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Interleukin 8 and CK-6 chemokines specifically attract rainbow trout (*Oncorhynchus mykiss*) RTS11 monocyte–macrophage cells and have variable effects on their immune functions

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Summary
In the current work, we have demonstrated that both rainbow trout (*Oncorhynchus mykiss*) interleukin 8 (IL-8), a CXC chemokine, and CK-6, a CC chemokine, are able of efficiently attracting RTS11, a rainbow trout established macrophage–monocyte-like cell line. Interestingly, two sub-populations of non-adherent cells are distinguishable by flow cytometry that could be identified as immature monocyte- and mature macrophage-like populations, respectively, and the two chemokines studied exert their effects on different populations. Although IL-8 specifically attracts the monocyte-like sub-population, CK-6 specifically attracts the macrophage-like cell sub-population. We have also determined the effects of both of these chemokines on RTS11 phagocytosis, respiratory burst and the expression of other immune-related genes. We found that IL-8 inhibited the phagocytosis capacity of RTS11 cells belonging to the macrophage-like profile. No effect was observed, however, on the respiratory burst, immune function that has been considerably affected throughout the establishment of the cell culture. Concerning the effect that IL-8 and CK-6 have on the expression of other immune genes, we found that IL-8 significantly induced the levels of expression of CK-6, IL-8, pro-inflammatory cytokines such as IL-1β and tumour necrosis factor α (TNF-α) of RTS11 cells. On the other hand, CK-6 induced the levels of expression of IL-8, iNOS and the integrin CD-18, while it had very faint effect on pro-inflammatory cytokines. These results constitute one of the very few studies in which the effect of IL-8, a CXC chemokine, on monocyte-like cells is described. Moreover,

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it demonstrates that different monocyte–macrophage sub-populations have different reactivity to different chemokines.
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Introduction

Among cytokines, the chemokine superfamily (cytokines with chemoattractant properties) is defined by the presence of four conserved cysteine residues that form a Greek-key structure by disulphide bonding [1]. Depending on the arrangement of the first two conserved cysteines in their sequence, chemokines are divided into four sub-families: CXC (α), CC (β), C and CX_3C classes, which in mammals have 17, 28, 2 and 1 known members, respectively. The CXC sub-family can be further subdivided into those that contain the amino acid motif ELR and those few that do not [2]. Generally, CXC chemokines with the ELR motif attract neutrophils but not monocytes/macrophages, whereas CC chemokines attract monocytes and not neutrophils [3]. Both types of chemokines can further attract other cell types such as lymphocytes [4]. However, in the literature, we can find some exceptions to this general rule, as some studies have pointed out to a chemoattractant and immunomodulatory capacity of interleukin 8 (IL-8), a typical ELR CXC chemokine on monocytes. Although the effects are always less rapid and less pronounced than those observed in neutrophils [5], probably due to the low binding affinity of the CXCR1 and CXCR2 IL-8 receptors expressed in monocytes [6], IL-8 was shown to induce a rise of the intracellular cytosolic-free calcium and the respiratory burst [5]. Although not exclusively mediated by chemotaxis, IL-8 has also been shown to trigger the firm adhesion of monocytes to the endothelium [7], suggesting a major role in the recruitment of blood monocytes into tissues. In this sense, IL-8 produced in the intestinal mucosa has been shown to be responsible for the recruitment of blood monocytes, which maintain the macrophage population in the mucosa [8]. Also, IL-8 produced inside some types of tumours has been shown to be responsible for the retention of monocyte-derived dendritic cells inside the tumour [9], and moreover, IL-8 has been shown to induce changes in macrophages to decrease their susceptibility to mycobacteria, demonstrating the interaction of IL-8 with receptors in mature macrophages as well [10].

In rainbow trout (*Oncorhynchus mykiss*), different CXC chemokine genes have been identified up to date: IL-8 [11–13], a non-ELR CXC chemokine related to the IFN-γ inducible chemokines named gammaIP [14], and the recently described CXCd family in which a novel gene CXCd1 and a duplicate CXCd2 have been identified [15]. Concerning trout CC chemokines, Laing and Secombes [16], searching within EST databases, identified 15 new CC chemokine sequences, bringing the total to 18 including the previously described CK1 [17], CK2 [18] 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. Some of these genes (CK4, CK5, CK7, CK8 and CK12) were found to be either closely related or duplicated (A and B), so in fact the different CC chemokines go from CK1 to CK12 [16]. Up to date, neither C nor CX_3C chemokines have been identified in fish, although in fish species such as zebrafish, channel catfish and pufferfish a great number of chemokine genes have been identified [19].

