 September 2020; Received in revised form 20 December 2020; Accepted 20 December 2020
Available online 23 December 2020
0145-305X/

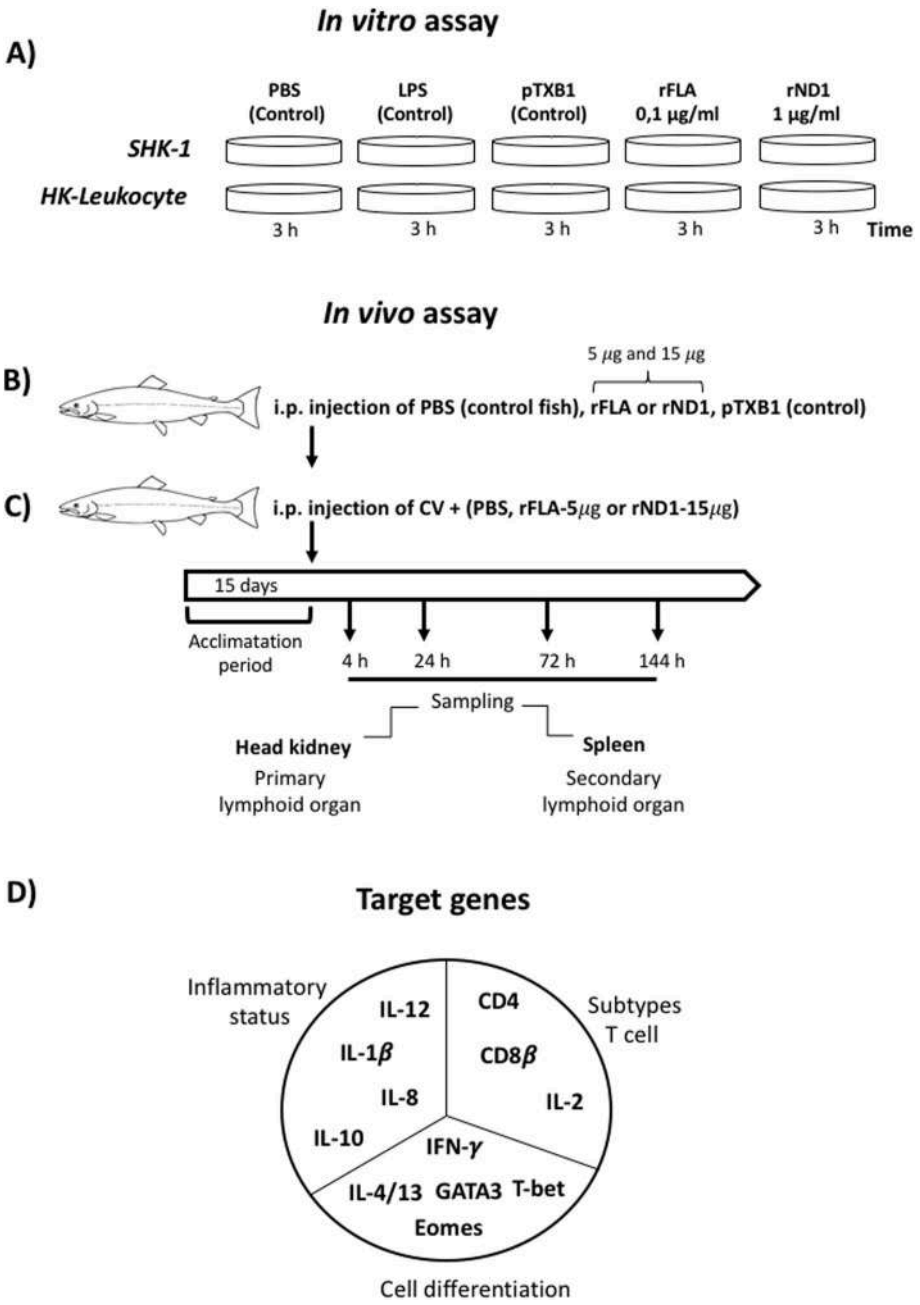

Fig. 1. Experimental design for an *in vitro* and *in vivo* study of flagellin's recombinants. Evaluation of *in vitro* biological activity of rFLA and rND1 in SHK-1 cell and HK-leukocyte (A). Evaluation of effective doses for rFLA and rND1 (B). Determination of an adjuvant effect of rFLA or rND1 in combination of commercial CV (C). Target genes evaluated for determination of inflammatory status, subtypes T cell and cell differentiation (D). More details are provided in the Material and Methods section.

Several studies have evaluated recombinant flagellin from various bacteria strains in different fishes. *In vitro* and *in vivo* assays have suggested a possible use for this protein as an immunostimulant or vaccine adjuvant (Jia et al., 2013; González-Stegmaier et al., 2015; Wangkahart et al., 2016, 2019; Jiang et al., 2017; Liu et al., 2017).

In previous *in vitro* studies in head kidney macrophages of gilthead seabream and rainbow trout, we have demonstrated the immunomodulatory role of two recombinant proteins of *Vibrio anguillarum*, a full length flagellin B (rFLA) and the amino-terminus of the D1 domain (rND1) of the same protein, the region mainly responsible for binding TLR5 and for the immunostimulatory activity of flagellin (Gonzalez--Stegmaier et al., 2015).

The present study aims to demonstrate the functionality of both recombinant proteins to promote the activation of proinflammatory pathways to induce a more effective immune response against pathogens after vaccination. We demonstrated that rFLA and rND1 separately, or each one combined with a commercial vaccine, can induce at least two functional pathways to promote a quick acute inflammatory state and a long-term immune differentiation profile in leucocytes from head kidney and spleen from *S. salar*. We observed that both molecules may improve the efficiency of a commercial vaccine against *P. salmonis* through a differential up regulation of critical transcripts for an innate and adaptive immune response, promoting an increased IgM production.

2. Materials and methods

2.1. Fish

Atlantic salmon (*S. salar*) were provided and maintained by the Salmon Clinical Trials Laboratory of Universidad Austral de Chile, Valdivia, Chile. A total of four hundred fish of 60 ± 20 g in weight were used in this study, which were distributed and kept in shaded tanks with 15 g L^{-1} salinity, equipped with a recirculation unit at 9-12 °C and were fed daily with a commercial diet. Following an adaptation period, one set of the untreated fish were used for *in vitro* assays and another set was divided for all the *in vivo* assays. Before any experiment, two animals per group were randomly tested by PCR and examined by histology to ensure that they were enzootic pathogen-free. The PCR panel included piscine reovirus (PRv) (Palacios et al., 2010), infectious pancreatic necrosis virus (IPNv) (Skjesol et al., 2011), infectious salmon anaemia virus (ISAv) (Snow et al., 2006), *Renibacterium salmoninarum* (Suzuki and Sakai, 2007) and *Piscirickettsia salmonis* (Karatas et al., 2008). All fish with external signs of disease or skin lesions were discarded and not considered for experimentation. All experiments complied with the requirements of the Chilean National Commission of Scientific and Technological Research (CONICYT) and the Bioethical Committee of Universidad Austral de Chile for the use of laboratory animals (certificate number 279/2017 and 47/2017).

