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# Immune effects observed after the injection of plasmids coding for rainbow trout (*Oncorhynchus mykiss*) CK5B, CK6 and CK7A chemokines demonstrate their immunomodulatory capacity and reveal CK6 as a major interferon inducer

Jana Montero, Elena Chaves-Pozo, Alberto Cuesta, Carolina Tafalla *

*Centro de Investigación en Sanidad Animal (CISA-INIA), Carretera de Algete a El Casar km. 8.1, Valdeolmos 28130, Madrid, Spain*

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ABSTRACT

In the current study, we have determined the immune effects of the intramuscular injection of eukaryotic expression plasmids coding for rainbow trout (*Oncorhynchus mykiss*) CK5B, CK6 or CK7A CC chemokines (pCK5B, pCK6 and pCK7A) as a first step towards the establishment of their biological role. We have studied the levels of expression of several immune genes in the spleen and head kidney by real-time PCR in comparison to the levels observed in animals injected with the empty plasmid. Concerning the levels of expression of these CC chemokines and the CXC chemokine, interleukin 8 (IL-8), each plasmid induced up-regulation on expression levels of its coded chemokine in the head kidney and spleen, but also affected the expression of other chemokines. Both pCK6 and pCK7A induced the expression of the other two CC chemokines, while pCK5B induced CK7A but not CK6. Both pCK5B and pCK7A induced IL-8 as well. pCK6 was the only plasmid that induced IL-1β in the head kidney, whereas in the spleen, this occurred only with pCK5B. Different effects on the head kidney and spleen were also visible for tumour necrosis factor α (TNF-α), since the three plasmids induced this cytokine in the head kidney, but only pCK5B and pCK6 in the spleen. Concerning the effects on type I interferon (IFN), again pCK6 induced the strongest enhancement in the head kidney, while in the spleen it was pCK5B. However, the levels of expression of the Mx gene, know to be induced by type I IFN correlated with the CK6-induced IFN levels in the head kidney, but not with the CK5B-induced IFN in head kidney or spleen, suggesting an inhibition of Mx mRNA levels independent of IFN due to CK5B. The clear effect of pCK6 on the levels of expression of IFN-γ and its strong effects on type I IFN, in contrast with its recent adscription to the CCL17/22 group linked to Th2 responses, were verified by studying the *in vitro* effects of recombinant CK6 on head kidney leukocytes. Again in this case, recombinant CK6 strongly induced type I IFN, Mx and IFN-γ to a lesser extent, revealing CK6 as a potent IFN inducer in contrast to its mammalian homologues. Finally, effects on major histocompatibility complex (MHC)-IIα, CD4 and CD8α expression demonstrate that the three chemokines are able to mobilize antigen-presenting cells, $CD4^+$ and $CD8^+$ lymphocytes.



## 1. Introduction

Chemokines are a family of cytokines, produced by different cell types, that have among other functions, chemoattractant properties. In mammals, they are divided into four subfamilies, depending on the arrangement of the first two conserved cysteines in their sequence: CXC, CC, C and $CX_3C$ classes [1]. The CC family with 28 members in mammals constitutes the largest chemokine group, which primarily attracts mononuclear cell types [2]. Chemokines not only act as chemoattractants, but also have many immunomodulatory actions, thus modulating the immune reactions of their target cells [3].

In rainbow trout (*Oncorhynchus mykiss*), 15 CC chemokine sequences were identified within EST databases [4], bringing the total to 18 including the previously described CK1 [5], CK2 [6] and CK3 (EMBL accession number AJ315149). Due to the previous naming of these three previously described molecules, all trout CC chemokines have been named accordingly. Up to date, very little is known about the regulation of expression and biological effects of these chemokines, and even their chemoattractant capacity has only been demonstrated for CK1 [7] using total blood leukocytes and CK6 using the RTS11 rainbow trout monocyte-macrophage cell

* Corresponding author. Tel.: +34 91 6202300; fax: +34 91 6202247.
*E-mail address:* tafalla@inia.es (C. Tafalla).

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line [8]. Moreover, correlations between these rainbow trout chemokines and their mammalian orthologs are very difficult to establish since, as in all the other piscine CC chemokines identified to date, rapid divergence and independent duplication events make it difficult to establish accurate branching patterns [2,9]. In mammals, CC chemokines have been divided in "inflammatory" CC chemokines expressed only after an immune stimulation and "homeostatic" CC chemokines which are produced under normal physiological conditions [10], division also used to classify rainbow trout CC chemokines into "inducible" and "constitutive" chemokines [4]. However, as more information becomes available concerning the immune roles of CC chemokines, and many chemokines appear to have a dual role, this division seems simplistic. Recently, seven large groups of fish CC chemokines have been established through phylogenetic analysis: the CCL19/21/25 group, the CCL20 group, the CCL27/28 group, the CCL17/22 group, the macrophage inflammatory protein (MIP) group, the monocyte chemotactic protein (MCP) group and a fish-specific group [2]. However, further studies concerning functional and expression studies need to be performed in fish as in mammals to clarify this classification and the immune role of the different members of this diverse chemokine family.

In this work, we have used eukaryotic expression plasmids coding for rainbow trout CK5B, CK6 and CK7A to study the biological function of these chemokines, previously catalogued as inducible or inflammatory CC chemokines [4]. CK5 and CK7 rainbow trout chemokines are two of the five CC chemokines for which two sequences identified have been designated as variants of the same gene (A and B) [4]. However, even though they share a high identity these two variants are differently regulated [4,11]. For this study, we chose CK5B instead of CK5A and CK7A instead of CK7B, since CK5B and CK7A and not the other two have been shown to be constitutively expressed in head kidney and in the rainbow trout established monocyte-macrophage cell line RTS11 [4]. Rainbow trout CK5B groups with human regulated on activation, normal T cells expressed and secreted (RANTES) and CCL5 molecules from diverse species. All these CCL5-like chemokines now fall within the MIP group [2]. CK6 is closely related to the flounder MIP1α [12] but has now been ascribed to the CCL17/22 group [2], whereas CK7A seems to belong to the MCP group, although it is not related in particular to any member of the family [4]. In order to contribute to the elucidation of the immunological role of CK5B, CK6 and CK7A, we have studied the effect of the injection of plasmids coding for these chemokines on the levels of expression of several immune genes in the head kidney and spleen. Their potential future use as molecular adjuvants for intramuscular DNA vaccination, an efficient vaccination strategy for many fish viruses that may be optimised through the co-administration of plasmids coding for chemokines will also be discussed, as has been widely assayed with beneficial results in mammalian DNA vaccination systems [13–16].