In the current work, we have used the rainbow trout monocyte–macrophage-established cell line RTS11 [20] to assay the chemoattractant capacity of two chemokines, representative of the CXC and CC families. As a model CXC chemokine, we have used IL-8. The chemoattractant capacity of rainbow trout IL-8 for neutrophils has been previously demonstrated indirectly, since the intramuscular injection of fish with an expression vector coding for IL-8 induced a massive neutrophil infiltration at the injection site [21]. Recently, the rainbow trout recombinant IL-8 was produced and used to establish the capacity of this chemokine to attract head kidney leukocytes *in vitro* and *in vivo* [22]. However, in that study, while the migration of neutrophils towards IL-8 was clearly demonstrated, no evidences of the attraction of monocytes or lymphocytes were elucidated. Thus, the capacity of IL-8 to attract and immunomodulate monocytes remained unclear in fish. On the other hand, as a model CC chemokine, we have used CK-6, a rainbow trout CC chemokine previously identified [16], which based on the phylogenetic studies performed with all vertebrate CC chemokines fell into the "inducible" category thought to play a more active role in inflammation, in contrast to "constitutive" CC chemokines. Trout CK-6 did not show a distinct phylogenetic relation to any human chemokine when a phylogenetic tree showing all vertebrate CC chemokines was performed [16], and formed a fish-only clade in which its sequence appeared alone with what had been named as flounder MIP1α [23]. Both the phylogenetic grouping and the fact that its expression was induced in RTS11 cells in response to tumour necrosis factor α (TNF-α) suggested a possible role in the inflammatory process [16], which we confirm in our study. These studies point out the utility of the established monocyte–macrophage RTS11 cell line to study chemokine activities. In addition to the chemoattractant capacities, we have also studied the effect that these two chemokines have on other immune functions of these RTS11 monocytes: the phagocytic capacity, the respiratory burst and the expression of other immune genes.

Materials and methods

Cell culture

RTS11, a continuous rainbow trout monocyte–macrophage-like cell line, originally isolated from a long-term spleen hematopoietic culture [20] was maintained at 18 °C in Leibovitz medium (L-15, Gibco, Invitrogen, UK) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and 15% foetal calf serum (FCS, Gibco). Cells were grown at

a high cell density and passaged at a 1:2 ratio approximately once a month as described previously [20].

Flow cytometric analysis of RTS11 cells

RTS11 cells were analysed by flow cytometry using a FACS Calibur flow cytometer (Becton Dickinson) using CellQuest™ software (Becton Dickinson). The cells were suspended in L-15 medium supplemented with 15% FCS, and the instrument settings were held constant throughout all experiments. The two sub-populations of non-adherent cells that could be identified based on forward and side scatter parameters were sorted using the FACS Aria cell sorter (Becton Dickinson), and stained with Giemsa according to standard procedures.

Recombinant rainbow trout chemokine production

The coding sequences of both rainbow trout IL-8 [14] and CK-6 [16] were cloned into the pRSET A (Invitrogen) plasmid using designed primers in which EcoRI and BamHI restriction sites were included. The pRSETA vectors obtained produced fusion proteins in which an N-terminal 6x His-tag allowed the purification of the recombinant chemokines over a Ni-sepharose affinity column. The plasmids were transformed into *Escherichia coli* DH5α competent cells (Invitrogen), the plasmid DNA extracted using an Invitrogen miniprep kit, and sequenced to confirm that no errors had been introduced.

These constructs were used to transform BL21(DE3)pLysS chemically competent *E. coli* cells (Invitrogen). Cultures were grown in TB broth with ampicillin (50 mg/ml) at 37 °C with constant agitation. For chemokine expression, exponentially growing cultures received 1 mM IPTG following the manufacturer's instructions and the cultures were incubated overnight at 28 °C. The resulting bacterial pellets were resuspended in lysis buffer (20 mM Na_2HPO_4, 0.5 M NaCl, 6 M guanidine HCl, pH 7.8) and sonicated until clear lysates were obtained. The lysates were passed twice through a 3 ml Ni affinity column (ProBond™, Invitrogen), washed and eluted using 250 mM imidazole. The 280 nm absorbance peak (5–10 ml) was pooled and dialysed against distilled water (four changes of 1 l each over 1–2 days). Under these conditions, a precipitate appeared that was separated from the supernatant by centrifugation at 10,000*g* × 10 min. The pellets of precipitates were resuspended in distilled water at 0.5–1 mg of protein per ml and kept frozen until used. Protein concentrations were determined by absorbance at 280 nm and the purity and size of the chemokines confirmed by SDS-PAGE. The protein concentrations of these precipitates were determined by absorbance at 280 nm and the purity and size of the chemokines confirmed by SDS-PAGE. In both IL-8 and CK-6, a band corresponding to the monomer chemokine was the predominant chemokine form, although bands that may correspond to dimer and tetramer forms of the chemokines were also observed.

