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# Co-injection of interleukin 8 with the glycoprotein gene from viral haemorrhagic septicemia virus (VHSV) modulates the cytokine response in rainbow trout (*Oncorhynchus mykiss*)

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**Abstract**

Since previous results showed that interleukin 8 (IL-8) was induced in rainbow trout (*Oncorhynchus mykiss*) in response to viral hemorrhagic septicemia virus (VHSV) infection, we have cloned IL-8 in an expression vector (pIL8+) and studied its possible adjuvant effect on the early response to a VHSV immunization model, focusing on the early response of several cytokines induced by a vector coding for the glycoprotein of VHSV (pMCV1.4-G) in the spleen and head kidney. First, we demonstrated that the pIL8+ successfully transcribed IL-8, by induction of IL-8 transcription in the muscle and blood, and by a massive infiltration of neutrophils at the muscle inoculation site. We have studied the effect of pIL8+ co-administration on the expression of two pro-inflammatory cytokines, such as IL-1β and tumour necrosis factor α (TNF-α); cytokines that have mainly an inhibitory role, IL-11 and transforming growth factor β (TGF-β); and a Th1 type cytokine, IL-18. We demonstrated that the co-administration of pIL8+ with pMCV1.4-G modulates the cytokine response that is induced, mainly by having its effect increasing pro-inflammatory cytokines (IL-1β and TNF-α1), with a greater impact on the spleen, and to a lesser extent in the head kidney. All these data suggest that IL-8 is able to modulate the early cytokine immune response that is produced in response to a DNA vaccine, and therefore, might be a potential immune adjuvant in fish viral vaccination. More work should be done to determine if this modulation has a beneficial effect on protection as seen in other mammal viral models.


*Keywords:* Interleukin 8 (IL-8); Viral hemorrhagic septicaemia virus (VHSV); DNA vaccine; Adjuvant

## 1. Introduction

In the past years, genetic vaccination (DNA vaccines) against fish rhabdovirus, both infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicaemia virus (VHSV), has proved very effective using expression plasmids coding for the glycoprotein (G) of these viruses [1–3]. Even though, the mechanisms through which they confer protection are still unclear [4,5], non-specific defence mechanisms, that could be up-regulated through the use of adjuvants, are thought to have an important role [5–7]. Furthermore, there are some aspects such as the route of delivery or the cost of vaccination that could be considerably reduced through the use of adjuvants.

In mammals, chemokines are among the adjuvants more widely used for vaccination against viruses [8–10]. Chemokines are a superfamily of cytokines, produced by different cell types, that have among other functions, chemoattractant properties. The chemokine superfamily is divided into four subfamilies, depending on the arrangement of the first two conserved cysteines in their sequence: CXC, CC, C and $CX_3C$ classes, which in mammals have 28, 16, 2 and 1 known members, respectively [11]. They have been recently catalogued as a new class of "intelligent" adjuvants for vaccines, being able to finely tune protective immune responses by recruiting specific cell types to the site of immunization [9]. These chemokines may be administered in independent

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plasmids, together with the DNA vaccine plasmid [12–15], or fused to the antigen [16].

Interleukin 8 (IL-8) is a CXC chemokine produced by numerous cell types. In mammals, it is known to be produced by macrophages/monocytes, epithelial cells, neutrophils, fibroblasts, and endothelial cells upon infection or stimulated by cytokines such as IL-1β and tumour necrosis factor α (TNF-α) [17,18]. Being a CXC chemokine, IL-8 predominantly promotes the recruitment of neutrophils, inducing also their activation characterized by the activation of the leukotriene pathway [19], by the release of their granular content [20,21], and by their increased adherence to endothelial cells and nitric oxide production [19,22]. IL-8 is also a chemoattractant for other cell types such as basophils, T lymphocytes, and NK cells, and also enhances permeability of endothelial cells [18,22,23–25]. In mammals, some studies have focused on the potential use of IL-8 as an adjuvant for DNA vaccination. For example, Kim et al. [12] determined that IL-8 administration increased the antibody response and enhanced T helper proliferation. In a further study, IL-8 co-administration significantly increased the levels of Th1 cytokines such as IL-2 and IFN-γ, and of other chemokines such as RANTES (regulated on activation, normal T cells expressed and secreted) or monocyte chemotactic protein 1 (MCP-1) [26]. All these results demonstrate the capacity of IL-8 to modulate cytokine production *in vivo* and thus drive the immune response to either a Th1 or a Th2 response, which is of great importance for protection.

In fish, IL-8 has been characterized in lamprey, *Lampetra fluviatilis* [27], Japanese flounder, *Paralichthys olivaceous* [28] and rainbow trout, *Oncorhynchus mykiss* [29–31]. However, an understanding of the biological role of IL-8 has not been yet achieved in any of these species. It is known that, as in mammals, IL-8 is induced in response to LPS [28,29], Poly I:C [29] or virus infection [32].

In the current work, we have focused on the effect that rainbow trout *O. mykiss* IL-8 co-administration has on the non-specific immune response to a construct coding for the glycoprotein of VHSV (pMCV1.4-G), by studying its effect on the expression of different pro-inflammatory cytokines (IL-1β and TNF-α1), cytokines that have mainly an inhibitory role (IL-11 and transforming growth factor β, TGF-β), and a Th1 type cytokine, IL-18.

## 2. Materials and methods

### 2.1. Fish

Rainbow trout (*O. mykiss*) of approximately 8–10 cm obtained from Lillogen (Leon, Spain) were maintained at the Centro de Investigaciones en Sanidad Animal (CISA-INIA) laboratory at 14 °C and fed daily with a commercial diet (Trow, Leon, Spain). Prior to the vaccination experiments, fish were acclimatised to laboratory conditions for 2 weeks.

### 2.2. Plasmid constructions

A PCR product encoding the entire open reading frame of IL-8 (excluding the stop codon) was cloned into the expression vector pcDNA3.1/V5-His-TOPO according to manufacturer's instructions (Invitrogen). Primers full-IL-8F (5′-AAG**ATG**AGCATCAGAATGTCAGCCAG-3′) and full-IL-8R (5′-TTTGTTGTTGGCCAGCATCTTCTCAA-3′) were used to obtain a PCR product encoding the entire open reading frame of rainbow trout IL-8 from a cDNA sample obtained from the spleen of a VHSV-infected trout obtained as previously described [32]. The PCR product (8 μl) was visualised on a 2% agarose gel stained with ethidium bromide, and a single band of 294 bp corresponding to the expected amplified product was observed. The non-purified PCR product (4 μl) was directly ligated into pcDNA3.1/V5-His-TOPO according to manufacturer's instructions. The reaction was used to transform One Shot TOP10 *Escherichia coli* cells (Invitrogen). Clones containing a full-size insert were identified by PCR screening, and the proper orientation was verified by sequencing. The resulting construct was designated as pIL8+. A construct in which the IL-8 product was cloned in the opposite orientation was designated as pIL8− and used as a negative control.