## 2. Materials and methods

### 2.1. Fish

Rainbow trout (*O. mykiss*) of approximately 6–8 cm obtained from Lillogen (Leon, Spain) were maintained at the Centro de Investigación en Sanidad Animal (CISA-INIA) laboratory at 14 °C with a re-circulating water system. Fish were fed daily with a commercial diet (Trow, Leon, Spain). Prior to the experiments, fish were acclimatised to laboratory conditions for 2 weeks.

### 2.2. Plasmid constructions

PCR products encoding the entire open reading frame of CK5B, CK6 and CK7A, containing both start and stop codons, were cloned into the expression vector pcDNA3.1/V5-His-TOPO according to manufacturer's instructions (Invitrogen). Primers designed to obtain these products (Table 1) were used to obtain PCR products encoding the entire open reading frame of the different chemokines from a cDNA sample obtained from the spleen of a VHSV-infected trout obtained as previously described [17]. The PCR products (8 μl) were visualised on a 2% agarose gel stained with ethidium bromide, and single bands of the expected size were observed. The non-purified PCR products (4 μl) were directly ligated into pcDNA3.1/V5-His-TOPO according to manufacturer's instructions and the reactions used to transform One Shot TOP10 *Escherichia coli* cells (Invitrogen). Clones containing the full-size inserts were identified by PCR screening, and the proper orientation was verified by sequencing. The resulting constructs were designated as pCK5B, pCK6 and pCK7A. A religated empty pcDNA3.1/V5-His-TOPO plasmid (pcDNA) was used as a negative control.

**Table 1**
Primers used to generate expression plasmids designated as "Full" were used to amplify the full open reading frames of these chemokines that were then to be ligated into pcDNA3.1. Start and stop codon are underlined.

| Primer | Sequence (5′→3′) | Amplicon size (bp) |
|---|---|---|
| Full CK5B-F | AGCATGTTCACCCCTCGTCTTGCTATGCTGTC | 305 |
| Full CK5B-R | TTACAGGAGTGGTGTCTGCTCCCCAGACTTCTTGCTG | |
| Full CK6-F | AGAATGAAGGTCCTCCTCGCTCTCCCG | 518 |
| Full CK6-R | TTATAATAGCTGCATATTGAAAATATTCAAACA | |
| Full CK7A-F | ACCATGAAGACCCTGACTGCTCTAC | 348 |
| Full CK7A-R | TTAGAATAGACCTAGTTTACAACCCATT | |

### 2.3. Intramuscular injection of pCK5B, pCK6 and pCK7A plasmids in rainbow trout

In order to determine the effect that the intramuscular injection of the different chemokine plasmids produced, 16 trout in each group were intramusculary injected with either pCK5B, pCK6 or pCK7A (1 μg in 100 μl of PBS per fish); the same amount of the empty construct used as negative control (pcDNA); or with the same volume of PBS.

At days 1, 2, 5 and 7 post-injection, four trout from each group were sacrificed by overexposure to MS-222, and head kidney, spleen and muscle corresponding to the injection site removed and blood extracted from the caudal vein.

Muscle and blood samples (serum and cells) were only used to verify the correct transcription of the plasmids, determining only the levels of expression of the coded chemokine for each plasmid, whereas the head kidney and spleen samples were used to determine the effects on other immune genes.

### 2.4. cDNA synthesis

Total RNA of the different organs was extracted using Trizol (Invitrogen). Organs were homogenised in 1 ml of Trizol in an ice bath, and mixed with 200 μl of chloroform. The suspension was then centrifuged at 12,000 × *g* for 15 min. The clear upper phase was aspirated and placed in a clean tube. Five hundred microliters of isopropanol were then added, and the samples were again centrifuged at 12,000 × *g* for 10 min. The RNA pellet was washed with 75% ethanol, dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at −80 °C.

RNA from the different individuals (0.5 μg each) in each group were pooled and treated with DNAse I to remove any genomic DNA traces that might interfere with the PCR reactions. One microgram of this pooled RNA was used to obtain cDNA using the Superscript

III reverse transcriptase (Invitrogen). RNAs were incubated with 1 μl of oligo (dT)12–18 (0.5 μg ml$^{-1}$) and 1 μl 10 mM dinucleoside triphosphate (dNTP) mix for 5 min at 65 °C. After the incubation, 4 μl of 5× first strand buffer, 1 μl 0.1 M dithiothreitol (DTT) and 1 μl of Superscript III reverse transcriptase were added, mixed and incubated for 1 h at 50 °C. The reaction was stopped by heating at 70 °C for 15 min, and the resulting cDNA was diluted in a 1:10 proportion with DEPC-treated water and stored at −20 °C.

### 2.5. Effect of recombinant CK6 on head kidney leukocyte IFN production

In order to verify that CK6 directly induced type I and type II interferon (IFN), the levels of expression of IFN2 (type I) and Mx (type I-induced) and IFN-γ (type II) were also evaluated in head kidney leukocytes treated with recombinant rainbow trout CK6. For this, head kidney leukocytes were isolated following the method previously described [18]. Briefly, the anterior kidney was removed aseptically and passed through a 100 μm nylon mesh using Leibovitz medium (L-15, Gibco, Invitrogen, UK) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), heparin (10 units/ml) and 2% foetal calf serum (FCS, Gibco). The resulting cell suspension was placed onto 51% Percoll density gradients. The gradients were 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. Cells were resuspended in L-15 with 5% FCS at a concentration of $1 \times 10^6$ cells/ml and disposed in 24-well plates (1 ml per well). After an overnight incubation, head kidney leukocytes were treated with different concentrations of recombinant rainbow trout CK6 obtained as previously described and known to be active in chemotaxis experiments previously performed [8]. After a further 24 h of incubation, RNA was extracted from the cells using Trizol as previously described.