As a control, BL21(DE3)pLysS chemically competent *E. coli* cells were also transformed with the empty pRSET A plasmid. This culture was grown, lysated and passed through the column in the same conditions as those used to obtain the chemokines. The eluate (mock-eluate) was adjusted to the same protein concentration and also tested for chemoattractant activity.

Chemotaxis experiments

The chemotactic capacity of RTS11 cells towards rainbow trout CK-6 and IL-8 was assayed in chemotaxis chambers introduced in 24-well plates (Costar-Corning Life Sciences). For this, RTS11 cells were pelleted at 500*g* for 15 min and adjusted to a concentration of 1×10^6 cells/ml. Six hundred microliters of L-15 medium supplemented with 15% FCS containing varying amounts of the different recombinant chemokines were placed in the wells. Controls with media alone were always included in the experiments, whereas the mock-eluate control (eluate obtained after transfection of BL21 bacteria with the empty pRSET A plasmid) was included only in some of the preliminary experiments, until it was demonstrated that this eluate had no increased chemotactic activity than the control consisting in media alone at any of the protein concentrations tested (1500, 150, 15 and 1.5 ng/ml). After introducing the chemotaxis chambers in each of the wells, 100 μl of the RTS11 cell suspension was loaded to the upper part of the chamber. The upper and lower chambers are separated by a 5 μm pore-sized polycarbonate filter. After 60 min of incubation at 20 °C, the number of cells that had migrated to the bottom of the wells was quantified by flow cytometry (FACS Calibur, Becton Dickinson). Cell number was determined at constant flow time (1 min) of the medium in the lower chamber. The migrating cells were analysed based on forward and side light scatter parameters. All experiments were performed in duplicate, and were repeated twice.

In some experiments, the same concentration of either IL-8 or CK-6 in the lower compartment was added in the upper compartment where the RTS11 cells were located. As described previously [24,25], this enables us to distinguish between real chemotaxis and chemokinesis (random migration). In the case of a specific migration being mediated by a specific receptor, the expectation would be that the addition of the chemokine in the upper compartment would significantly block migration, since the chemokine in the upper compartment would bind to the receptors present in the cells, and moreover, the cells would not have a chemokine gradient to migrate to.

Effect of CK-6 and IL-8 on the RTS11 phagocytic capacity

The ability of the chemokines to modulate the capacity of RTS11 cells to phagocytose *Saccharomyces cerevisiae* (strain S288C) was studied under two different exposure regimens. In a group pf experiments, RTS11 cells were pre-incubated for 24 h with different doses of recombinant IL-8 or CK-6, while in another group of experiments the different chemokine doses were added simultaneously with the labelled yeast. In the first case, RTS11 cells were pelleted at 500*g* for 15 min and adjusted to a concentration of 1×10^6 cells/ml with L-15 supplemented with 15% FCS. After the addition of different doses of rainbow trout IL-8 (150 and 15 ng/ml) and CK-6 (1500 and 150 ng/ml), the cells were incubated at 20 °C for 24 h, and at this point the

phagocytosis assay conducted. In another group of experiments, the RTS11 cells were pelleted and adjusted to 1×10^6 cells/ml. At this point, immediately after the addition of the different chemokine concentrations, the labelled yeast was added and the phagocytic assay performed.

In all cases, phagocytosis samples consisted of labelled-yeast cells and RTS11 cells (10 yeast: cells). Samples were mixed, centrifuged (500*g*, 5 min, 20 °C), resuspended in L-15 and incubated at 20 °C for 30 min. At the end of the incubation time, the samples were placed on ice and 400 μl ice-cold PBS was added to each sample to stop phagocytosis. The fluorescence of the extracellular yeasts was quenched by adding 40 μl ice-cold trypan blue (0.4% in PBS). Standard samples of FITC-labelled *S. cerevisiae* or RTS11 cells were included in each phagocytosis assay. Controls also consisted of RTS11 cultures in which the yeast cells were added at the end of the incubation time and just before the readings. Samples were acquired using a FACS Calibur flow cytometer and the phagocytic ability was defined as the percentage of cells with ingested yeast cells (green-FITC fluorescent cells).