2.2. Expression and purification of recombinant proteins

The recombinant rFLA and rND1 proteins were generated and tested as previously reported (González-Stegmaier et al., 2015). Briefly, *E. coli* ER2566 competent cells harboring pTXB1-flagellin constructs (rFLA or rND1) were grown in an LB medium supplemented with 100 μg μL^{-1} ampicillin at 37 °C to an DO_{600} of 0.5 and recombinant proteins expression was induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 18 °C for 4 h in the case of rFLA and at 37 °C for 4 h in the case of rND1. The recombinant proteins were purified by affinity chromatography on a chitin resin (New England BioLabs, S6651S) following the manufacturer's recommendations and their purity was finally assessed by SDS-PAGE and Coomassie Brilliant Blue staining. The presence of contaminating LPS was removed from the purified recombinants proteins utilizing a detoxi-Gel Endotoxin removing Gel (Invitrogen, 20339) following the manufacturer's recommendations. The residual LPS content was determined using Chromogenic LAL Endotoxin Quantitation Kit (Pierce, 88282) and it was estimated <0.3 EU mL^{-1} for each molecule.

2.3. Cell culture and treatments

The SHK-1 cell line was cultured without antibiotics in an L-15 medium, supplemented with 10% FCS at 20 °C in 75 cm^2 flasks (Costar, Fisher Scientific, Ottawa, ON, Canada). The cells were dispensed into 24-well plates at a concentration of 1 x 10^5 cells per well in L-15 medium with 10% FCS for 24 h at 20 °C (confluent 90%) before stimulation.

Atlantic salmon head kidney leukocytes were isolated following the method previously described by Secombes (1990). Briefly, fish were killed through an overdose of MS-222, and the anterior kidney was then removed aseptically and passed through a 100 mm nylon mesh using the Leibovitz Medium (L-15, Gibco) supplemented with penicillin (100 IU mL^{-1}), streptomycin (100 mg mL^{-1}), heparin (10 units mL^{-1}), and 2% fetal calf serum (FCS). The resulting cell suspension was placed onto Percoll gradients with a density of 34%-51% and then centrifuged at 500 *g* for 30 min at 4 °C. The interface cells were collected and washed twice at 500 *g* for 5 min in L-15 containing 0.1% FCS. The viable cell concentration was determined by Trypan blue exclusion. The cells were re-suspended in L-15 with 10% FCS and dispensed into 24-well plates at a concentration of 5 x 10^5 cells mL^{-1}, macrophage monolayers were obtained after overnight culturing at 18 °C.

SHK-1 cells and head kidney leukocytes were exposed for 3 h to 0.1 μg mL^{-1} rFLA, 1 μg mL^{-1} rND1, 10 μg mL^{-1} LPS from *E. coli* 055:B5 (Sigma Aldrich), respectively in L-15 medium with 2% FCS (Fig. 1A). After treatment, total RNA was extracted from cells. The used dose for each molecule was previously assessed in isolated head kidney leukocytes (HKL) from gilthead seabream (*S. aurata* L., Sparidae) and in rainbow trout (*O. mykiss*, Salmonidae) (González-Stegmaier et al., 2015).

To exclude non-specific effects by potential contaminants from *E. coli*, total proteins extracted from *E. coli* transformed with the empty pTXB1 vector were fractionated by affinity chromatography as previously described. The elute fraction (inner control, pTXB1) was used to stimulate isolated cells and evaluate the ability to induce the overexpression of proinflammatory genes.

2.4. In vivo treatments

Six independent groups of 30 fishes per tank were used to assess two doses for each molecule. Each fish was intraperitoneally injected with 100 μL of phosphate buffer saline (PBS) alone or containing 5 μg or 15 μg of rFLA or rND1 per fish (Fig. 1B). Additionally, another group was injected with 100 μL of elute fraction from affinity chromatography as a control of endotoxins from bacteria debris (pTXB1). This elute fraction corresponds to the total proteins extracted from transformed *E. coli* with the empty pTXB1 vector. The required amount of each recombinant for an *in vivo* assay was estimated between 1 μg and 100 μg according to previous published reports (Montero et al., 2014; Hynes et al., 2011; Jiang et al., 2017).

For testing the adjuvant effect, three groups of 30 individuals per tank were used to assess the effectivity of each molecule in combination with a commercial vaccine (CV) (provisional record SAG 1868, Centrovet). This vaccine is an injectable emulsion against Salmon Rickettisal Syndrome (SRS). Fishes were intraperitoneally injected with 100 μL of CV alone or in combination with 5 μg of rFLA or 15 μg of rND1 (Fig. 1C). Phosphate buffer saline (PBS) was the vehicle; 100 μL were used as a procedure control.

For both assays, three animals from each group were anaesthetized and euthanized using MS-222 (Sigma) at 4, 24, 72- and 144-h post-injection. Head kidney and spleen samples were collected for total RNA extraction and RT-qPCR analyses (Fig. 1B and C). 30 days post treatment blood samples were collected from artery caudal using a 1 mL sterile syringe and placed in centrifuge tubes at room temperature for 2 h. Then serum was collected by centrifugation (3000 rpm, 4 °C, 10 min) and stored at −80 °C until use.

2.5. RNA extraction and cDNA synthesis

All cell cultures (SHK-1 cells, HK leukocytes) and tissues (head kidney and spleen) were individually placed in microtubes with 1 mL of TRIzol and ceramic beads and homogenized in the Precellys Evolution Homogenizer (Bertin Instruments, France) at room temperature. Then, 200 μL of chloroform was added, and the sample was vigorously mixed and allowed to stand for 2 min before centrifugation at 4 °C for 15 min at maximum speed. Supernatant was transferred to a new tube and mixed with 400 μL of 70% ethanol. This mixture was passed through the columns provided by the E.Z.N.A. Tissue RNA Kit (Omega Bio-Tek Inc., USA) according to the manufacturer's instructions. RNA concentrations and purity were measured using a spectrophotometer (NanoDrop, ND-1000) and stored at −80 °C.

For differential gene expression assays 0.5 μg of total RNA was used for cDNA synthesis, which was performed using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara Bio, USA) according to the manufacturer's instructions. A negative control was included using total RNA from a sample pool subjected to DNAse treatment but without reverse transcriptase to check for genomic DNA contamination.

Table 1
Primer and sequences used for gene expression analysis by quantitative real time PCR.