### 2.6. Analysis of gene expression

In order to evaluate the transcriptional activity of each chemokine plasmid, the levels of expression of each of the administered chemokine were evaluated in muscle and blood cDNA samples from fish injected with PBS, the empty plasmid, or the plasmid coding for this specific chemokine. To evaluate the effect that the injection of the different chemokine coding plasmids may have on the expression of other immune genes, the mRNA levels were determined in both head kidney and spleen. In all cases, real-time PCR was performed with an Mx3005P™ QPCR instrument (Stratagene) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 μl of 2× SYBR Green supermix, 5 μl of primers (0.6 mM each) and 5 μl of cDNA template) were incubated for 10 min at 95 °C, followed by 40 amplification cycles (30 s at 95 °C and 1 min at 60 °C) and a dissociation cycle (30 s at 95 °C, 1 min 60 °C and 30 s at 95 °C). For each mRNA, gene expression was corrected by the elongation factor 1α (EF-1α) expression in each sample. The primers used are shown in Table 2. All amplifications were performed in duplicate and the experiment was repeated once to confirm the results. Finally, the levels of expression of the IFN-related genes in head kidney leukocytes in response to recombinant CK6 were also evaluated through real-time PCR as described above.

### 2.7. Statistical analysis of gene expression

First, the expression obtained for each gene in all animals was referred to the level of expression of the EF-1α as previously described [19]. Then, the relative levels of expression of the different gene obtained in the head kidney and spleen of fish injected with either the empty plasmid or the expression plasmids were normalized by the corresponding results obtained in the PBS injected group. In all cases, Student-*t*-tests were performed to determine differences between the empty plasmid and each chemokine expression plasmid treated groups ($P < 0.05$).

**Table 2**
Primers used for real-time PCR analysis.

| Gene | Acc. number | Primer sequence (5′→3′) |
|---|---|---|
| EF-1α | AF498320 | F: GATCCAGAAGGAGGTCACCA<br>R: TTACGTTCGACCTTCCATCC |
| CK7A | CA343117 | F: CCGAGAATCCCTCTTCAACA<br>R: TCATCGTCGTCTTGGCAGTA |
| CK5B | CA374135 | F: TTTGCTGATCGTCAGATACCC<br>R: GTGTCTGCTCCCCAGACTTC |
| CK6 | CA355962 | F: TGAAAGGCCTACGAATCTGC<br>R: GTTGTTGTTGGCTGGTTGTG |
| IL-8 | AJ279069 | F: ATTGAGACGGAAAGCAGACG<br>R: CTTGCTCAGAGTGGCAATGA |
| IL-1β | AJ223954 | F: CTGAAGCCAGACCTGTAGCC<br>R: GCAACCTCCTCTAGGTGCAG |
| TNF-α | AJ277604.2 | F: CCACACACTGGGCTCTTCTT<br>R: GTCCGAATAGCGCCAAATAA |
| IFN2 | AJ582754 | F: AGTTCCTGTGTATCACCTGTCG<br>R: GATGCTCAGTACATCTGTCCCA |
| IFN1 | AJ580911 | F: AAAACTGTTTGATGGGAATATGAAA<br>R: CGTTTCAGTCTCCTCTCAGGTT |
| Mx | U30253,<br>U47945, U47946 | F: AGCGTCTGGCTGATCAGATT<br>R: AGCTGCTCGATGTTGTCCTT |
| IFN-γ | AJ616215 | F: GAAGGCTCTGTCCGAGTTCA<br>R: TGTGTGATTTGAGCCTCTGG |
| MHC-IIα | AJ251432 | F: ACACCCTTATCTGCCACGTC<br>R: TCTGGGGTGAAGCTCAGACT |
| CD4 | AY973028 | F: CCTGCTCATCCACAGCCTAT<br>R: CTTCTCCTGGCTGTCTGACC |
| CD8 α | AF178053 | F: AGTCGTGCAAAGTGGGAAAG<br>R: GGTTGCAATGGCATACAGTG |

## 3. Results

### 3.1. Transcriptional activity of the pCK5B, pCK6 and pCK7A plasmids

After obtaining the eukaryotic expression plasmids coding for CK5B, CK6 and CK7A (pCK5B, pCK6 and pCK7A), we first evaluated the transcriptional activity of the plasmids after their intramuscular injection through the determination of the expression levels of the coded chemokine in muscle and blood (Fig. 1). As expected, the plasmids produced an increased expression of the coded chemokine significantly higher than the mock-injected controls, and the controls injected with the empty plasmid in the muscle indicating a correct transcription, meaning that after their intramuscular injection, they are correctly expressed in the muscle. This increased chemokine transcription was also clearly visible in the blood for CK5B and CK6 implying strong systemic immune effects, but was less clear for CK7A, maybe in correlation with the weaker immunostimulary effects that were observed later.

### 3.2. Effect of the intramuscular injection of the CC chemokine coding plasmids on the expression of chemokine genes

The expression of CC (CK5B, CK6, CK7A) and CXC (interleukin 8, IL-8) chemokines was studied in rainbow trout injected with