Effect of rainbow trout CK-6 and IL-8 on the RTS11 respiratory burst

Two types of experiment were conducted. In a group of experiments, RTS11 cells were pre-incubated for 24 h with different doses of recombinant IL-8 or CK-6, while in another group of experiments the different chemokine doses were added simultaneously to the addition of phorbol myristate acetate (PMA, Sigma-Aldrich), used to trigger the respiratory burst.

The respiratory burst activity was measured using nitroblue tetrazolium (NBT, Sigma-Aldrich). In the case of RTS11 cells that had been previously incubated with different chemokine doses, RTS11 were washed with phenol-red free HBSS (pH 7.1, Gibco, Invitrogen) and then exposed to HBSS containing NBT (1 mg/ml) and PMA (1 μg/ml). Control cells treated with NBT in the absence of PMA were also included to verify that the respiratory burst had been effectively triggered. In the case of including the chemokines simultaneously to the addition of PMA, the different chemokine doses were added to the HBSS containing NBT and PMA. In all cases, the reaction was stopped by fixing the cells with 100% methanol for 5 min. After two washes in 70% methanol, the plates were air-dried. The reduced formazan in each well was dissolved using 120 μl KOH (0.112 g/ml) and 140 μl dimethyl sulfoxide (Sigma-Aldrich). The reduction of NBT was then measured at 620 nm using a plate reader. All experiments were carried out in triplicate.

Effect of CK-6 and IL-8 on the expression of other immune-related genes

The expression of different immune genes was determined by RT-PCR. RTS11 cells were treated with different concentrations of CK-6 or IL-8 and incubated for 18 h at 18 °C. Control wells not treated with the chemokines were also included. Total RNA was extracted from RTS11 cells using Trizol (Invitrogen) following the manufacturer's instructions. One microgram of RNA was 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 deoxynucleotide triphosphate (dNTP) mix for 5 min at 65 °C. After incubation, 4 μl of 5 × first-strand buffer (250 mM Tris–HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) and 2 μl 0.1 M dithiothreitol 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 diluted 1:10 and stored at −20 °C.

All amplification reactions contained 200 μM of each dNTP, 1 unit of Taq polymerase (Invitrogen), 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 1 μM of each primer and 1 μl of cDNA in a final volume of 25 μl. A PCR with primers for glyceraldehyde 3-phosphate dehydrogenase (GADPH) was performed with all samples as a positive control for RT-PCR, to confirm that all samples contained the same amount of cDNA.

Primers used for gene amplification and references are shown in Table 1. All PCRs were carried out in a Perkin Elmer 2400 cycler and all samples were amplified twice to verify the results. PCR products (8 μl) were visualised on 1–2% agarose gels stained with ethidium bromide. Samples to be compared were always run in the same agarose gel and a

Table 1 Summary of primers used in this study.

Gene	Primers	Reference
GAPDH	F: 5′ ATGTCAGACCTCTGTGTTGG 3′ R: 5′ TCCTCGATGCCGAAGTTGTCG 3′	[38]
IL-8	F: 5′ GAATGTCAGCCAGCCTTGTC 3′ R: 5′ TCCAGACAAATCTCCTGACCG 3′	[11]
CK-6	F: 5′ CGAATCTGCTCTGACACTTCC 3′ R: 5′ TGGTGAGTTGTTGACCATTGA 3′	[16]
IL-8R	F: 5′ GGTGTTAGGAGAATGTCTTG 3′ R: 5′ CAGGGACTGTTGACTGAAGC 3′	[36]
IL-1β	F: 5′ AGGGAGGCAGCAGCTACCACAA 3′ R: 5′ GGGGGCTGCCTTCTGACACAT 3′	[39]
iNOS	F: 5′ CATACGCCCCCAACAAACCAGTGC 3′ R: 5′ CCTCGCCTTCTCATCTCCAGTGTC 3′	[40]

100 bp ladder was used as a size marker. The intensity of the amplification bands was estimated using ImageJ 1.33 software. Semi-quantitative analysis of mRNA transcription for each gene was performed relative to the GADPH expression of the same sample using the formula: intensity of target gene band/intensity of its corresponding GADPH band.

Statistics

Data were statistically analysed by one-way analysis of variance (ANOVA) and Tukey's comparison of means when necessary. Differences were considered statistically significant when $P<0.05$.