Primer and sequences used for gene expression analysis by quantitative real time PCR in *Salmo salar*				
gene	name		Sequence (5′- 3′)	Accession No.
elf1α	Elongation factor 1 alpha	F	CCCCTCCAGGACGTTTACAAA	NM_001123629.1
		R	CACACGGCCCACAGGTACA	
il1β	Interleukin 1 beta	F	CAAGCTGCCTCAGGGTCT	NM_001123582.1
		R	CGGCACCCTTTAACCTCTCC	
il8	Interleukin 8	F	GCAACAGCGGTCAGGAGATT	NM_001140710.2
		R	TGGAATGATTCCCCTTCTTCA	
il2	Interleukin 2	F	CATGTCCAGATTCAGTCTTCTATACACC	AM422779
		R	GAAGTGTCCGTTGTGCTGTTCTC	
il4/13	Interleukin 4/13	F	ACCACCACAAAGTGCAAGGAGTTCT	FN820501
		R	CACCTGGTCTTGGCTCTTCACAAC	
il10	Interleukin 10	F	CGCTATGGACAGCATCCT	EF165029
		R	AAGTGGTTGTTCTGCGTT	
il12β	Interleukin-12 subunit beta	F	CTGAATGAGGTGGACTGGTATG	BT049114
		R	ATCGTCCTGTTCCTCCG	
infγ	Interferon gamma	F	CTAAAGAAGGACAACCGCAG	AY795563
		R	CACCGTTAGAGGGAGAAATG	
cd4	T-Cell Surface Glycoprotein CD4	F	GAGTACACCTGCGCTGTGGAAT	NM_001124539
		R	GGTTGACCTCCTGACCTACAAAGG	
cd8β	T-cell surface glycoprotein CD8 beta chain	F	CGCACACACCTCAACAACTC	AY693394
		R	ATTGATGCGCAGTGTGAAAG	
eomes	T-box family transcription factor eomesodermin	F	ACCTCTCGTCGTCAGATACTG	EU418014
		R	GGACCGGTGAGTCTTTTCTTC	
Tbet	T-box family transcription factor T-bet	F	GGTAACATGCCAGGGAACAGGA	FM863825
		R	TGGTCTATTTTTAGCTGGGTGATGTCTG	
gata3	Trans-Acting T-Cell-Specific Transcription Factor GATA-3	F	CCCAAGCGACGACTGTCT	EU418015
		R	TCGTTTGACAGTTTGCACATGATG	

2.6. Analysis of gene expression

Gene expression analyses were performed on the SHK-1 cell, HK leukocytes and tissues (head kidney and spleen) from treated *S. Salar* using specific primers described in Table 1 and Fig. 1D. The qPCR amplification efficiency for each primer pair was determined using 1:5 serial cDNA dilutions from head kidney samples. The amplification efficiency was calculated considering the value of the slope from each trend lines; cycle threshold (CT) results were plotted as a function of the $\log_{10}$ values for each dilution according to the equation $E = 10^{(1/slope)}$ (Bustin, 2002). The specificity of the qPCR products for each pair of primers was confirmed by melting curve analysis. Amplification efficiencies resulted between 92% and 105% which were suitable for gene expression analyses (data not shown).

Real-time PCR analyses were performed at the StepOnePlus instrument (Applied Biosystems) using the Brilliant II SYBR Green qPCR Master Mix kit (Agilent Technologies). Each amplification reaction was performed in a final volume of 15 μL consisting of 7.5 μL of buffer, 250 nM–750 nM primers (depending on the gene), 300 nM ROX (50 nM) and 2 μL of cDNA diluted 1:10. The PCR program consisted of a 10-min activation and denaturation step at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at the annealing temperature of the corresponding primers and an additional 15 s extension at 72 °C. The melting curve was analyzed to examine the specificity of the reaction in each well and to verify the absence of primer dimers and non-specific amplification products. The samples were heated from 60 °C to 90 °C in increments of 0.3 °C with a dwell time of 5 s at each temperature for continuous monitoring of fluorescence.

Three biological replicates were used, and each qPCR was performed in duplicate. The reactions included a negative control without reverse transcriptase to check for genomic DNA contamination and a negative control without a template to check for the presence of primer dimers. All genes were quantified from the same batch of cDNA. CT values obtained using StepOne version 2.3 software (Applied Biosystems) were transformed into relative expression units using comparative Ct method ($2^{-\Delta\Delta Ct}$, where Ct is a cycle threshold (Pfaffl et al., 2002). Elongation factor 1 alpha (*elf1α*) was selected as housekeeping gene for normalization. Fold change was defined based on a control group (3 animals each time) collected in parallel with each experimental point.

2.7. Indirect ELISA

An indirect ELISA test was developed based on total proteins extract from *P. salmonis* for the semi quantification of IgM levels in serum. 96-well microtiter plates (Nunc Immunoplate MaxiSorp, 439454) were coated with a whole protein extract obtained from freshly cultured *P. salmonis* (Oliver et al., 2016), 100 μL per well of 2 μg mL^{-1} protein solution and incubated overnight at 4 °C. After *P. salmonis* coating, the plates were washed three times with 0.05% Tween-20, pH 7 (PBS-T) and incubated with 100 μL of 5% skimmed milk in PBS (blocking solution) for 2 h at room temperature. After washing, wells were loaded with 100 μL of 1/20 diluted serum from each treated fish and incubated at 18 °C for 2 h. The plates were washed (three times each) and incubated with 100 μL of a 1:1000 diluted monoclonal antibody anti-IgM of Salmon (Ango, FM-190AZ-5) for 1 h at room temperature. The anti-IgM antibody was washed and the anti-mouse IgG horseradish peroxidase (Thermo) was added at a 1:2000 dilution by 1 h at room temperature. Finally, the plates were washed and 3,3′,5,5′-tetramethyl-benzidine substrate (TMB) was added and incubated for 15 min. Absorbance was measured at 450 nm with a VERSA max microplate reader.

2.8. Statistical analysis

All data were statistically analyzed using the nonparametric Mann-Whitney Test to determine differences between cells and/or fish treated with the recombinant proteins and the untreated control. Two-way ANOVA and Tukey's multiple comparisons tests were performed for statistical analysis between various variables. The critical value for statistical significance was established as $p \leq 0.05$. Values marked with asterisks mean the following: *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$. All statistical analyses were carried out using the GraphPad Prism 8 program. The scale for each graph was adjusted to log10.