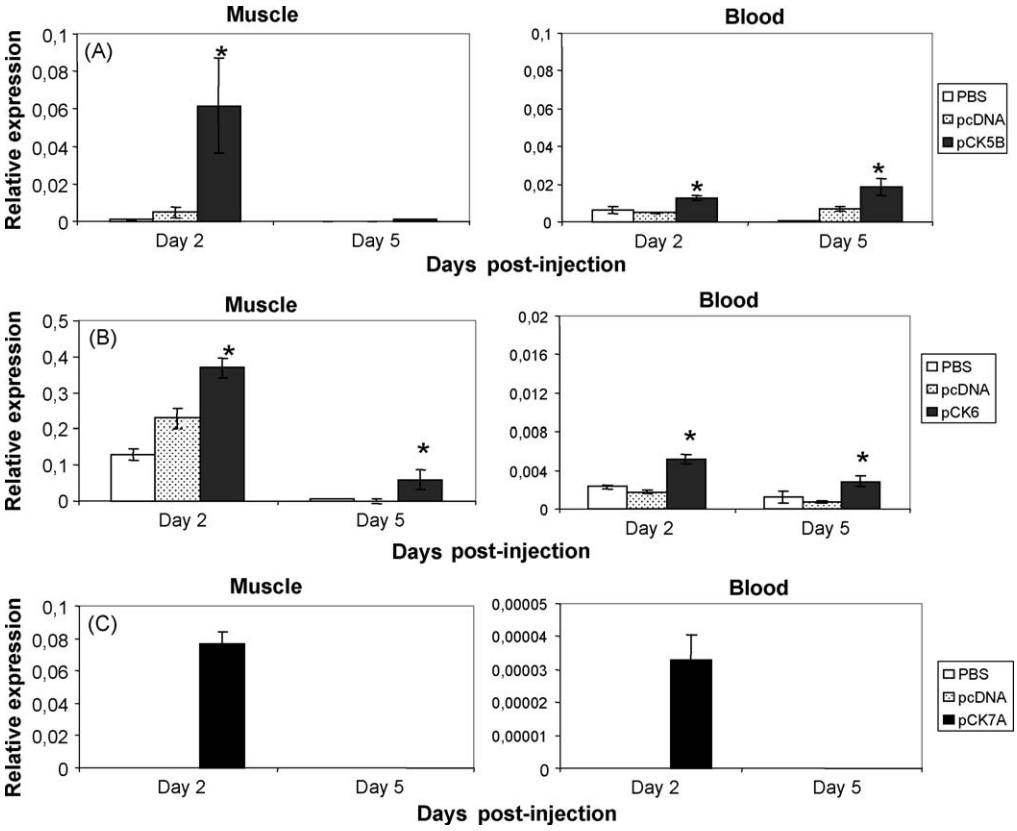


**Fig. 1.** Expression of CK5B (A), CK6 (B) and CK7A (C) transcripts in the muscle and blood of rainbow trout intramuscularly injected with either pCK5B, pCK6 or pCK7A plasmids. Trout were either mock-injected (PBS), injected with the empty plasmid (pcDNA) or with the corresponding expression plasmid. The expression of each chemokine was assayed through real-time PCR after 2 or 5 days post-injection. Data are shown as the mean chemokine gene expression relative to the expression of endogenous control EF1-α ± S.D. Then, Student-*t*-tests were performed between the values obtained with the chemokine plasmid and the corresponding values obtained with the empty plasmid. $^{*}P < 0.05$ compared to empty plasmid.

pCK5B, pCK6 and pCK7A (Fig. 2). First, as occurred in the blood, we observed that each of the three plasmids induced the expression of the chemokine they coded in the head kidney (at day 5 with pCK5B, day 2 with pCK6 and pCK7A) and spleen (at day 2 with pCK5B, day 7 with pCK6 and day 2 with pCK7A).

In head kidney, the levels of expression of CK5B not only were induced by pCK5B, but also by pCK6, while in the spleen, higher CK5B mRNA levels were induced by all three chemokine coding plasmids.

The levels of expression of CK6 in head kidney were up-regulated in response to pCK6 at day 2 post-injection but then decreased to levels lower than those of controls, suggesting a negative feedback effect. In this organ as in the spleen, pCK7A also induced CK6 mRNA expression.

All three chemokine expression plasmids induced CK7A in the spleen, whereas in the spleen the increased levels of expression were only observed in response to pCK7A and pCK6.

Concerning the CXC chemokine IL-8, pCK5B in the head kidney and spleen and to a lesser extent pCK7A only in head kidney, induced the expression of this chemokine belonging to a different subfamily.

In summary, pCK5B induced CK5B, CK7A and IL-8; pCK6-induced CK5B, CK6 and CK7A while pCK7A induced CK5B, CK6, CK7A and IL-8.

### *3.3. Effect of the intramuscular injection of the CC chemokine coding plasmids on the expression of genes related to the pro-inflammatory response*

In order to study the effect of the three chemokine expression plasmids on the pro-inflammatory response, the levels of expression of the IL-1β and tumour necrosis factor α (TNF-α) genes were determined after their intramuscular injection (Fig. 3). In the head kidney, an enhancement on the levels of IL-1β expression was only observed after the injection of pCK6 at days 1, 5 and 7, whereas in the spleen, only pCK5B induced the expression of this cytokine at day 2 post-injection. Significant down-regulations in the levels of expression of IL-1β were observed both in head kidney and spleen after pCK7A injection. All three plasmids enhanced the expression of TNF-α in the head kidney, while only pCK5B and pCK6 induced its expression in the spleen.

### *3.4. Effect of the intramuscular injection of the CC chemokine coding plasmids on the expression of genes related to the IFN response*

Regarding the effect of the three chemokine expression plasmids on the type I IFN response, we studied the levels of expression of Mx, IFN1 and IFN2 (two different isotypes of rainbow trout type I IFN) (Fig. 4), but the levels of expression of IFN1 in this experiments were undetectable through real-time PCR indicating that none of the plasmids induced its expression (data not shown). The three chemokine expression plasmids induced an increased IFN2 expression in the head kidney, although the greater effects were observed in response to pCK6 at day 2. In the spleen, however, only pCK5B enhanced the levels of IFN2, and pCK6 and pCK7A even significantly down-modulated its expression at day 2. In the case of Mx, pCK6 and pCK7A induced its expression both in the head kidney and spleen.