Results

Flow cytometric analysis of the different RTS11 sub-populations

Before performing any of the studies concerning the effects of IL-8 and CK-6 on the RTS11 cell line, we analysed the presence of different sub-populations within the cultures. As described before [26], some adherent cells are always observed in the cultures and contrast with the major non-adherent population (Figure 1A). The proportion of adherent cells decreases with the passage of cell cultures, and can be increased through the supplementation with additional FCS [26]. However, due to the small relative proportion of these adherent cells, all our future studies were performed with the non-adherent population. Within these cells, two different sub-populations could be identified by flow cytometric analysis based on forward and side light scatter analyses (Figure 1B). These sub-populations were designated as R1 and R2, where R1 cells have a typical profile of immature monocytes and R2 cells are more complex cells which could be considered as mature macrophages, as has been done previously in other macrophage–monocyte fish cultures [27,28]. The viability of these two sub-populations was confirmed by the addition of propidium iodide and cell viabilities of more than 95% were always obtained for both sub-populations. In addition, we performed Giemsa staining of sorted cells by FACS based on forward and side light scatter profile. R1 cells, which constitute around 13.8% of the population (SD $\pm$ 2.5), have a typical progenitor monocyte morphology. They are small cells (3–8 μm) with a high nucleus to cytoplasm ratio. By contrast, the R2 population represents 71.2% of the non-adherent cells (SD $\pm$ 5.2), with a typical mature macrophage morphology (large irregular cells with abundant cytoplasm and extensive vacuolisation) (Figure 1B). This finding allowed us to further analyse the chemoattractant properties of the two chemokines on the two different sub-populations, R1 and R2.

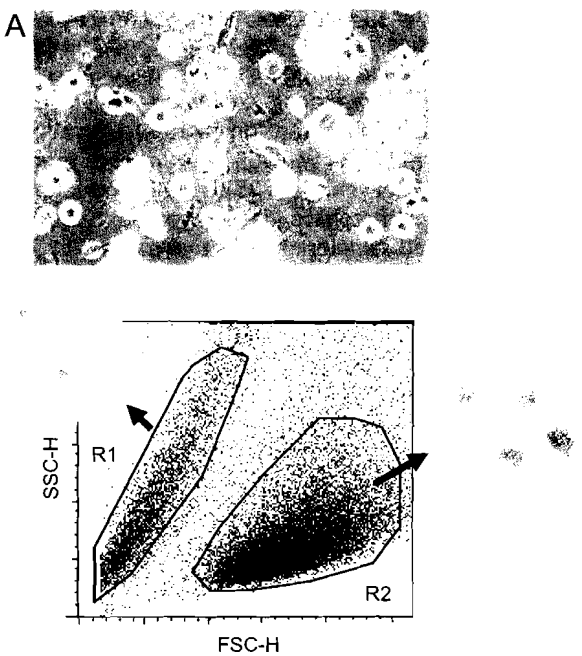

Figure 1 Different sub-populations present in RTS11 cell cultures. (A) Two populations can be clearly distinguished by microscopy analyses: a major round non-adherent cell population that fails to adhere to bare plastic and a population that strongly adheres to bare plastic and shows a mixture of round and spreading cells. (B) FACS analysis of the non-adherent cells shows two different sub-populations based on forward and side scatter parameters. The morphology of the two non-adherent sub-populations was also examined by microscopy in sorted sub-populations after a Giemsa staining. Representative photomicrographs of cells are shown in flow cytometry gates for each sub-population.

Rainbow trout IL-8 and CK-6 specifically attract different RTS11 sub-populations

Rainbow trout IL-8 significantly attracted RTS11 cells (Figure 2A) in a dose-dependant manner. Both the 150 and 15 ng/ml doses had a significant effect ($p< 0.05$) on the migration of RTS11 cells. Higher doses had a lower effect on migration (data not shown). The eluate obtained after transfection of BL21 bacteria with the empty pRSET A plasmid (mock-eluate) did not shown a chemoattractant capacity different from that observed for culture media alone, thus in all the following experiments only culture media was included as a control. When the two non-adherent sub-populations in RTS11 cultures were examined for their migratory capacity towards IL-8, only R1 cells were found to be significantly attracted by IL-8 (Figure 2B). This sub-population corresponds to a more immature monocyte cell type. Moreover, when IL-8 was also added in the upper compartment of the chemotaxis chambers, to desensitise the receptors, the migration of this R1 sub-population was significantly inhibited, indicating a real chemotaxis activity in contrast to chemokinesis (random migration).