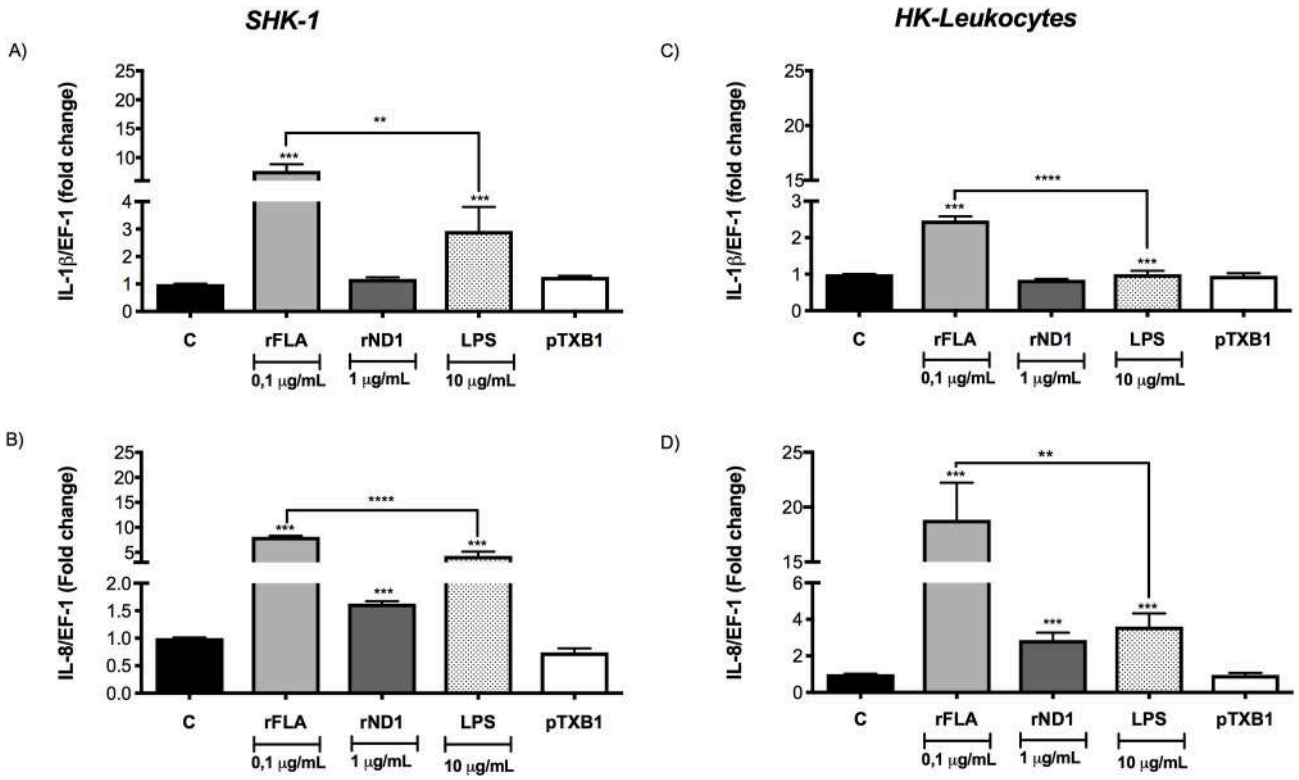

Fig. 2. IL-1β and IL-8 expression in SHK-1 and HK-Leukocytes. IL-1β (A, C) and IL-8 (B, D) overexpression induced after 3 h stimulation with rFLA and rND1 in SHK-1 cell line and HK-Leukocytes. Fold change values were normalized to the expression of ELF1α mRNA and the results are represented as the increase of mRNA for each gene compared to the unstimulated control. C: control without stimulation; rFLA: recombinant *V. anguillarum* flagellin; rND1: recombinant amino-end of the D1 domain of *V. anguillarum* flagellin; LPS: lipopolysaccharides from *E. coli* 055:B5 (Sigma Aldrich); pTXB1: expression vector without the insert, used as inner control. All results are presented as the mean ± SEM of triplicate samples. Statistical differences from the Mann-Whitney test are shown.

3. Results

The present study aimed to demonstrate the functionality of a recombinant flagellin and a small recombinant peptide from one of its domain to promote the activation of proinflammatory pathways to induce a more effective immune response against pathogens after vaccination (Fig. 1).

3.1. IL-1β and IL-8 transcripts are highly upregulated by rFLA in leukocytes of Salmo salar

The optimal concentrations of rFLA (0.1 μg mL^{-1}) and its peptide rND1 (1 μg mL^{-1}) necessary to induce the expression of cytokines were determined previously in isolated head kidney leukocytes (HKL) from gilthead seabream (*S. aurata* L., Sparidae) and in rainbow trout (*O. mykiss*, Salmonidae) (González-Stegmaier et al., 2015). In this study, we demonstrated that SHK-1 cells and head kidney leukocytes from *Salmo salar* were able to induce the expression of IL-1β or IL-8 after 3 h stimulation with 0.1 μg mL^{-1} rFLA, increasing both by 7 and 8-fold, respectively, compared to the unstimulated control (Fig. 2A). SHK-1 cells stimulated with the peptide rND1 showed a rise of IL-8 by 1.6-fold compared to the unstimulated control (Fig. 2B). In head kidney leukocytes IL-1β and IL-8 transcripts increased 2.4 and 18.8-fold, respectively, using rFLA compared to the unstimulated control, and only 2.8-fold for IL-8 when rND1 was used (Fig. 2C and D). In both cell types (SHK-1 and HKL), rFLA effects were more powerful than rND1, which can be explained by the complexity of the whole protein compared with the small recombinant peptide. Furthermore, rFLA showed a much greater stimulatory activity compared with LPS alone, which was used as a positive control to demonstrate the ability of induction of IL-1β and IL-8 transcripts in the cells. A significantly higher *in vitro* upregulation of IL-1β and IL-8 was observed using 100-fold less amount of rFLA (0.1 μg mL^{-1}) than LPS (10 μg mL^{-1}) at the same time of incubation (Fig. 2), which may be explained by the differential intracellular signaling and ligand-receptor affinities. Further studies are necessary to understand that phenomenon. Additionally, a basal upregulation induced by the pTXB1 control was detected for IL-1β and IL-8 in SHK1 and HKL, which may be explained by the presence of traces of bacterial debris, no proteins were observed by SDS-PAGE analysis (data not shown). However, this slight increase induced by the empty vector was not statistically significant.

3.2. rFLA is more active than rND1 to promote an in vivo proinflammatory response in Salmo salar

For this purpose, 30 animals per group were intraperitoneally injected with 5 and 15 μg of rFLA or rND1, respectively. Tissue samples from head kidney and spleen were obtained 4 h (Fig. 3), 24 h, 72 h and 144 h (Supplementary Fig. S1) post-injection. The results showed that both rFLA and rND1 induced a time-dependent acute pro-inflammatory response (Fig. 3). An upregulation of 20-fold for IL-1β and around 10-fold for IL-8 after 4 h of stimulation with rFLA-5μg in head kidney and spleen when compared to PBS-control (Fig. 3). Expression of both genes decreased progressively after 24 h of stimulation in both tissues (Supplementary Fig. S1). Unexpectedly, a significant lower upregulation for both transcripts was detected in head kidney from animals treated with 15 μg rFLA (Fig. 3A and C) which was not observed in spleen samples (Fig. 3B and D). When rND1-15μg was applied, a 19-fold and 14-fold upregulation of IL-1β and IL-8 was observed in head kidney and this effect was significantly higher than rND1-5μg (Fig. 3A and C). In spleen, rND1 (5 or 15 μg) induced a significant increase of IL-1β and IL-8 (4–10-fold) compared to PBS control, however rND1-15μg effect was