The pMCV1.4-G plasmid used, consisted in the glycoprotein gene of VHSV under the control of the long cytomegalovirus (CMV) promoter, previously described [33]. The pMCV1.4-G contained additional 218 bp upstream of the 687 bp enhancer sequences of the regular CMV promoter and an intron. pMCV1.4 was smaller than its precursor pMOK by deleting ∼1 Kbp of unnecessary bacterial sequences (Ready Vector, Madrid, Spain).

### 2.3. pIL8+ transcription and histology

The transcriptional activity of pIL8+ was determined by studying the expression of IL-8 in different organs after the injection of the plasmid, and through histological techniques.

For determining in which organs IL-8 was transcribed after the injection of the pIL8+ plasmid, fish were intramuscularly injected with either the pIL8+ construct (0.5 μg in 100 μl of PBS per fish); the same amount of the construct used as negative control, pIL8−; or with the same volume of PBS. After 1 and 3 days post-injection, fish were killed and blood, muscle, head kidney, spleen and liver sampled for RNA extraction. An RT-PCR procedure previously described [32] was used to detect the transcription of IL-8.

Muscle samples were also taken from these fish for histology. Samples from the inoculation point, including skin and dorsal musculature were taken and fixed in Bouin's fixative during 18 h for histopathological study. After fixation, samples were rinsed in 70% ethanol and dehydrated through a graded series of ethanol to xylol and embedded in paraffin wax. Sections of 4 μm-thick were cut and stained routinely with hematoxilin and eosin (H & E).

### 2.4. Injection of pMCV1.4-G and pIL8+ into rainbow trout muscle

In order to determine whether IL-8 could alter the immune response to pMCV1.4-G, fish were intramuscularly injected in the presence or absence of pIL8+. For this, fish were divided into five groups with 20 trout each and intramuscularly injected with one of the following treatments: (group 1) 100 μl of PBS; (group 2) 100 μl of PBS containing 0.5 μg pMCV1.4-G plasmid DNA per fish; (group 3) 100 μl of PBS containing 0.5 μg pMCV1.4-G and 0.5 μg pIL8− per fish; (group 4) 100 μl of PBS containing 0.5 μg pMCV1.4-G and 0.5 μg pIL8+ per fish or (group 5) 100 μl of PBS with 0.5 μg pIL8+ per fish.

At days 3, 7 and 10 post-injection, five trout from each group were killed and spleen and head kidney removed. No fish were sampled from group 5 at day 10, since a previous experiment showed that injection of pIL8+ had no effect at this point on the level of cytokines.

### 2.5. 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.

Two micrograms of RNA were used to obtain cDNA using the Superscript II reverse transcriptase (Invitrogen). Briefly, RNA was incubated with 1 μl of oligo (dT) 12–18 (0.5 μg/ml) 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 and 2 μl 0.1 M dithiothreitol (DTT) were added, mixed and incubated for 2 min at 42 °C. Then, 1 μl of Superscript II reverse transcriptase was added and the mixture incubated at 42 °C for 50 min. The reaction was stopped by heating at 70 °C for 15 min, and the resulting cDNA was diluted in a 1:5 proportion with DEPC-treated water and stored at −20 °C.

### 2.6. PCR of IL-1β, TNF-α1, IL-11, TGF-β and IL-18

All amplification reactions were performed using 0.5 μl dNTP mix (10 mM each), 0.2 μl Taq polymerase (5 units/μl, Invitrogen), 2.5 μl Taq 10× buffer, 0.75 μl $MgCl_2$ 50 mM, 0.5 μl of each primer (50 μM) and 1 μl of cDNA in a final volume of 25 μl. First, a PCR with primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed with all samples as a positive control for RT-PCR, since GAPDH is constitutively expressed in all organs. These PCR products also allowed the verification that equivalent amounts of cDNA were present in the different samples and therefore amplifications of the different immune genes were comparable among samples.

Primers used for gene amplification, and the sizes of the different PCR products are shown in Table 1. All PCRs were

Table 1
Primer sequences, sizes of PCR products and amplification conditions for the different genes studied

| Gene | Primers | Size of PCR product (bp) | Number of cycles | Cycling conditions | Reference |
|---|---|---|---|---|---|
| GAPDH | F: 5′-ATGTCAGACCTCTGTGTTGG-3′<br>R: 5′-TCCTCGATGCCGAAGTTGTCG-3′ | 514 | 25 | 94 °C 30 s<br>58 °C 30 s<br>72 °C 1 min | [34] |
| IL-1β | F: 5′-AGGGAGGCAGCAGCTACCACAA-3′<br>R: 5′-GGGGGCTGCCTTCTGACACAT-3′ | 353 | 28 | 94 °C 30 s<br>60 °C 30 s<br>72 °C 30 s | [35] |
| TNF-α1 | F: 5′-TTCGGGCAAATATTCAGTCG-3′<br>R: 5′-GCCGTCATCCTTTCTCCACT-3′ | 433 | 10<br><br><br>25 | 94 °C 1 min<br>60 °C 1 min<br>72 °C 20 s<br>94 °C 1 min<br>60 °C 1 min<br>72 °C 20 s + 1 s per cycle | [36] |
| IL-11 | F: 5′-TCAACTCCCTTGAGATGAGACC-3′<br>R: 5′-TCCTGGGAAGACTGTAACACATC-3′ | 271 | 33 | 94 °C 30 s<br>52 °C 30 s<br>72 °C 30 s | [37] |
| TGF-β | F: 5′-AGACTCTGAATGAGTGGCTGCAAG-3′<br>R: 5′-CTCCAAGACCTGTGGAACACAGCA-3′ | 482 | 29 | 94 °C 30 s<br>60 °C 30 s<br>72 °C 1 min | [36] |
| IL-18 | F: 5′-AGCAGCTCCGAATGTAAGGTG-3′<br>R: 5′-AGGCAAAGGTTGCTCCAGTG-3′ | 384 | 35 | 94 °C 20 s<br>60 °C 20 s<br>72 °C 20 s | [38] |

carried out in a Perkin-Elmer 2720 cycles and amplification conditions always consisted in a denaturing step of 94 °C for 5 min followed by the different specific cycling conditions shown in Table 1 followed by a final extension of 7 min at 72 °C. For each gene, after optimising the conditions following protocols described previously and referenced in Table 1, at least two PCRs with different number of cycles were performed in order to determine at which point of the amplification differences were evident among samples. Once the optimal number of cycles (Table 1) was determined, all samples were amplified twice to verify the results. The PCR products (8 μl) were visualised on a 2% agarose gel stained with ethidium bromide. Samples that were to be compared were always run in the same agarose gel. A 100 bp ladder was used as a size marker. The intensity of the amplification bands was estimated using Image Gauge version 4.0 software (Fujifilm). Semi-quantitative analysis of mRNA transcription for each gene was performed relative to the GAPDH expression of the same sample using the formula: intensity of target gene band/intensity of its corresponding GAPDH band. Data were then analysed using Student's *t*-test and differences were considered statistically significant at $p < 0.05$.