Regarding type II IFN, IFN-γ mRNA levels were enhanced in head kidney with pCK5B, pCK6 and pCK7A and with pCK6 and pCK7A in spleen, although as it occurred with the type I IFN genes,

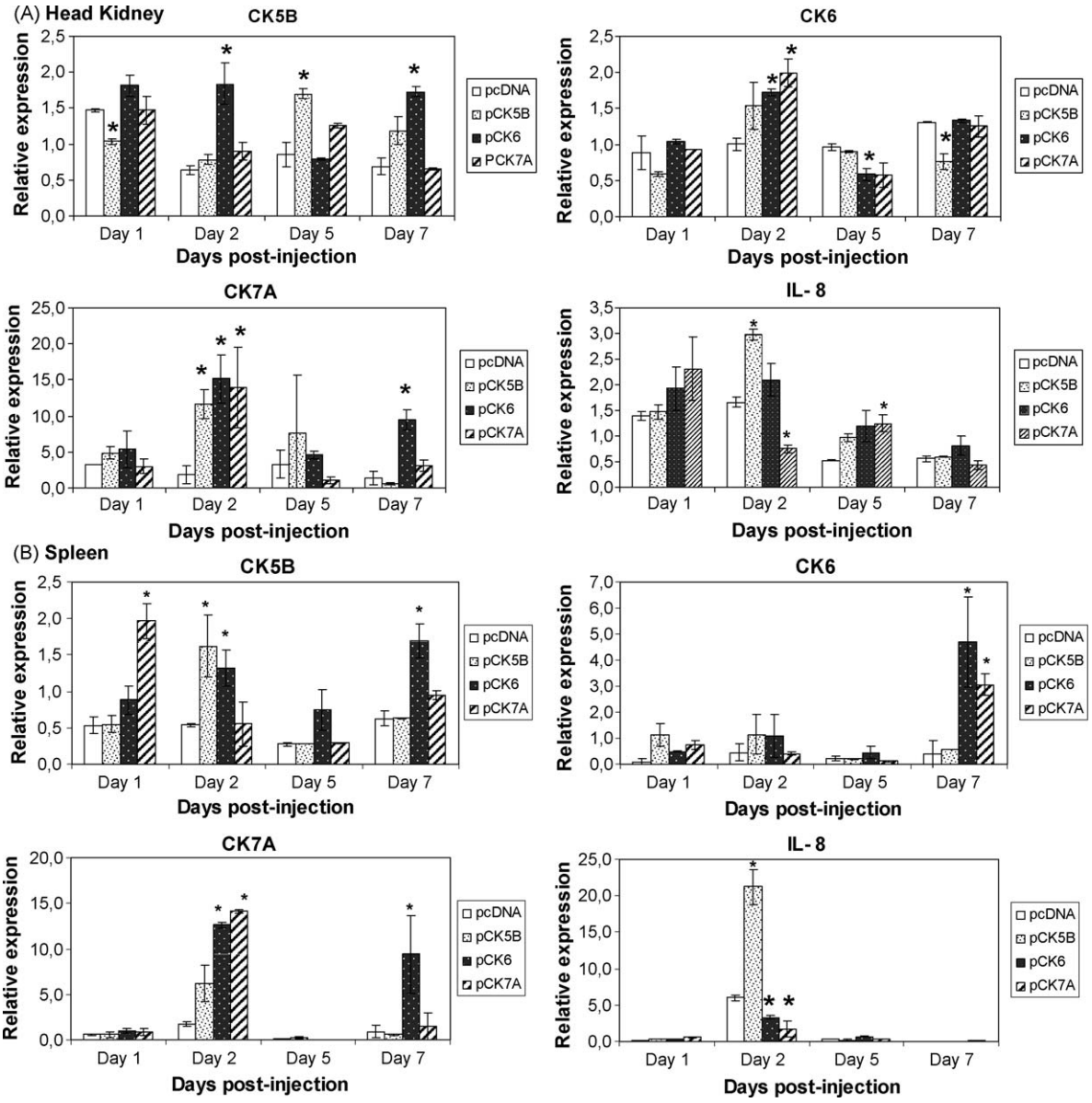


**Fig. 2.** Chemokine gene expression in head kidney (A) and spleen (B) of rainbow trout intramusculary injected with pCK5B, pCK6 and pCK7A compared to levels obtained in trout injected with the empty plasmid. The relative expression shown was normalized by dividing the expression in all the plasmid-injected groups by that obtained in the PBS injected group. Then, Student-*t*-tests were performed between the values obtained with the chemokine plasmid and the corresponding values obtained with the empty plasmid. $^{*}P < 0.05$ compared to empty plasmid.

a much stronger response was observed in response to the pCK6 plasmid in both organs (Fig. 4).

### 3.5. Effect of the intramuscular injection of the CC chemokine coding plasmids on the expression of markers of antigen-presenting cells and T lymphocyte subpopulations

We studied the effect of the plasmids on the levels of expression of the alpha chain of major histocompatibility complex (MHC)-II as a marker for antigen-presenting cells and CD4 and the alpha chain of CD8 as markers for different lymphocyte subsets. No significant stimulatory effects on the expression of MHC-IIα were observed in the head kidney, and moreover, pCK5B, pCK6 and pCK7A plasmids provoked significant down-modulations, especially at days 1 and 5 (Fig. 5). While the levels of MHC-IIα expression were enhanced upon pCK5B and pCK7A injection in the spleen, significant down-modulations were also observed in response to pCK6 at day 1 and to pCK5B at day 5.

Concerning the effect of the chemokine plasmids on the expression of markers for T lymphocyte subpopulations, CD4 was slightly induced by the action of pCK5B and CK7A plasmids in both head kidney and spleen at day 5 post-injection, but significant down-modulations were observed in the head kidney with pCK5B and pCK6 and in the spleen with pCK6 and pCK7A. The expression of CD8 was significantly reduced in response to pCK5B and pCK7A in the head kidney as in spleen. In spleen, however, all three plasmids induced a significant up-regulation of CD8 expression, especially at day 2 post-injection.