CK-6 at 150 and 1500 ng/ml also induced a significant migration of RTS11 cells (Figure 3A). When the migratory capacity of R1 and R2 sub-populations in RTS11 cultures were compared, both sub-populations migrated towards CK-6 (Figure 3B). However, when the receptors were desensitised by the addition of CK-6 in the upper compartment of the chemotaxis chambers, only the migration of the R2 sub-population was significantly inhibited, whereas the migration of the R1 population was not affected by the desensitisation. This result indicates that the R2 population is the one being specifically attracted by CK-6

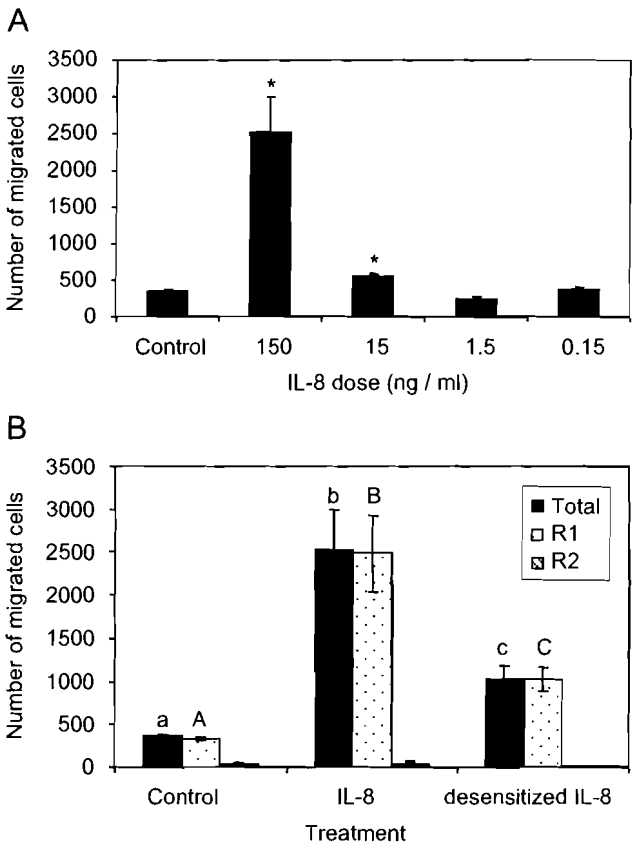

Figure 2 The effect of rainbow trout IL-8 on the migration of non-adherent RTS11. The chemotaxis assay was performed as described in Materials and methods and FACS analysis used to enumerate the number of migrating cells, which were recorded as total non-adherent RTS11 (A and B) or as RTS11 sub-populations, R1 (immature monocytes) and R2 cells (mature macrophages) (B). In (A) migration towards different IL-8 concentrations in the lower chamber is shown and in (B) migration towards 150 ng IL-8/ml in the lower chamber (IL-8) or in both lower and upper chambers (desensitized IL-8) is shown. The mean number of migrated cells (*y*-axis) with standard deviation for 2 wells of each treatment (*x*-axis) is plotted for one of two similar experiments. ANOVA on the data in A and on the three sets of data in B was significant ($p<0.05$). The means were then compared with the control in A and asterisks show those means significantly different from the control, or among each other in B by Tukey's comparisons test. In this case, different letters (lower case for total cells and upper case for R1) identify treatment means that were significantly different from each other ($p< 0.05$).