## 3. Results

### 3.1. Transcriptional activity of IL-8 after pIL8+ intramuscular injection

After intramuscular injection of pIL8+, an enhanced IL-8 mRNA expression could be detected in the muscle (three to four-fold) and blood (>five-fold) of the injected trout (Fig. 1). These increases, observed after 1 and 3 days, were not observed in either mock-infected controls or in animals injected with the pIL8− control plasmid. In the case of head kidney, spleen and liver, the levels of IL-8 mRNA expression were not different in pIL8+ treated animals than controls.

Infiltration of leukocytes was observed surrounding muscle fibres nearby the inoculation points (Fig. 2). The infiltration was significantly higher in fish inoculated with the pIL8+ plasmid (Fig. 2B) than that observed in those inoculated either with PBS or with the pIL8− control plasmid (Fig. 2A). These infiltrating cells were mainly neutrophils but also some erythrocytes and lymphocytes could be observed (Fig. 2C). In all cases, some muscle fibres showed necrosis due to the needle injury.

### 3.2. Effect of the injection of pIL8+ on the IL-1β and TNF-α1 response to pMCV1.4-G

In the spleen, pMCV1.4-G by itself, at the concentration used, was not capable of significantly inducing IL-1β expression, neither alone nor in combination with the control plasmid pIL8− (Fig. 3A). However, when pIL8+ was administered together with pMCV1.4-G, an increase of IL-1β was observed at days 3 and 7. This increase was significant when compared to mock-injected controls and when compared to the group injected with pMCV1.4-G in combination with the pIL8− control plasmid. At day 3, a significant induction of IL-1β was also observed in fish treated with the pIL8+ plasmid alone.

In the head kidney, a significant IL-1β expression compared to the control group was observed in the groups treated with pMCV1.4-G in combination with pIL8− or with pIL8+ at day 10 (Fig. 3B). The effect observed was not significantly different between these two groups.

The transcription of TNF-α1 was also studied in the injected groups. In the spleen, a significant induction of TNF-α1 was observed at day 3 only in fish injected with pMCV1.4-G and pIL8+, as well as in fish treated with the pIL8+ alone (Fig. 4A). At days 7 and 10, there was still some induction, although it was not significant due to high individual variations.

No effect on TNF-α1 expression was observed in the head kidney at days 3 and 7 post-injection (Fig. 4B). At day 10, all groups treated with pMCV1.4-G showed a significantly higher TNF-α1 expression than controls, although no significant differences were observed among them.

### 3.3. Effect of the injection of pIL8+ on the IL-11 and TGF-β response to pMCV1.4-G

The effect of pMCV1.4-G alone or in combination with IL-8 on two typically inhibitory cytokines (IL-11 and TGF-β) was assayed in the spleen and head kidney of the injected trout. In the spleen, a significant induction of IL-11 mRNA expression, when compared to controls, was observed in all groups at day 3 post-injection (Fig. 5A). Although a higher IL-11 expression was observed when pMCV1.4-G was administered together with pIL8+, this expression was not significantly different than that observed in the group in which pMCV1.4-G was administered with the pIL8− con-

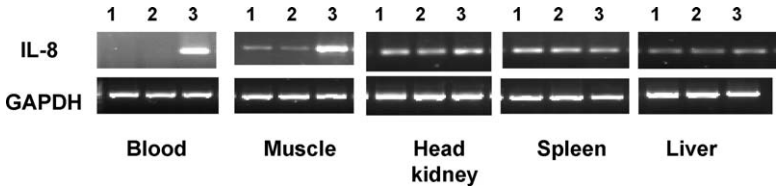


Fig. 1. Expression of IL-8 transcripts in different organs after intramuscular injection of pIL8+ plasmid. Trout ($N = 4$ in each group) were either mock-infected: (1) treated with the pIL8− control plasmid; (2) or with the pIL8+ plasmid; (3) and after either 1 or 3 days, the expression of IL-8 was assayed by RT-PCR. The figure shows the results obtained in a representative fish after 3 days of treatment that were identical to those obtained 1 day post-treatment.

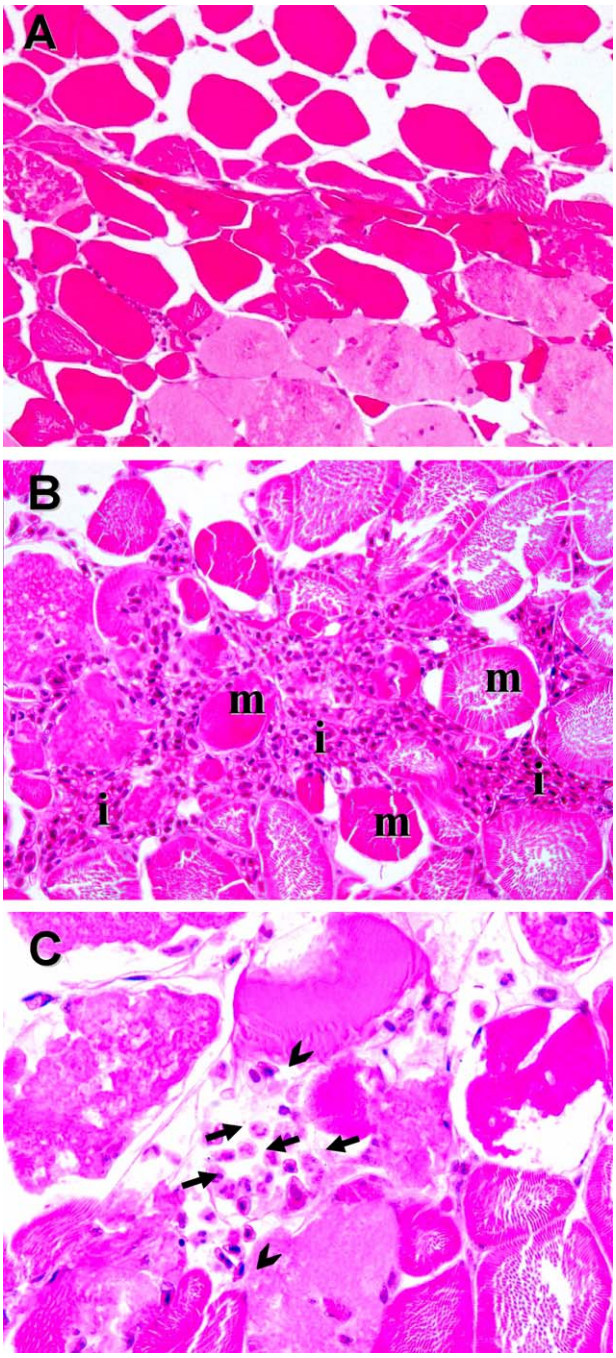


Fig. 2. Histological staining (H&E) showing muscle tissue at the injection site of fish inoculated with pIL8+ plasmid: (A) Control fish sampled at day 3 post-injection. Several necrotic muscular fibres can be observed due to the needle injury but no leukocyte infiltration. Original magnification 200×. (B) Inflammatory infiltration (i) surrounding muscular fibres (m) in the dorsal musculature (inoculation point) of an animal inoculated with pIL8+ and sampled at day 3. Original magnification 200×. (C) Neutrophil (arrows) infiltration together with some erythrocytes (arrowheads) in the dorsal musculature (inoculation point) of an animal inoculated with pIL8+ plasmid after 3 days. Original magnification 400×.