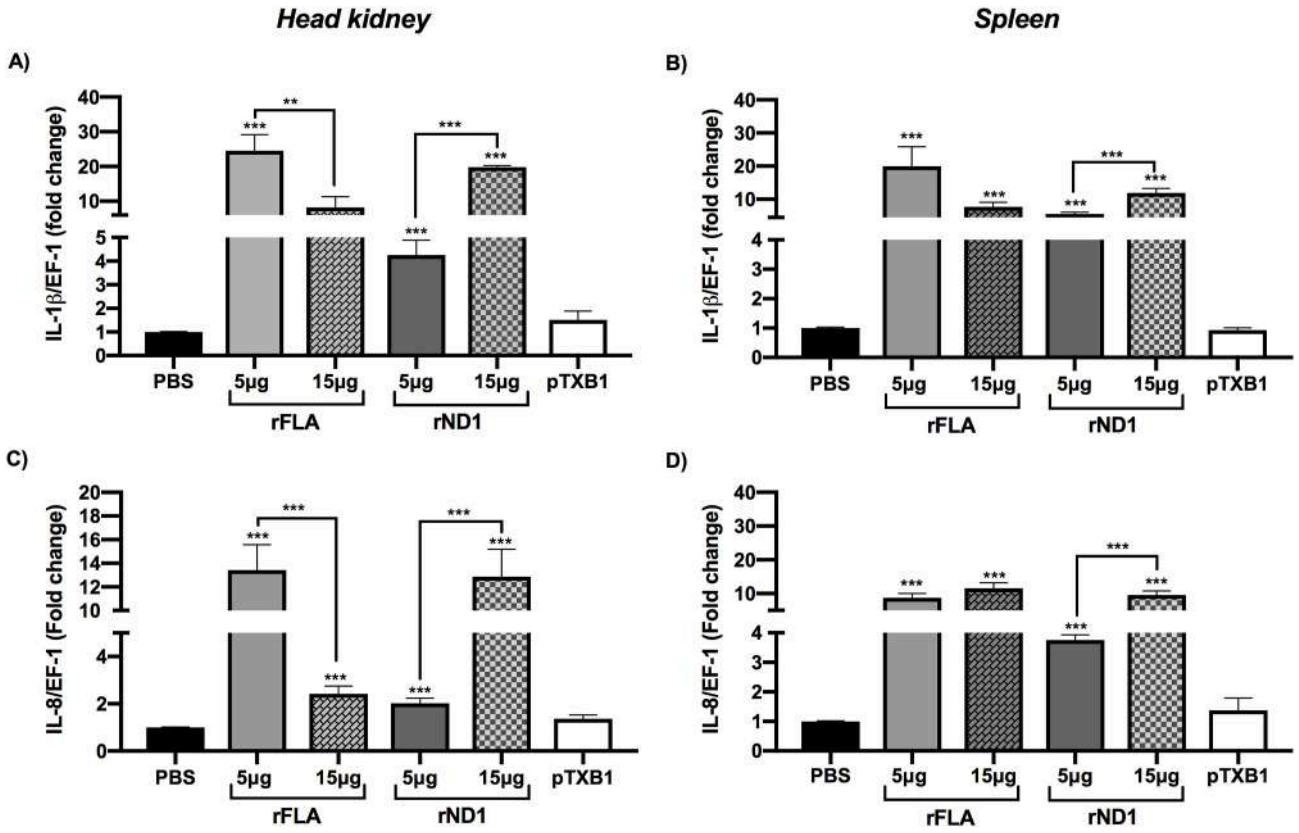

Fig. 3. Effective dose for flagellin's recombinants and *in vivo* cytokine expression in *Salmo salar* at 4 h post injection. Optimal dose analysis through IL-1β (A, B) and IL-8 (C, D) expression in head kidney and spleen of *S. salar* after i.p. injection with rFLA or rND1. Fold-change values were normalized to ELF1α mRNA expression, and the results are represented as mRNA increase for each gene, compared to the control (PBS). rFLA: recombinant *V. anguillarum* flagellin; rND1: recombinant amino-end of the D1 domain of *V. anguillarum* flagellin; pTXB1: expression vector without the insert used as inner control. PBS: vehicle control. All results are presented as the mean ± SEM of triplicate samples. Statistical differences from the Mann-Whitney test are shown.

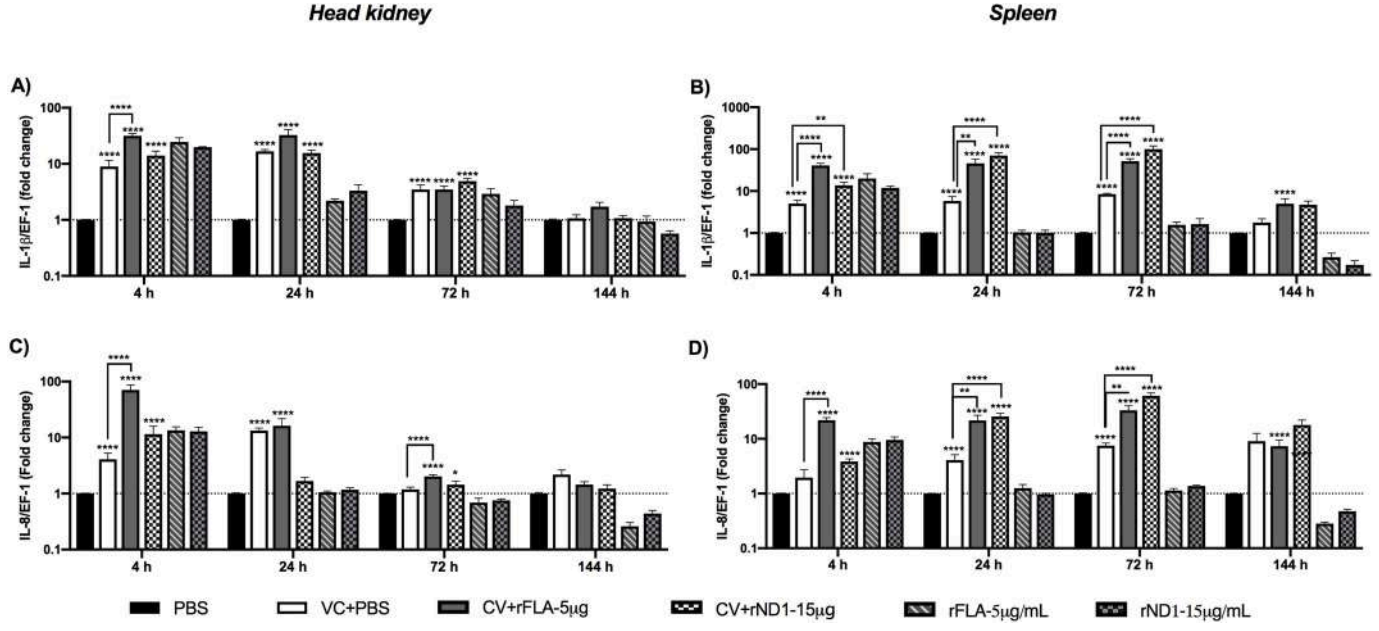

Fig. 4. Recombinant of flagellins increase the expression of IL-1β and IL-8 when incorporated to commercial vaccine (CV). IL-1β (A, B) and IL-8 (C, D) expression in head kidney and spleen of *S. salar* after i.p. injection with 100 μL of CV in combination with 100 μL of PBS alone (CV + PBS) or containing 5 μg or 15 μg of rFLA (CV + rFLA-15μg) or rND1 (CV + rND1-5 μg) per fish. Fold-change values were normalized to ELF1α mRNA expression, and the results are represented as mRNA increase for each gene, compared to the control (PBS). CV: Commercial vaccine; rFLA: recombinant *V. anguillarum* flagellin; rND1: recombinant amino-end of the D1 domain of *V. anguillarum* flagellin; PBS: vehicle control. All results are presented as the mean ± SEM of triplicate samples. Statistical differences from Two-way ANOVA and Tukey's multiple comparisons test are shown.