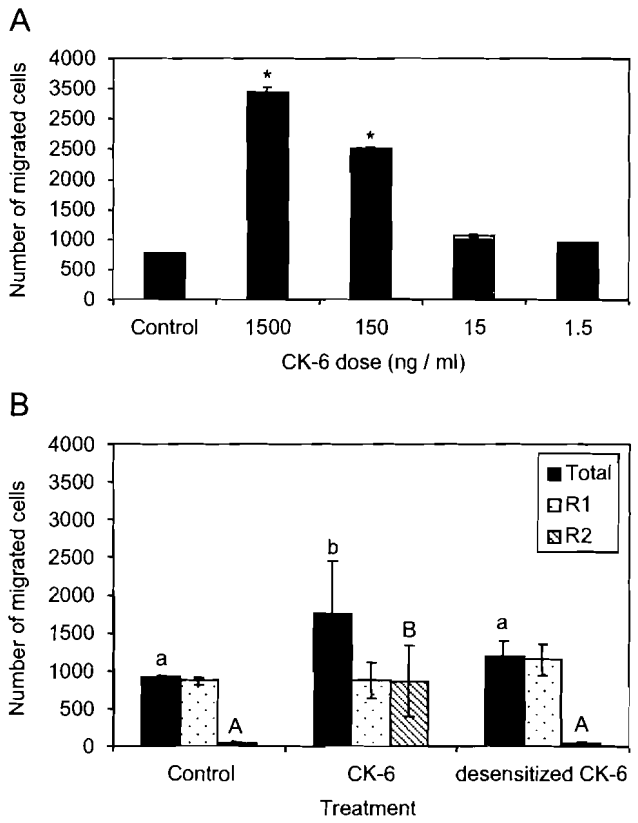

Figure 3 The effect of rainbow trout CK-6 on the migration of non-adherent RTS11. The chemotaxis assay was performed as described in Materials and methods and FACS analysis used to enumerate the number of migrating cells, which were recorded as total non-adherent RTS11 (A and B) or as RTS11 sub-populations, R1 (immature monocytes) and R2 cells (mature macrophages) (B). In (A) migration towards different CK-6 concentrations in the lower chamber is shown and in (B) migration towards CK-6/ml in the lower chamber (CK-6) or in both lower and upper chambers (desensitized CK-6) is shown. The mean number of migrated cells (*y*-axis) with standard deviation for two wells of each treatment (*x*-axis) is plotted for one of the two similar experiments. ANOVA on the data in A and on the three sets of data in B was significant ($p<0.05$). The means were then compared with the control in A and asterisks show those means significantly different from the control, or among each other in B by Tukey's comparisons test. In this case, different letters (lower case for total cells and upper case for R2) identify treatment means that were significantly different from each other ($p< 0.05$).

(real chemotaxis), and the migration of R1 cells seen in this case is due to random chemokinesis. Thus, IL-8 attracts monocyte-like cells, whereas CK-6 attracts macrophage-like cells.

Rainbow trout CK-6 and IL-8 inhibit the RTS11 phagocytic capacity

We examined the effect that these two chemokines might have on the phagocytic capacity of RTS11 cells. When characterising the phagocytic capacity of these cells, what we observed is that only the R2 sub-population was phagocytosing significantly. After 30 min of incubation of the cell cultures with the yeast particles, the percentage of phagocytic cells within the R1 sub-population was 13–25% depending on the experiments; however, incubating further the cells with the yeast particles did not increase the uptake (data not shown).

Once this was established, we determined the effect of the two chemokines on the phagocytic capacity of RTS11 cells. Concerning IL-8, what we observed is that the phagocytic capacity of the R2 sub-population of RTS11 cells