trol plasmid. At day 10, a significant induction of IL-11 was again observed only in the group treated with pMCV1.4-G and pIL8+.

In the head kidney, at day 3, only the group injected with pMCV1.4-G in combination with pIL8+ expressed IL-11 higher than controls (Fig. 5B). At day 10, all groups treated with pMCV1.4-G showed an IL-11 expression significantly higher than controls, although no significant differences were observed among groups.

In the case of TGF-β, after 3 and 7 days, a significant increase in the levels of mRNA expression in the spleen was observed in all groups compared to the control group, including the group treated with pIL8+ alone (Fig. 6A). The TGF-β expression observed at these points in the group in which pMCV1.4-G was co-administered with pIL8+ was significantly higher than that observed in the group in which pMCV1.4-G was administered with pIL8−.

Neither the effect of pMCV1.4-G nor the IL-8 administration was observed in the head kidney at any of the time points screened (Fig. 6B).

### *3.4. Effect of the injection of pIL8+ on the IL-18 response to pMCV1.4-G*

Only the group co-injected with pMCV1.4-G and pIL8+, and the group treated with pIL8+ alone showed a significant up-regulation of IL-18 mRNA expression in the spleen when compared to controls at days 3 and 7 post-vaccination (Fig. 7A).

On the other hand, a significant decrease of IL-18 mRNA levels in comparison to control levels was observed in the head kidney of fish co-injected with pMCV1.4-G in combination with either pIL8− or pIL8+, as well as in the group treated with the pIL8+ plasmid alone (Fig. 7B). At day 10, a significant up-regulation was in all groups injected with pMCV1.4-G.

## 4. Discussion

During the past years, a great number of cytokine sequences have become available in fish allowing not only the study of their regulation at a molecular level, but also their use as molecular adjuvants or immunostimulants. In addition, DNA vaccinology has proven to be very effective in controlling some of the most devastating viral diseased such as rhabdovirus [1–3], however, up to date, there are no studies related to the capacity of fish cytokines to act as molecular adjuvants in viral vaccination. Concerning bacterial vaccination, recombinant carp IL-1β has shown to enhance the antibody response to an *Aeromonas hydrophyla* vaccine [39]. In mammals, the use of chemokines as molecular adjuvants has had a special interest due to their chemoattractant properties [8–10,12–15].

In the current work, we have focused on the effect of the co-administration of a rainbow trout recombinant IL-8