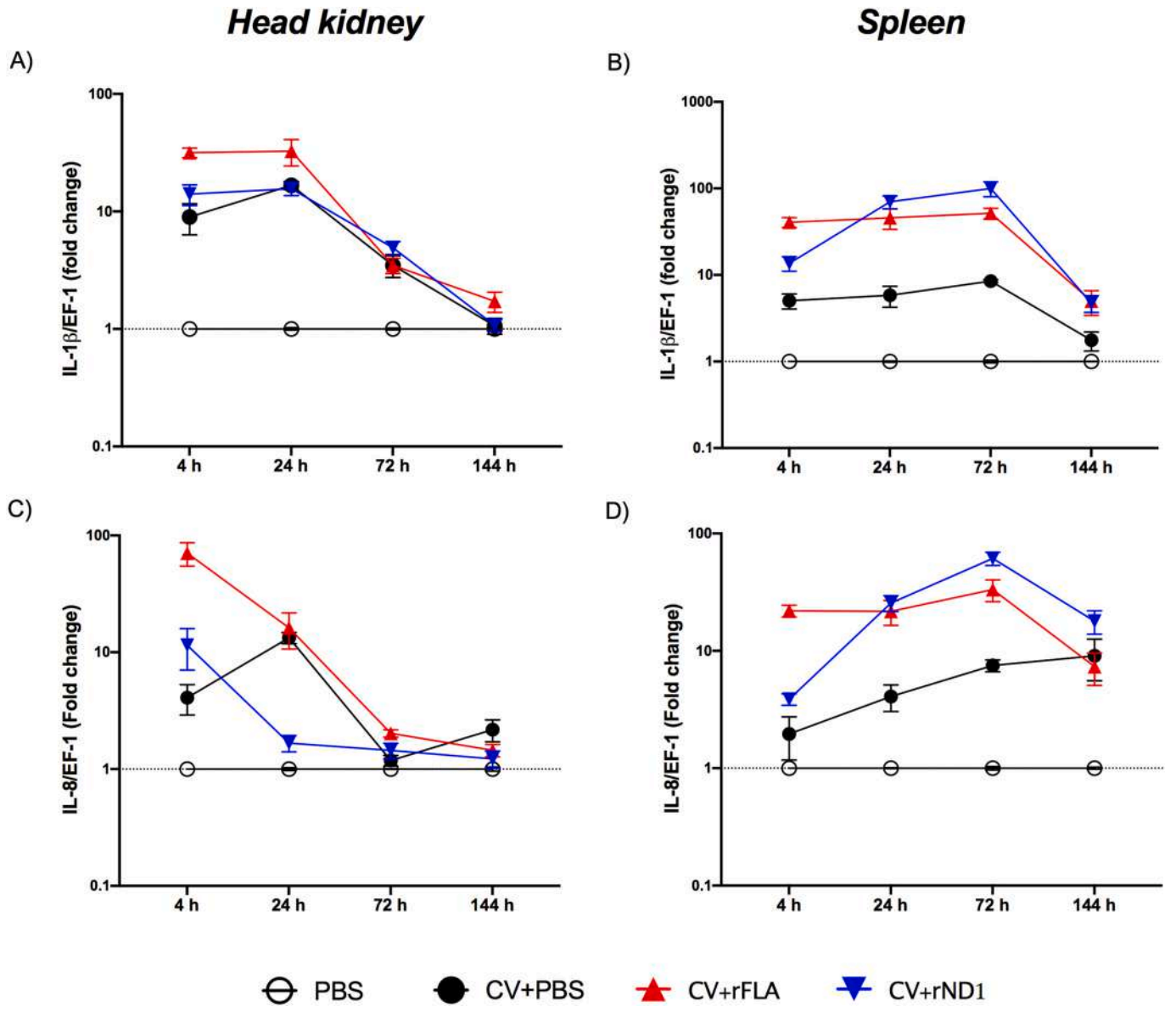

Fig. 5. Timecourse of vaccination-induced IL-1β and IL-8 after using rFLA and rND1. IL-1β (A, B) and IL-8 (C, D) expression in head kidney and spleen of *S. salar* after i.p. injection with 100 μL of CV in combination with 100 μL of PBS alone (CV + PBS) or containing 5 μg of rFLA (CV + rFLA-5μg) or 15 μg of rND1 (CV + rND1-15μg) per fish. Fold-change values were normalized to ELF1α mRNA expression, and the results are represented as mRNA increase for each gene, compared to the control (PBS). CV: Commercial vaccine; rFLA: recombinant *V. anguillarum* flagellin; rND1: recombinant amino-end of the D1 domain of *V. anguillarum* flagellin; PBS: vehicle control. All results are presented as the mean ± SEM of triplicate samples.

statistically higher (Fig. 3B and D).

3.3. rFLA or rND1 in combination with an anti-P. salmonis vaccine promotes a strong pro-inflammatory signature in a tissue dependent manner

30 animals were grouped by treatment, and each fish was intraperitoneally injected with 200 μL of an inoculum composed by: 1) commercial vaccine (CV) and vehicle phosphate buffer saline (CV + PBS); 2) commercial vaccine (CV) combined with 5 μg rFLA (CV + rFLA), 3) CV plus 15 μg rND1 (CV + rND1), and 4) PBS alone. The amount of rFLA and rND1 was defined based on the results in Fig. 3. Samples of head kidney and spleen were obtained 4 h, 24 h, 72 h and 144 h post-injection. The results showed that when the fishes are i.p. injected with VC + PBS, there is an increase in the cytokines expression as expected. In addition, both rFLA and rND1 in combination with CV were able to induce a pro-inflammatory response (Figs. 4 and 5). rFLA in combination with the vaccine promoted in head kidney an acute upregulation after 4 h for both IL-1β and IL-8 transcripts by around 30-fold and 70-fold, respectively (Figs. 4 and 5, A, C). The CV + rFLA stimulatory effect was significantly higher than the vaccine alone or the combination CV + rND1. Even though the observed response decreased progressively after 24 h, the effect on IL-8 expression seemed to be sustained across the time when CV + rFLA was used (Figs. 4C and 5C). In spleen, the combination of the commercial vaccine with any of both molecules induced an acute response which was sustained at least for 72 h after injection (Figs. 4 and 5, B, D). The IL-1β upregulation induced by both combinations, rFLA or rND1, was significantly higher compared with both controls (PBS or CV + PBS), reaching 10-fold and 3-fold over the vaccine alone (CV + PBS), respectively (Figs. 4B and 5B). IL-1β levels slightly increased after 24 h and kept stable for 72 h.

The effect of both molecules on IL-8 gene expression in spleen was highly significant, overall 5 to 60-fold over the PBS control or compared with the vaccine alone (CV + PBS) at any tested time (Figs. 4D and 5D).