### 3.6. Effect of recombinant CK6 on head kidney leukocyte IFN production

Since the results obtained after the injection of the expression plasmids coding for the different rainbow trout chemokines pointed to a role for CK6 in regulating the levels of expression of type I and type II IFN, we studied the levels of mRNA expression of

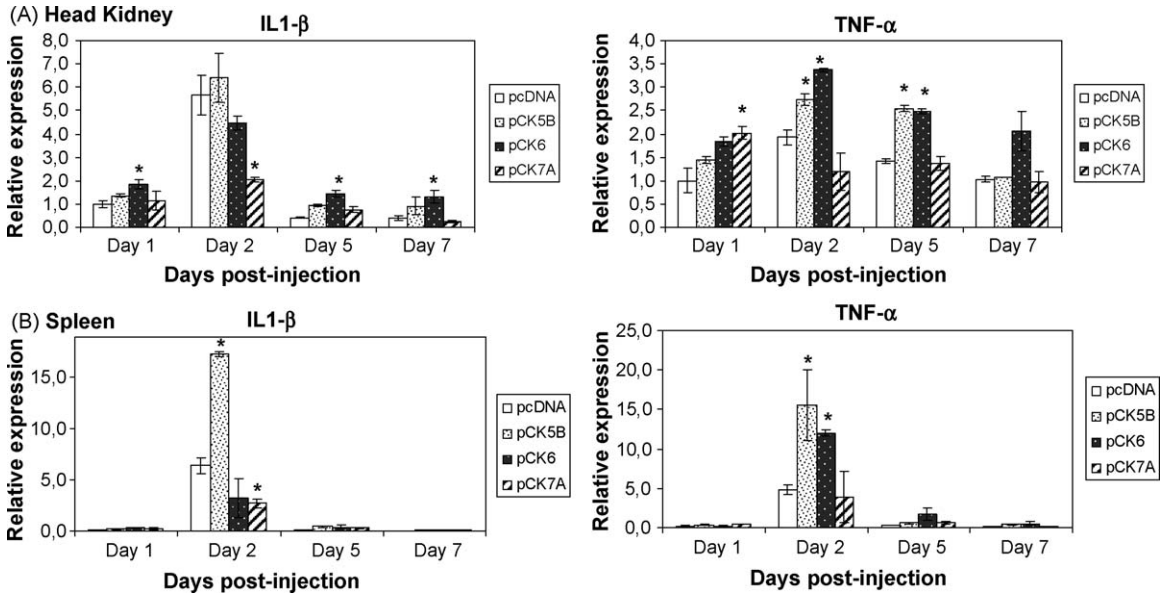


**Fig. 3.** Levels of expression of pro-inflammatory genes in head kidney (A) and spleen (B) of rainbow trout intramuscularly injected with pCK5B, pCK6 and pCK7A. The relative expression shown was obtained by dividing the expression in all the plasmid-injected groups by that obtained in the PBS injected group. $^{*}P < 0.05$ compared to empty plasmid.

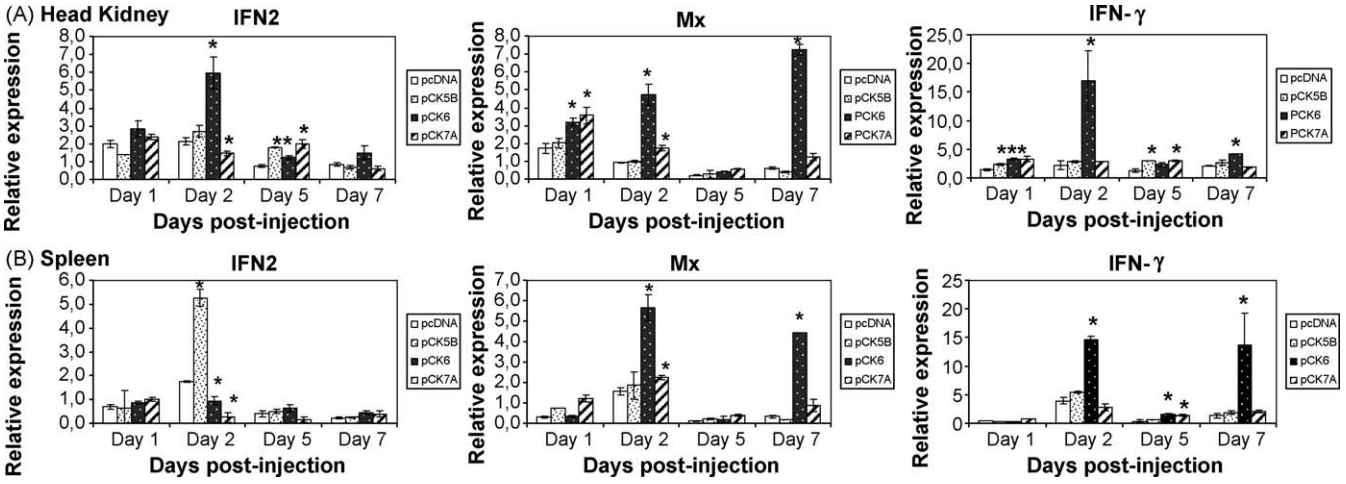


**Fig. 4.** Levels of expression of genes related to the type I and type II IFN response in head kidney (A) and spleen (B) of rainbow trout intramuscularly injected with pCK5B, pCK6 and pCK7A. The relative expression shown was obtained by dividing the expression in all the plasmid-injected groups by that obtained in the PBS injected group. $^{*}P < 0.05$ compared to empty plasmid.