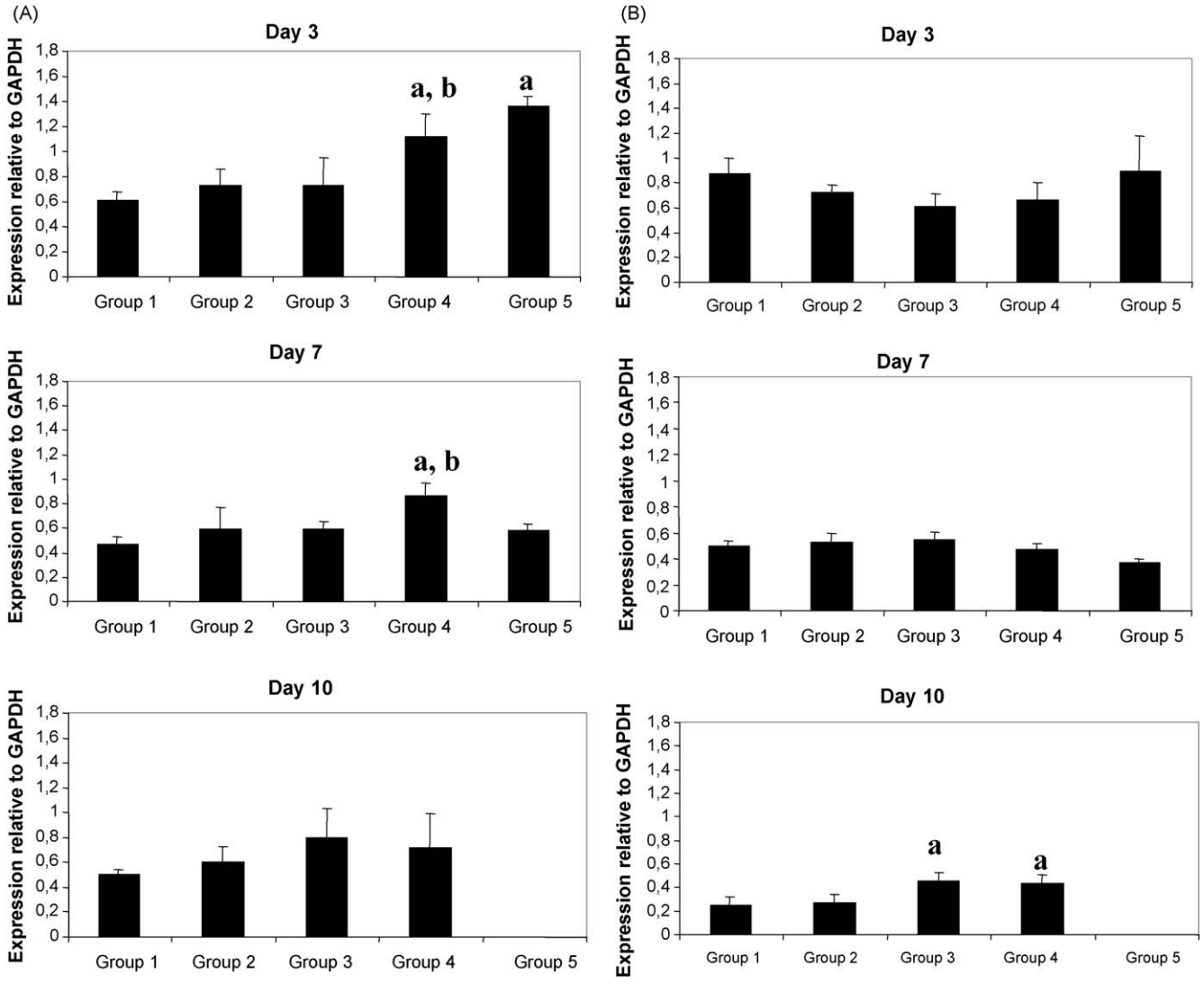


Fig. 3. Effect of IL-8 co-administration on IL-1β expression in response to pMCV1.4-G. Levels of expression of IL-1β in spleen (A) and head kidney (B) of mock-injected controls (group 1), fish intramuscularly injected with 0.5 μg of pMCV1.4-G (group 2), fish injected with 0.5 μg of pMCV1.4-G and 0.5 μg of pIL8− control plasmid (group 3), injected with 0.5 μg of pMCV1.4-G and 0.5 μg of pIL8+ plasmid (group 4) or treated with pIL8+ plasmid alone (group 5). Data are presented as mean relative expression ± S.D. for five individuals from each group. Mean values with an "a" are significantly different than that of control mock-injected fish, while mean values with "b" are significantly different than the group treated with both pMCV1.4-G and the control plasmid pIL8− (group 3).

gene on the non-specific early immune response towards an VHSV DNA vaccine construct. Previous results indicated that IL-8 is induced in response to VHSV infection [32], thus suggesting its role in the defence against this virus. The fact that IL-8 would be capable of modulating the early cytokine response would suggest that, as seen in mammals, this would have consequences on the immunogenicity of a vaccine. We have focused on the effect of IL-8 on two typically pro-inflammatory cytokines such as IL-1β and TNF-α, and a mainly inhibitory cytokine like TGF-β; but we also wanted to include some of the most recently described rainbow trout cytokines such as IL-11 [37], which is also typically inhibitory, and IL-18 [38], since the effect that a DNA vaccine may have on the regulation of these two cytokines has never been studied.

We first determined whether the pIL8+ construct was transcribed only in the muscle or it could travel to other organs. DNA vaccines, once injected intramusculary, are thought to be confined to the muscle [7]. We found that after intramuscular injection of pIL8+, IL-8 transcription could be detected in the muscle, but also in the blood of injected trout for up to 3 days, as was described for other intramuscularly administered chemokines [40], something that would lead to a greater systemic response. We constructed the control plasmid pIL8− in which the IL-8 ORF was cloned in the opposite orientation. This plasmid was tested in parallel, and used as a control for stimulatory effects that could come from CpG motifs that may be present in the plasmid. pIL8− did not have an effect on IL-8 transcription, and therefore constituted a good negative control for further experiments. We have also verified that

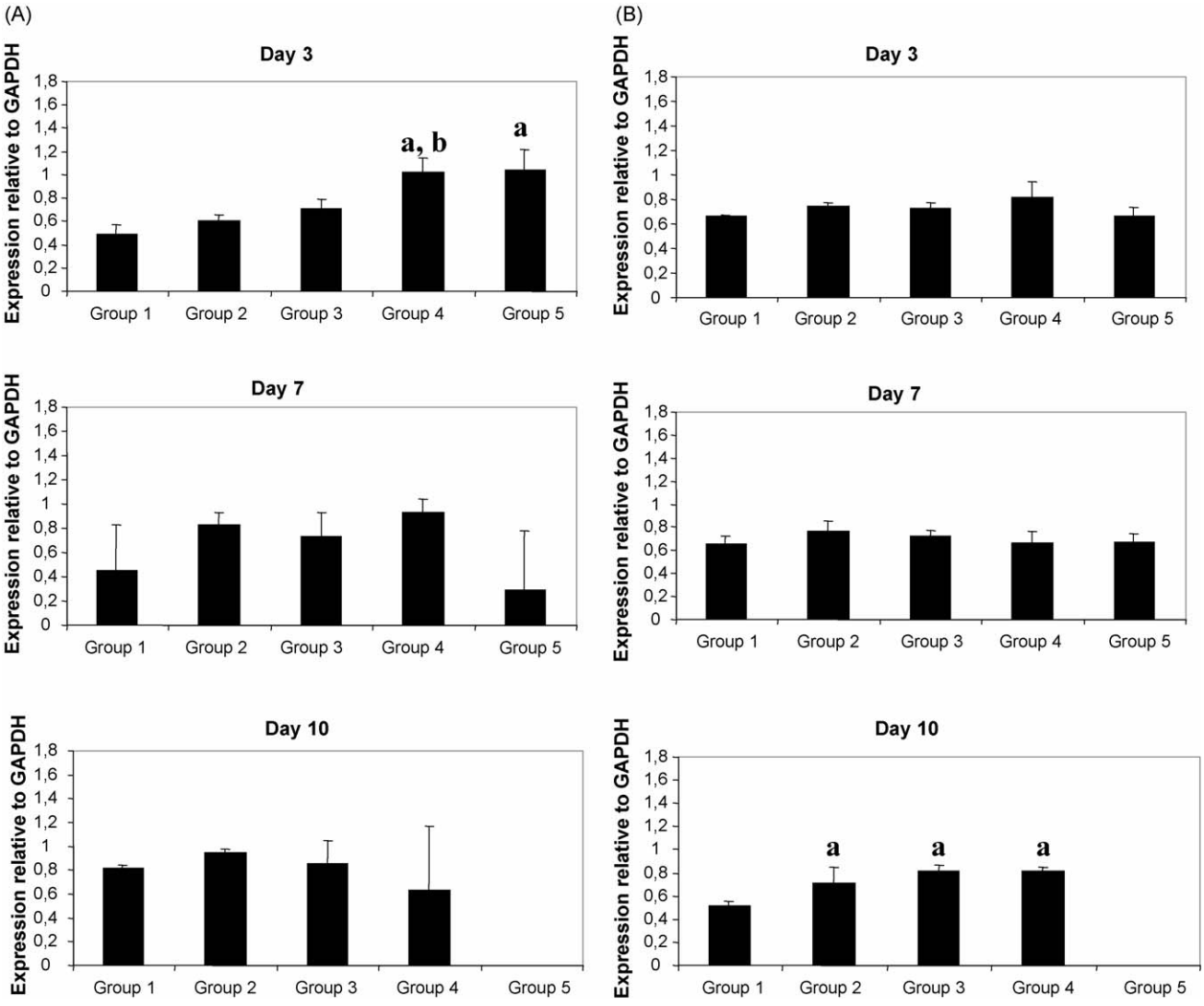


Fig. 4. The effect of IL-8 co-administration on TNF-α1 expression in response to pMCV1.4-G was determined as described in the legend of Fig. 3. Mean values with an "a" are significantly ($p < 0.05$) different than that of control mock-injected fish, while mean values with "b" are significantly ($p < 0.05$) different than the group treated with both pMCV1.4-G and the control plasmid pIL8− (group 3).

when pIL8+ was injected a greater cellular infiltration was produced at the site of inoculation. Again, increased cellular infiltration was not observed with the pIL8− control plasmid. So far, the cells for which IL-8 is chemoattractant have not been identified in fish. In our experiments, a great number of neutrophils were attracted to the site of pIL8+ inoculation as expected, however, we can not conclude that other cell types such as T lymphocytes are not also attracted by this chemokine as seen mammals [23]. Both the transcription of pIL8+ and the attraction of neutrophils to the site of injection suggest that IL-8 is being correctly expressed in trout muscle. Furthermore, fish injected with pIL8+ alone showed a significantly higher IL-1β, TNF-α1, IL-11, TGF-β and IL-18 expression in the spleen than controls at day 3 and also at day 7 in the case of TGF-β and IL-18.