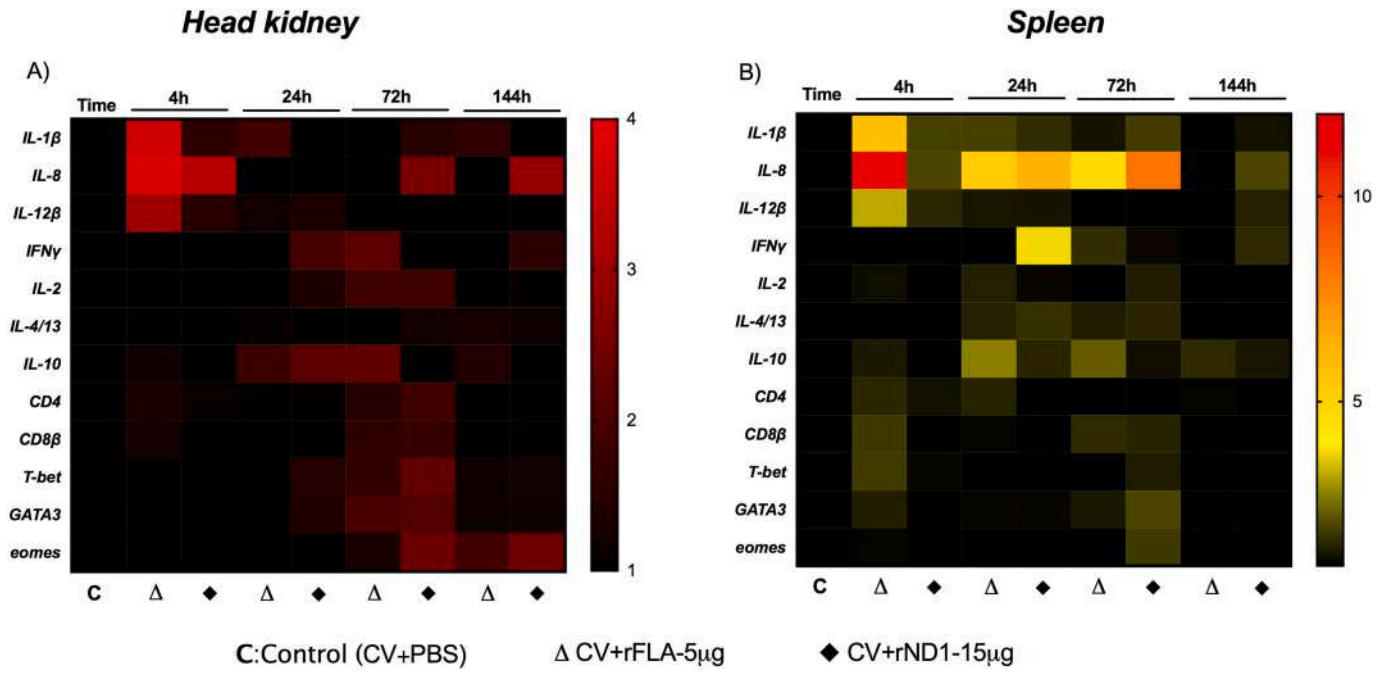

Fig. 6. A differential cytokine profile in spleen and head kidney is induced by rFLA or rND1 as adjuvant during vaccination against *P. salmonis*. The expression levels of transcripts involved in inflammation, cellular activation and differentiation in head kidney (A) and spleen (B) are shown in two color maps. The fishes were i.p. injected with 100 μL of CV (Commercial vaccine) in combination with 100 μL of PBS alone or containing 5 μg of rFLA (CV + rFLA-5μg) or 15 μg of rND1 (CV + rND1-15μg) per fish. The samples were extracted at 4 h, 24 h, 72 h and 144 h post injection. Fold-change values were normalized to ELF1α mRNA expression, and the results are represented as mRNA increase for each gene, compared to the control (CV + PBS). All results are presented as the mean ± SEM of triplicate samples. For more details check the Supplementary Figs. S2 and S3. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The combinations of CV + rFLA and CV + rND1 induced a dramatic increase of the specific messenger, around 5-fold for the vaccine alone (Figs. 4D and 5D). Both combinations promoted a high expression of IL-8 after 4 h, progressively increasing and remaining high for at least 72 h.

3.4. A differential cytokine profile in spleen and head kidney is induced by rFLA or rND1 as adjuvant during vaccination against P. salmonis

The immune signature induced by flagellin was evaluated through the detection of 10 transcripts related to inflammation, differentiation and subtype immune cells after vaccination (Fig. 1). The differentially regulated genes were graphically represented using a heat map for each molecule and tissue, and its p-values were indicated. Overall, the ten tested genes showed a broad range of increment as a very early (4 h) and a late response (72 h) to the cocktail vaccination (Fig. 6). Additionally, to the strong *in vivo* IL-1β and IL-8 upregulation induced by the combination of CV + rFLA in head kidney and spleen (Figs. 5 and 6), rapidly after 4 h IL-12β significantly increased its levels by 3-fold above the vaccine alone (CV + PBS) in head kidney (p-value 0.0001) and spleen (p-value 0.01). Both tissues have shown a differential response to the treatment. The most significant observed changes in head kidney tissue with CV + rFLA were the induction of IL-2, CD4 and CD8β mRNA levels at 72 h after treatment and the very late increase of EOMES transcript (p-value 0.0001) at 144 h of vaccination (Supplementary Fig. S2). On the other hand, in spleen a significant increase of CD4 (p-value 0.0001), CD8β (p-value 0.0001), and T-bet (p-value 0.0001) was observed 4 h post inoculation (Supplementary Fig. S2), then at 24 h IL-2 and CD4 slightly increased (p-value 0.01 and 0.0001 respectively) and later, 72 h after treatment IL-4/13 and IL-10 cytokines increased significantly (p-value 0.0001) without any relevant change after 144 h. The signature induced by CV + rND1 was prominently observed from the 72 h after treatment in both of the analyzed tissues (Supplementary Fig. S3). In head kidney the transcript levels for IL-2, CD4, CD8β, T-bet, GATA3 and EOMES were statistically upregulated at 72 h post injection (p-value 0.0001), and only EOMES kept increased after 144 h after. A similar pattern was observed in spleen only after 72 h with CV + rND1 with an increased level of IL-2, IL-4/13, CD8β, T-bet, GATA3 and EOMES (p-value 0.001).

3.5. IgM antibodies are induced by rFLA or rND1 as adjuvant during vaccination against P. salmonis

Preliminarily, a humoral immune response induced by rFLA or rND1 as adjuvant during vaccination against *P. salmonis* was indirectly assessed as the level of IgM after vaccination. By the 30th day post injection, an increase in serum IgM levels was detected in fishes injected with both vaccine cocktails, CV + rFLA or CV + rND1, compared to the group inoculated with the vaccine alone (CV + PBS) (Supplementary Fig. S4). Further studies on this matter are necessary to demonstrate the specificity of the flagellin-induced IgM.