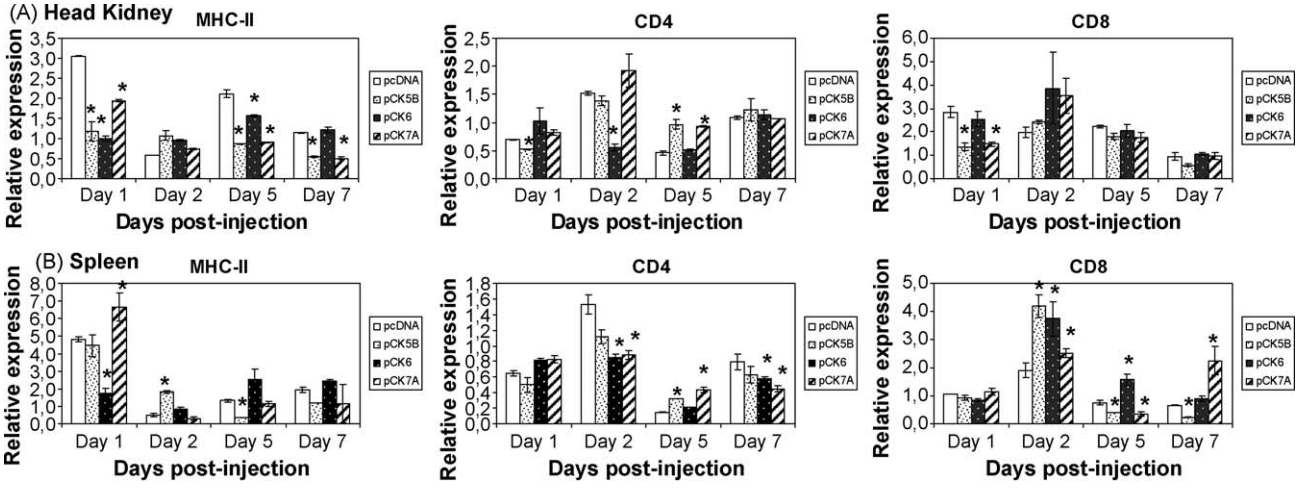


**Fig. 5.** Levels of expression of markers for antigen-presenting cells and T lymphocyte subpopulations in head kidney (A) and spleen (B) of rainbow trout intramuscularly injected with pCK5B, pCK6 and pCK7A. The relative expression shown was obtained by dividing the expression in all the plasmid-injected groups by that obtained in the PBS injected group. $^{*}P < 0.05$ compared to empty plasmid.

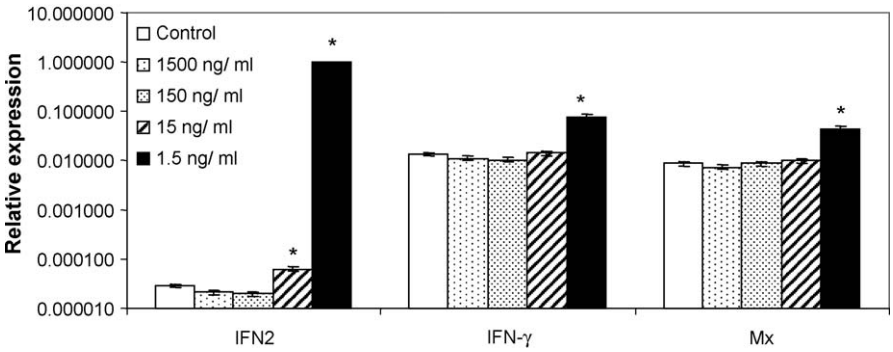


**Fig. 6.** Effect of recombinant CK6 on head kidney leukocyte IFN production. Real-time PCR analysis of the levels of IFN2, Mx and IFN-γ mRNAs in rainbow trout head kidney leukocytes in response to different concentrations of recombinant CK6. After 24 h of incubation with the different chemokine concentrations, RNA was extracted and the levels of expression of these genes evaluated through real-time PCR. Results are shown as the mean relative expression referred to that of EF-1α ($N = 2$).

IFN2, Mx and IFN-γ in head kidney leukocytes exposed to recombinant rainbow trout CK6. The results obtained in vivo were confirmed since CK6 was also capable of up-regulating in head kidney leucocytes the levels of IFN2, Mx and IFN-γ mRNA, even though it was the lowest CK6 dose the one that produced the greatest effects (Fig. 6).

## 4. Discussion

We have determined the immune effects of the intramuscular injection of eukaryotic expression plasmids coding for rainbow trout CK5B, CK6 or CK7A as a first step towards the establishment of their biological role and the evaluation of their potential use as molecular adjuvants in intramuscular fish DNA vaccination. First, after the construction of the expression plasmids, we evaluated the correct transcription in the muscle and blood. As described for other cytokine coding plasmids [20], the intramuscular injection provoked the chemokine transcription not only in the injection site, but also in the blood, specially for CK5B and CK6, leading to strong systemic immune effects.

To determine the effects that these plasmids produced on the expression of different immune genes in the head kidney and spleen, we first focused on the expression of CK5B, CK6 and CK7A, as well as the CXC chemokine, IL-8, to establish relations among the different chemokines. Among CC chemokines, CK6 and CK7A induced the expression of the other two CC chemokines. CK5B, however, induced CK7A but not CK6. Concerning the relations between the CC and CXC groups, we had previously demonstrated that CK6 and CK7A, but not CK5B were significantly induced by rainbow trout IL-8 [11]. In the current study, we have demonstrated that CK5B and CK7A are capable of inducing the expression of IL-8, while CK6 has no significant effect, although at some points slight stimulatory effects were also observed.

Regarding the effects that these chemokines have on the levels of mRNA of genes which have a role in the immune response, we can conclude that CK5B induces IL-1β, TNF-α, IFN2, and IFN-γ; CK6 induces IL-1β, TNF-α, IFN2, Mx and IFN-γ; and CK7A stimulates TNF-α, IFN2, Mx and IFN-γ. Overall, when comparing the effects of the chemokine plasmids on the head kidney and spleen, many differences were observed and in many occasions the plasmids induced genes in one organ that were not induced in the other. This was especially evident in the case of the effects that CK5B and CK6 had on IL-1β and IFN2 production. The pCK6 was the only plasmid that induced a significant increase of IL-1β and IFN2 in head kidney, while in the spleen this occurred only with pCK5B. These results suggest that CK6 induces the expression of IL-1β and IFN2 in a cell population present only in the head kidney, and CK5B stimulates another cell population present in the spleen to produce these same cytokines or on the other hand, the same cell population is differently regulated in both tissues. Thus, a possible benefit derived from the presence of up to 18 different CC chemokines, could be that chemokines have similar effects on different cell types present in different tissues or are involved in different pattern of regulation of the same cell population in different tissues implying tissue-specific regulation of the immune system. Another example of these differences between the effects observed in head kidney and spleen, is the fact that all three chemokine plasmids induced CD8 in the spleen but not in the head kidney. In mammals, it is know that CCL5, MCP and MIP are chemoattractant for $CD8^+$ T lymphocytes [21], and although at some time points in the spleen there are also significant inhibitions in the levels of expression of CD8, at least at day 2 there is a significant increase in response to all three chemokines. In general, the effect that the plasmids provoke in the levels of expression of these genes that we have catalogued as "markers" for antigen-presenting cells and lymphocyte subsets are inhibitory, and apart from CD8 levels in the spleen, significant enhancements were only observed in the case of the levels of MHC-IIα in response to pCK7A in the spleen, and CD4 in the spleen and head kidney in response to pCK5B and pCK7A. The changes in the levels of mRNA that are observed in this type of genes is most probably associated with migration of cell populations to or from the different immune organs, since at least for CD4 and CD8, up-regulation in the number of molecules per cell is not usual in mammals [22]. Therefore overall, all three chemokines seem to be attractant for all cell types studied, even though the factors which condition the fact that cells are recruited into the immune organs or mobilized from them, are probably due to subtle differences in the concentrations of these and other different chemokines that may affect the migration of each cell type.