Once its bioactivity was verified, we studied the capacity of pIL8+ to modulate the early cytokine response produced towards a VHSV DNA vaccine construct. We focused on the effect observed in the spleen and head kidney, since these two organs are major sites of VHSV replication [41] and are also the two main lymphoid organs in fish. Since, similar rhabdoviral DNA vaccines [7] nor the pIL8+ are transcribed in these organs, these effects will constitute part of a systemic immune response, that will most probably have effects on protection.

pMCV1.4-G by itself induced IL-1β and TNF-α1 mRNA expression in the head kidney at day 10 post-injection, although for IL-1β, it was only when administered with the control plasmid pIL8−. In previous publications, the induction of IL-1β in the spleen of rainbow trout was demonstrated in response to Poly I:C, a typical INHV DNA vaccine, IHNV [7] or VHSV [32], whereas TNF-α has only been demonstrated to be induced in the spleen of rainbow trout in response to IHNV [7]. In our experiments, pMCV1.4-G by

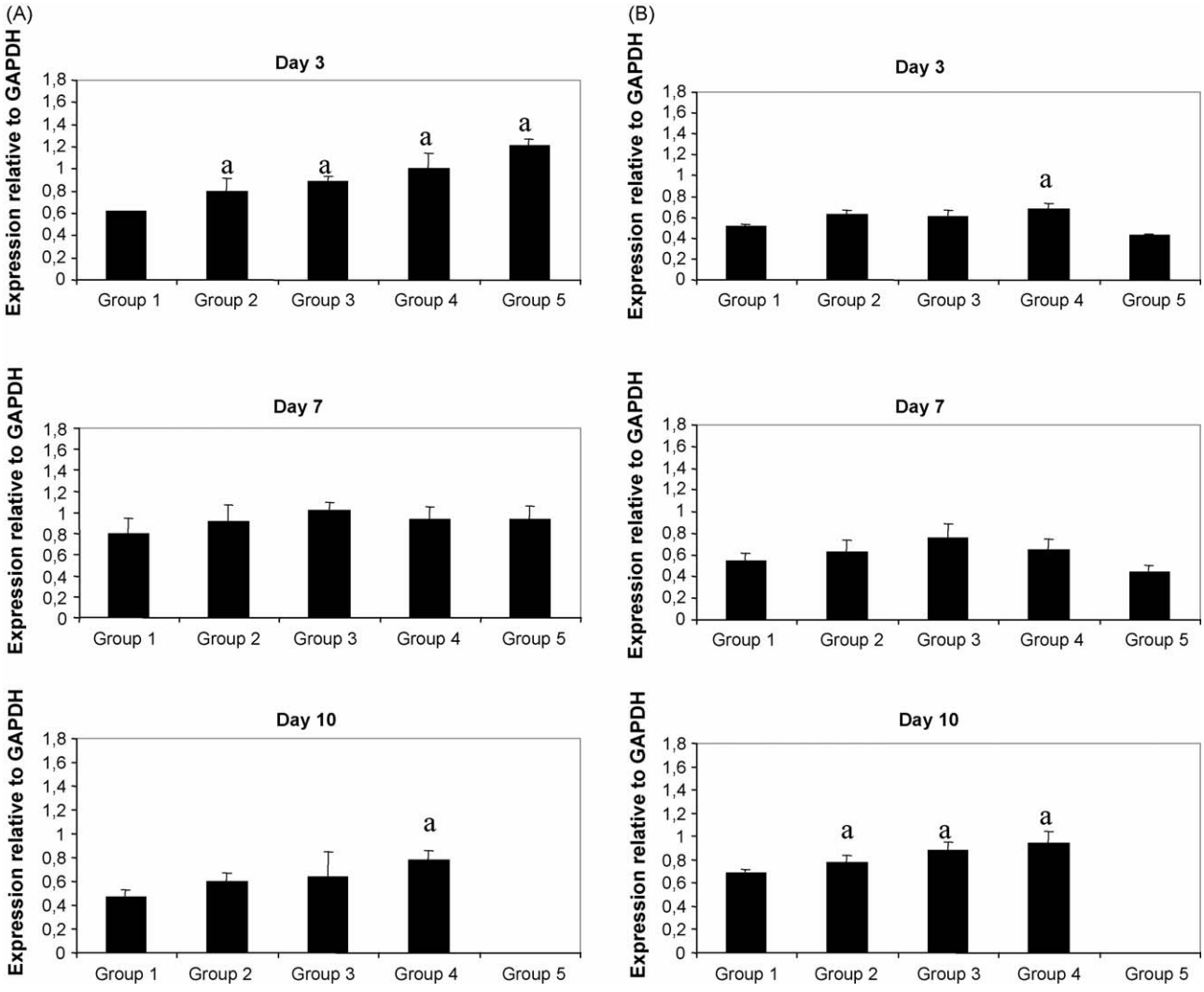


Fig. 5. The effect of IL-8 co-administration on IL-11 expression in response to pMCV1.4-G was determined as described in the legend of Fig. 3. Mean values with an "a" are significantly ($p < 0.05$) different than that of control mock-injected fish, while mean values with "b" are significantly ($p < 0.05$) different than the group treated with both pMCV1.4-G and the control plasmid pIL8− (group 3).

itself was not capable of inducing significant levels of IL-1β. This may be due to differences in promotor and glycoprotein sequences, but it seems probable that this difference is explained by the fact that in our work we reduced the amount of vaccine construct injected from 1 μg to 0.5 μg per fish. However, this lower response allowed us to better interpret whether IL-8 is able to modulate the expression of this and the other cytokines.

We also found an effect of pMCV1.4-G on the expression of IL-11, a cytokine recently identified in rainbow trout *O. mykiss* [37]. Up to date, there is no information concerning the role that this cytokine may play in antiviral defence in fish. In mammals, IL-11 is an anti-inflammatory cytokine, that inhibits the production of key immunostimulatory cytokines by macrophages, including IL-1β, TNF-α and IL-12 [42]. IL-11 is produced in response to virus [43], bacteria [44] and pro-inflammatory cytokines such as IL-1β and TNF-α, or TGF-β [45]. IL-11 also interacts with T cells, mainly decreasing Th1 type cytokines such as IL-12 and interferon-γ (IFN-γ) [46] while enhancing Th2 cytokines such as IL-4 and IL-10 [46,47].