4. Discussion

In this study we showed that a non-commercial recombinant flagellin B from *Vibrio anguillarum* (rFLA) and one recombinant amino-terminus peptide from its D1 domain (rND1) induced *in vitro* and *in vivo* an effective pro-inflammatory state able to promote a cellular and humoral immune response in Atlantic salmon (*S. salar*). The results *in vitro* are comparable to those obtained previously and reported by our group in gilthead seabream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*) macrophages (González-Stegmaier et al., 2015). Other authors also have showed a pivotal pro-inflammatory effect in teleost fish when other sources of flagellin have been used (Jia et al., 2013; Wangkahart et al., 2016, 2019; Jiang et al., 2017; Liu et al., 2017). Our work suggests that rFLA or rND1 have the ability to increase the inflammatory profile of a commercial vaccine against *P. salmonis* and it may improve its

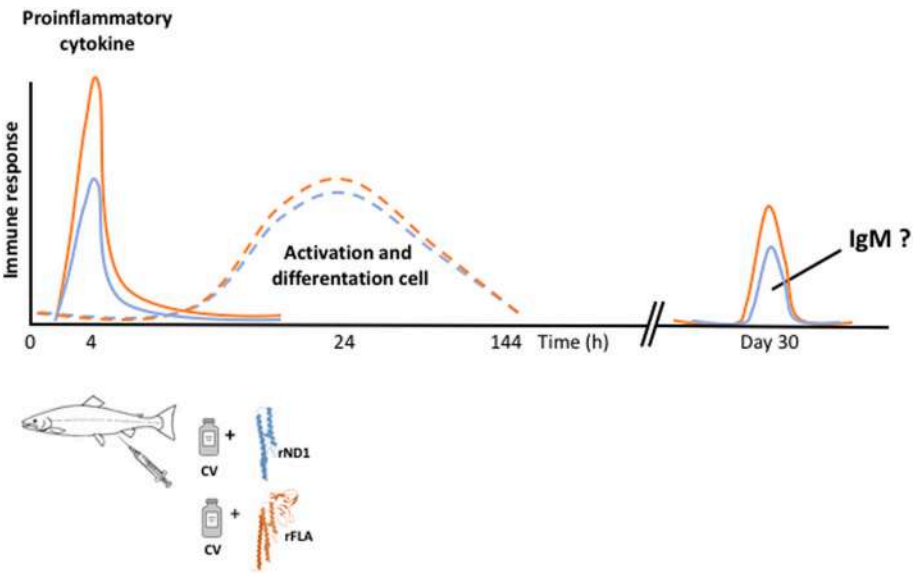

Fig. 7. Proposed model of action for rFLA and rND1 as an immunostimulant/adjuvant when incorporated into a commercial vaccine. The application of recombinant molecules (rFLA or rND1) in combination with a commercial vaccine (CV) increases an early proinflammatory response 4 h post injection in both head kidney and spleen, followed by a greater cell activation and differentiation 72 h post injection when compared to the commercial vaccine applied alone. An increase in immunoglobulins was observed on day 30 in serum of fishes injected with molecules in combination of commercial vaccine. These results suggest that these recombinant molecules could be used to increase the effect of vaccine against *P. salmonis* or other fish pathogens.

performance, at least under the established conditions of this research. 5 μg de rFLA and 15 μg de rND1 are necessary to induce a significant expression of IL-1β and IL-8. These results could be compared with those obtained by Hynes et al. (2011), which evaluated two doses (15 μg and 50 μg) of a flagellin FlaD (FDL) and a truncated form (FDS), suggesting that Atlantic salmon appear to be very sensitive to flagellin, and no significant differences were observed between the transcript levels detected for IL-1β, TNFα and IL-8 when a lower or a higher dose of FDL or FDS was used (Hynes et al., 2011). Studies realized by Wangkahart et al. (2019) in rainbow trout showed that 10 μg of a full-length recombinant flagellin (YRF) from Y. ruckeri induced a significant expression of multiple proinflammatory cytokines. The authors reported a rapid induction in the overexpression of IL-8 in the spleen, which was sustained up to 24 h and then gradually decreased; these findings are consistent with those obtained at 4 h with our rFLA or rND1. When rFLA or rND1 were incorporated to the commercial vaccine, the overexpression of all key pro-inflammatory cytokines was prolonged in the spleen up to 72 h with both recombinants (CV + rFLA or CV + rND1). Several studies in higher vertebrates have demonstrated the capacity of flagellin to act as an adjuvant to promote the production of cytokines for a wide range of innate cell types, to trigger the recruitment of B and T lymphocytes to the lymphoid organs, and to activate TLR5^{+}dendritic cells (Mizel and Bates, 2010). Moreover, flagellin genes from many bacteria share highly conserved regions in the extreme amino- and carboxyl-terminals of the D1 domain; a region which is responsible for the immunostimulatory activity of flagellin (Eaves-Pyles et al., 2001; Takeda et al., 2003) and which has been amply studied in fish (Jiao et al., 2009, 2010; Hynes et al., 2011; Jia et al., 2013; Jiang et al., 2017; Wangkahart et al., 2019).

However, the mechanisms of the long-term protection elicited by vaccines in fish remain poorly understood Yamaguchi et al. (2019). We have evaluated several genes to generate a basic cytokine profile able to explain rFLA and rND1 activity. The differentiation of Th cells towards a Th1 profile is controlled by the transcription factor T-bet (Seder et al., 2003). These cells secrete effector cytokines such as interferon γ (IFNγ) and tumor necrosis factor α (TNF-α) to control intracellular infections, and interleukin 2 (IL-2) to induce lymphocyte proliferation (as CD4 or CD8 positive T cells). GATA3 is the transcription factor that mediates the differentiation of Th cells towards a Th2 profile (Kanhere et al., 2012). Th2 cells produce IL-4, IL-5, and IL-13 that stimulate B cells and control extracellular infections through the secretion of antibodies. For instance, the transcription of GATA-3 and IL4/13, a homologue of the mammalian Th2 cytokine genes IL-4 and IL-13 were examined in several rainbow trout tissues, among which transcription levels were higher in thymus, skin and gills (Takizawa et al., 2011). To date, there is not enough evidence of the presence of Th1 cells in fish; however, the upregulation of IFNγ, IL-12, and T-bet transcripts have been reported in rainbow trout as an indirect evidence of a Th1-like response induced by an infection model or vaccination (Ashfaq et al., 2019). The correlation between T-bet expression and IFNγ secretion could indicate events associated with Th1 regulation in other fishes (Ashfaq et al., 2019).

Eomes has been described in fish as a relevant transcription factor during early development (Mione et al., 2001; Bruce et al., 2003, 2005; Bjornson et al., 2005) but its function or role in immunity has not been studied in detail yet. In the mammalian immune system, Eomes is highly expressed in CD8^{+} T cells and functions redundantly with T-bet in the induction of IFN-γ production to prevent CD8^{+} T cells from differentiating into other T cell subtypes, regulating the differentiation and maturation of CD8^{+} T-cells into effector cells (Pearce et al., 2003; Lupar et al., 2015; Lino et al., 2017).

Two T-box transcription factors, Eomes and T-bet, are important for the development of effector and memory CTL (Bruce et al., 2005; Intlekofer et al., 2005) as well as Th1 cells (Zhu et al., 2010).

5. Conclusions

The present study has demonstrated that rFLA and rND1 induce the expression of genes involved in an inflammatory response, quickly and for a short time, and that effects can occur when the molecules are used alone or in combination with a commercial vaccine against *P. salmonis* in *Salmo salar*. Our results suggest that these molecules could be used to improve existing vaccines against *P. salmonis* by adding those in its current formulation, or in the creation of new vaccines against other fish pathogens (Fig. 7).

Acknowledgements

This work was supported by CONICYT/FONDECYT Postdoctoral Grant 3170356 (RGS), FONDAP 15110027. We also thank Dr. Alex Romero for facilitating his laboratory to develop this research. We also thank Gregory Dunne for the English review assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2020.103988.

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