Continuing with the effects observed in genes related to the immune response, a 15–20-fold increase in the levels of expression of IFN-γ produced by pCK6 alone in this case both in the head kidney and spleen indicate once more the distinct regulation and effects of pCK6 compared to the other two CC chemokines studied. Although the phylogenetic signal is very low with chemokine sequences making it rather impossible to establish orthology relationships [2,9], phylogenetically, CK6 belongs to the CCL17/22 group clustered together in mammals on a different chromosome than the MIP and MCP groups in which CK5B and CK7A are ascribed [2]. Moreover CCL17/22 chemokines are known to attract preferably Th2 lymphocytes [23,24] and thus are associated with the induction of Th2 cytokines such as IL-4 and IL-13, and not with Th1 cytokines such as IFN-γ [25]. To date, it is still not clear whether a distinct Th1/Th2 lymphocyte differentiation exists in teleost fish, but, in any case, this clear and unexpected effect on IFN-γ, as in the type I IFN genes IFN2 and Mx, could be a beneficial trait to be taken into account when choosing one of these

chemokine plasmids as potential adjuvants for DNA vaccination, since IFN-γ has a major role on cellular specific immune responses [26] which are thought to have a major role in conferring protection against fish viruses [27] and type I IFN is thought to correlate with protection conferred by DNA vaccines [28]. Therefore, in order to verify this clear effect of pCK6 on types I and II IFN, despite its adscription to the CCL17/22 group, we examined the effect of rainbow trout recombinant CK6 on the levels of expression of these genes in head kidney leukocytes. Through this experiment, we have verified that CK6 is a potent type I IFN inducer also capable of stimulating IFN-γ. On the other hand, a result that must also be taken into account derived from this study is the inhibitory effect that pCK7A had on many of the genes studied specially at day 2 after injection, since at this point this plasmid significantly suppressed IL-8, IL-1β and IFN2 mRNA levels in both the head kidney and spleen. *A priori*, these effects exclude pCK7A as a potential molecular adjuvant for antiviral vaccination and point to CK6 as the best candidate.

From the results shown in this paper dealing with the effects on the type I IFN response, some unexpected results were also observed. In the case of the effects of pCK6 and pCK7A in the head kidney, although not always coinciding in the intensity and times post-injection, the IFN2 induction correlated with Mx induction in this organ. For CK5B, however, we observed an increase in IFN2 that did not correlate with Mx induction. This is especially outstanding in the spleen, where trout injected with pCK5B had a 5-fold increase in the levels of expression of IFN2, and no increase at all in the levels of Mx. It has been previously shown that the rainbow trout type I IFN2 induces Mx [29], therefore, an inhibition of Mx mRNA independent of IFN due to CK5B seems to be taken place and should be further investigated. In mammals, CCL5/RANTES is in close relation to immune mechanisms triggered by viral infections, as it has been shown to be up-regulated in macrophages persistently infected with Theiler's murine encephalomyelitis virus, but restored to normal levels after abrogation of the infection by IFN [30] and is known to be required for the survival of virus-infected macrophages because of its antiapoptotic actions [31]. These previous studies, together with our results point to a suppressive effect of CK5B/CCL5 on IFN signalling. Also surprising is the fact that fish injected with pCK6 with levels of IFN1 and IFN2 equivalent to those of controls injected with the empty plasmid have levels of Mx mRNA in the spleen 5-fold higher than those of controls. However, this later case could be due to an effect of CK6 on a type I IFN molecule not studied in the current work. Up to date, four different type I IFN genes have been described in rainbow trout [29,32], IFN1 and IFN2 known to have antiviral activity and a common receptor (Group I) and IFN3 and IFN4 (Group II) for which antiviral activity has not been demonstrated yet [29,32]. In our work, we have chosen to focus on those type I IFN genes with a defined immune role, known to induce Mx antiviral genes, but the possibility that other group I type I IFN genes with effects on Mx are present on rainbow trout but not yet identified exists.

In conclusion, using expression plasmids coding for rainbow trout CK5B, CK6 and CK7A CC chemokines we have determined that CK5B induced IL-8, TNF-α and IFN2 in both head kidney and spleen, CK7A and IFN-γ only in the head kidney and IL-1β, MHC-IIα and CD8α only in spleen. In the case of CK6, we have demonstrated an stimulatory effect on the expression of CK5B, CK7A, TNF-α, Mx and IFN-γ in head kidney and spleen, of IL-1β and IFN2 only in the head kidney and CD8α in the spleen. For CK7A, we have demonstrated its capacity to induce CK6, Mx and IFN-γ both in the head kidney and spleen, IL-8, TNF-α and IFN2 in the head kidney and MHC-IIα and CD8α in the spleen. These results also reveal the potent effect of CK6 on types I and II IFN production despite its grouping with mammalian CCL17/22 and point to a suppressive role of CK5B on type I IFN signalling. This work constitutes one of the very few studies dealing with the role that chemokines have on the fish immune system, apart from their attractant capacity, studies that will help establish the differences among the members of the diverse chemokine family.

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