As expected from the results obtained with an G-based IHNV DNA vaccine [7], pMCV1.4-G also induced TGF-β expression in the spleen at days 3 and 7. As IL-11, TGF-β is mainly anti-inflammatory. In mammals, TGF-β inhibits B and T cell proliferation and differentiation, antagonises pro-inflammatory cytokines such as IL-1β, TNF-α and IFN-γ [48], and blocks the expression of IL-1β and IL-2 receptors. TGF-β also has inhibitory effects on neutrophils and macrophages [49,50]. Although mainly inhibitory, it is known that TGF-β, at early stages of infection, can facilitate CD8+T responses such as differentiation [51] and IL-2 secretion [52]. In a previous work, we demonstrated that VHSV at a non-lethal dose induces a similar pattern of TGF-β expres-

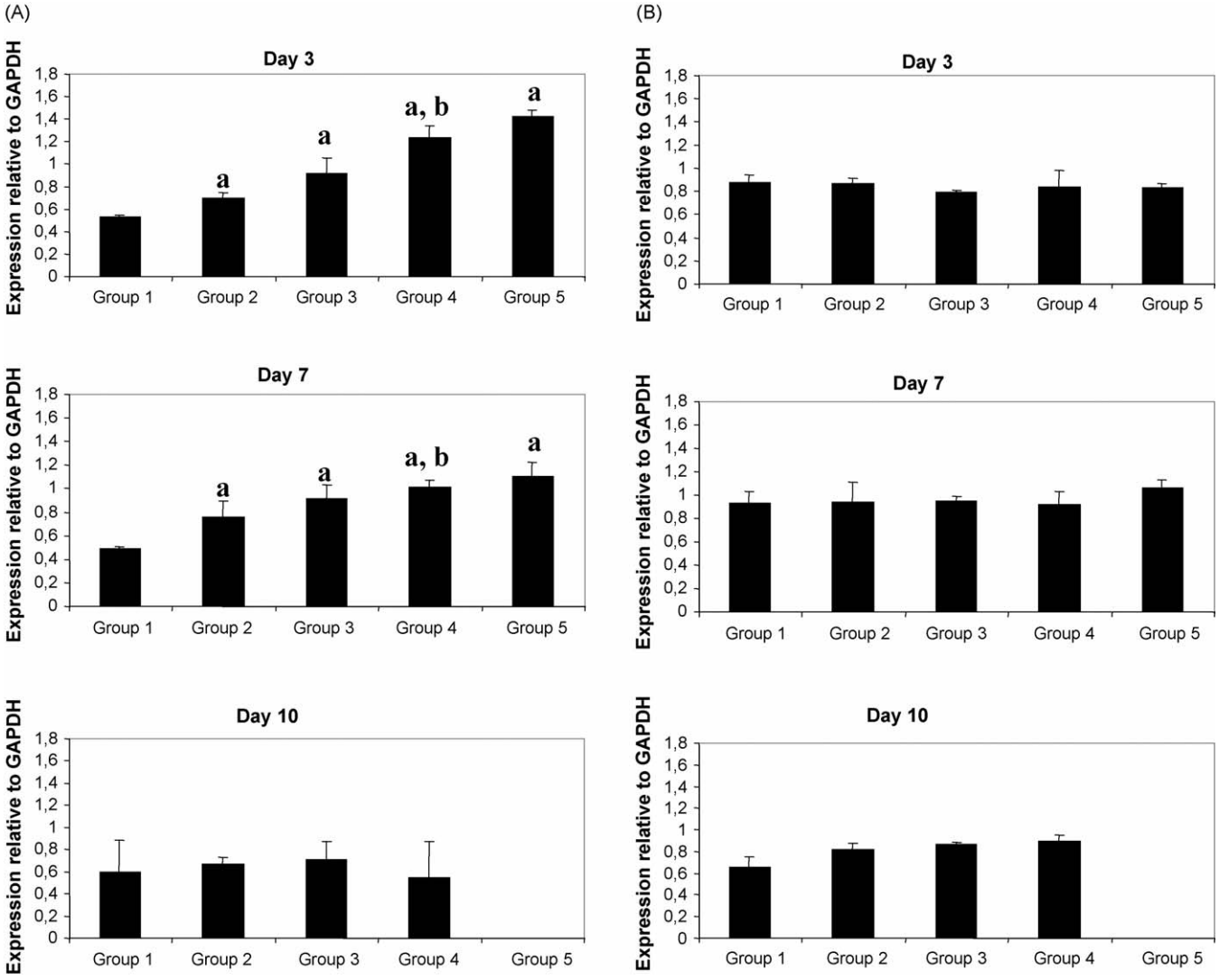


Fig. 6. The effect of IL-8 co-administration on TGF-β expression in response to pMCV1.4-G was determined as described in the legend of Fig. 3. Mean values with an "a" are significantly ($p < 0.05$) different than that of control mock-injected fish, while mean values with "b" are significantly ($p < 0.05$) different than the group treated with both pMCV1.4-G and the control plasmid pIL8− (group 3).

sion than pMCV1.4-G in rainbow trout [32], thus suggesting a positive effect of this cytokine on the protection conferred by the vaccine construct.

The role of IL-18 in antiviral defence has not been yet elucidated in fish [38]. In rainbow trout, an alternative spliced form to that constitutively expressed in the lymphoid organs was detected, and some differences in the pattern of expression were detected between the two forms. For our studies, we have focused on the predominant form of IL-18. Previously called IFN-γ-inducing factor, in mammals, this cytokine is known to induce IFN-γ, TNF-α and GM-CSF production and synergise with IL-12 [53], having effects mainly on Th1 lymphocyte subsets [54]. In the absence of IL-12, it can also stimulate Th2 immune responses [55]. IL-18 also influences neutrophil responses, and is know to induce IL-1, IL-8, TNF-α expression, respiratory burst activity and degranulation in these cells [56]. In mammals, IL-18 production is mostly regulated at a post-transcriptional level through the processing of an inactive precursor [55] and through the production of an inhibitory IL-18 binding protein [57]. In rainbow trout, although in head kidney cells and macrophages, IL-18 expression was not affected by either LPS, Poly I:C or rIL-1β, in the established fibroblastic cell line RTG-2, both Poly I:C and LPS significantly decreased IL-18 expression, while they enhanced the expression of the alternative spliced form, suggesting some kind of transcriptional regulation [38]. In our experiments, pMCV1.4-G by itself was not capable of altering IL-18 expression in the spleen, but significantly down-regulated its expression on head kidney at day 3, whereas it up-regulated it at day 10. The reduction of IL-18 transcription observed at day 3 would most probably go along with an increased transcription of the alternative spliced form, as it happened in RTG-2 cells [38]. In any case, all this data suggest that in the rainbow trout there is tran-

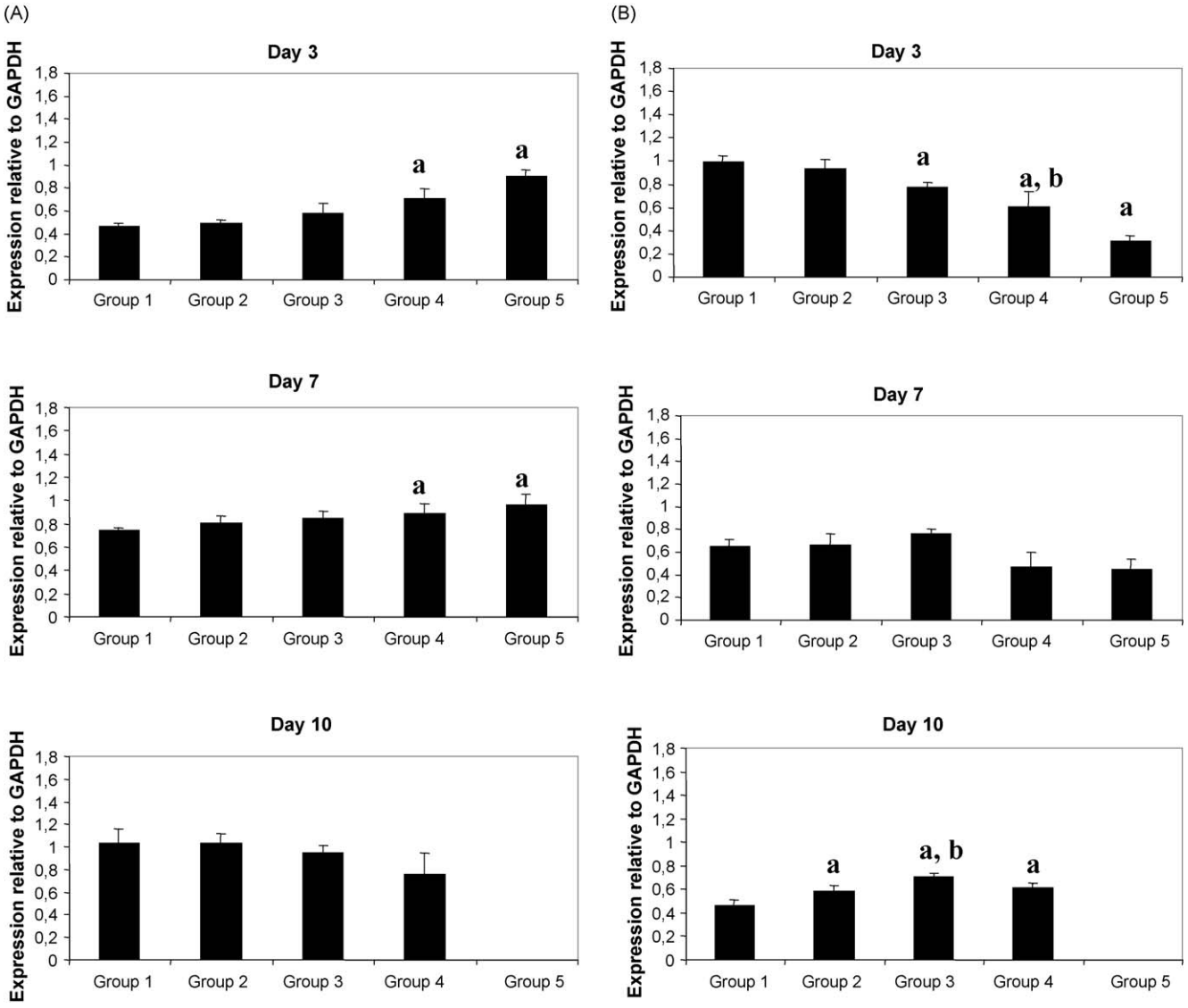


Fig. 7. The effect of IL-8 co-administration on IL-18 expression in response to pMCV1.4-G was determined as described in the legend of Fig. 3. Mean values with an "a" are significantly ($p < 0.05$) different than that of control mock-injected fish, while mean values with "b" are significantly ($p < 0.05$) different than the group treated with both pMCV1.4-G and the control plasmid pIL8− (group 3).

scriptional regulation of IL-18, as suggested by other authors [38]. The exact function of the two alternative spliced forms of IL-18 must be elucidated in order to understand its role in disease and vaccination.

This early cytokine response induced by pMCV1.4-G was altered when IL-8 was co-administered by the pIL8+ plasmid. On one side, we have the effects that are significantly higher than those obtained in the group in which pMCV1.4-G was co-administered with the control plasmid pIL8−. In this case, we have an up-regulation of IL-1β, TNF-α1 and TGF-β in the spleen at day 3, of IL-1β, and TGF-β in the spleen at day 7, and an up-regulation of IL-18 at day 10 in the head kidney. It would be expected that this higher expression of pro-inflammatory cytokines would have beneficial effects on protection. In the case of IL-1β, it has been shown to enhance the resistance towards VHSV infection [58]. In rainbow trout, two different TNF-α isoforms have been identified [59]. We studied the expression of TNF-α1, since a previous study obtained a higher up-regulation of TNF-α1 in response to IHNV [7]. In any case, similar functions have been observed for both isoforms, known to induce IL-1β, IL-8, cyclooxygenase (COX-2), and both TNF-α1 and TNF-α2 [35].

On the other hand, we have effects of pIL8+ that were not significantly higher than those observed in the group injected with pMCV1.4-G and pIL8−, but that were significantly higher than mock-injected controls at time points at which pMCV1.4-G with or without pIL8− was not capable of inducing a significant response. Although not statistically different, these results should be pointed out. In this sense, we have an up-regulation of IL-18 at days 3 and 7 in the

spleen, and an up-regulation of IL-11 at day 10 in the spleen and at day 3 in head kidney. In the case of IL-18, we still do not know whether an increase or decrease (as seen in the head kidney at day 3) goes along with a higher IL-18 activity, as the activity reported in mammals, since post-trascriptional regulation can not be excluded.

In conclusion, we have demonstrated that the co-administration of IL-8 with a viral DNA vaccine modulates the cytokine response that is induced, mainly having its effect over pro-inflammatory cytokines (IL-1β and TNF-α1), with a greater impact on the spleen, and to a lesser extent in the head kidney. More work should be performed to determine whether this modulation has a beneficial effect on protection as seen in other viral models in mammals [12,26].

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