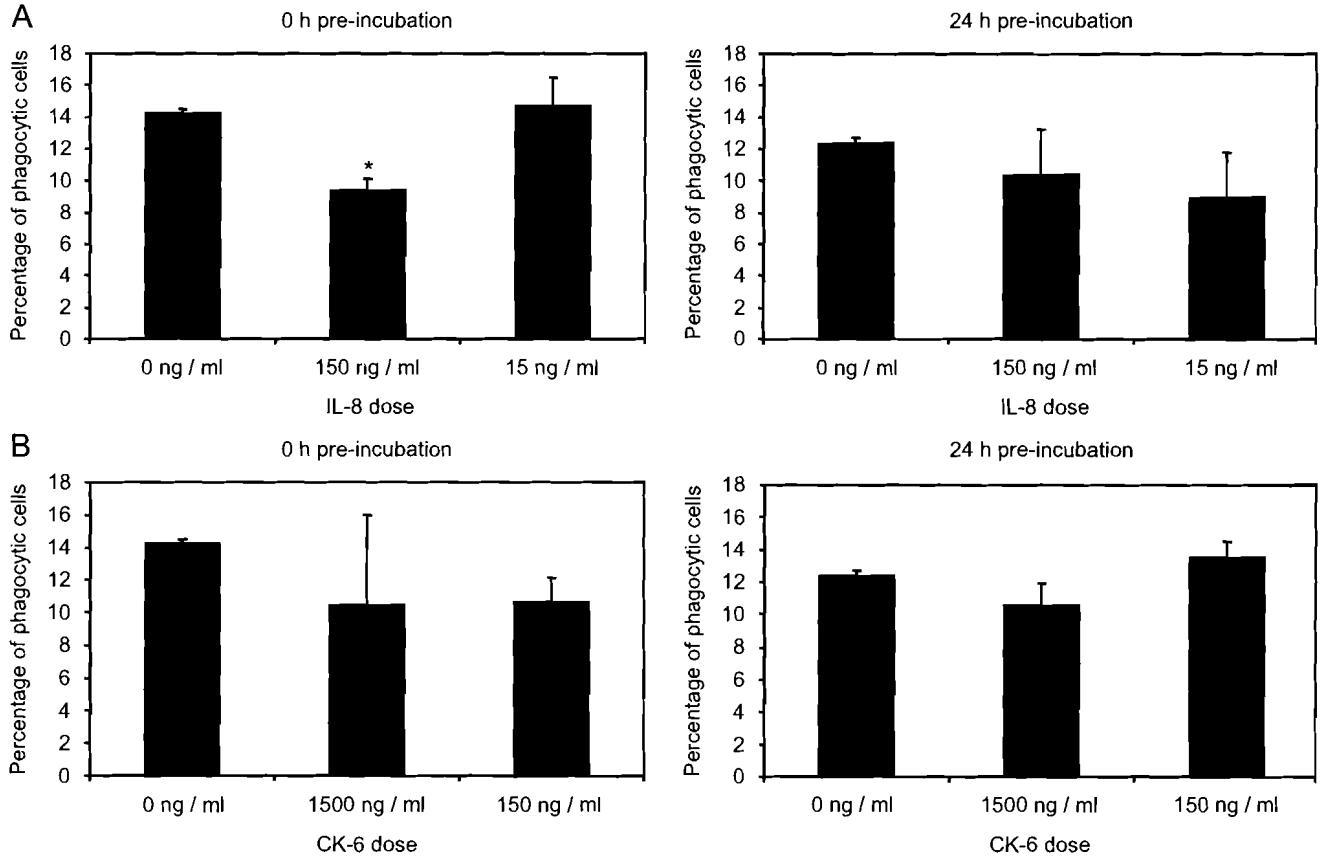

Figure 4 Effect of rainbow trout IL-8 and CK-6 on the phagocytic activity of RTS11 cells. The phagocytic ability was defined as the percentage of cells with ingested yeast cells (green-FITC fluorescent cells). Different doses of IL-8 (A) or CK-6 (B) were added to the RTS11 either simultaneously to the addition of the yeast particles (0 h pre-incubation) or 24 h before the phagocytosis assay was conducted (24 h pre-incubation). *Mean values significantly lower than those obtained in the controls not treated with the chemokine ($p < 0.05$).

is significantly inhibited by the addition of the chemokine simultaneously to the addition of the yeast particles (Figure 4A). The phagocytic capacity is restored when the phagocytosis assay is conducted after 24 h of pre-incubation of the cells with IL-8, indicating that the more severe effects on phagocytosis of this chemokine are due to contact between the cells and the chemokines, but then the negative effects are in part lost with time.

CK-6 also inhibited the phagocytic capacity of RTS11 cells, although not significantly (Figure 4B). When added simultaneously to the yeast, CK-6 significantly inhibited the phagocytic capacity of the R2 sub-population, but similar to IL-8, this negative effect is in part restored after 24 h.

Effect of rainbow trout CK-6 and IL-8 on the RTS11 respiratory burst

Neither IL-8 nor CK-6 had a significant effect on the respiratory burst activity of RTS11 cells (Figure 5). These results were observed when the RTS11 cells were pre-incubated for 24 h with the different chemokine doses or when the chemokines were added simultaneously to the PMA. In all experiments, the respiratory burst had been effectively triggered since the results obtained in the wells treated with NBT and PMA were significantly higher than those treated with NBT alone. However, the capacity of RTS11 to perform the respiratory burst is much lower than the respiratory burst capacity that can be triggered in primary monocytes–macrophages and studies using different methods for triggering and measuring a respiratory burst failed to detect one in RTS11 [20,29].

Effect of CK-6 and IL-8 on the expression of other immune-related genes

We determined whether the two recombinant chemokines produced an effect on the levels of expression of different immune genes on RTS11 cells (Figure 6). When RTS11 cells were exposed to different doses of recombinant IL-8 we found a significant increase in the levels of expression of both CK-6 and IL-8. No significant effect was found, however, for iNOS, and only a slight effect on CD-18. A significant induction of the levels of expression of the IL-1β and TNF-α genes was observed.

Recombinant CK-6 had no significant effect on the levels of expression of CK-6, indicating no feedback effect for this chemokine (Figure 6A). CK-6, however, significantly induced the levels of expression of IL-8, iNOS and CD-18. In contrast to what was observed with IL-8, only a low effect was observed on pro-inflammatory genes. IL-1β was slightly

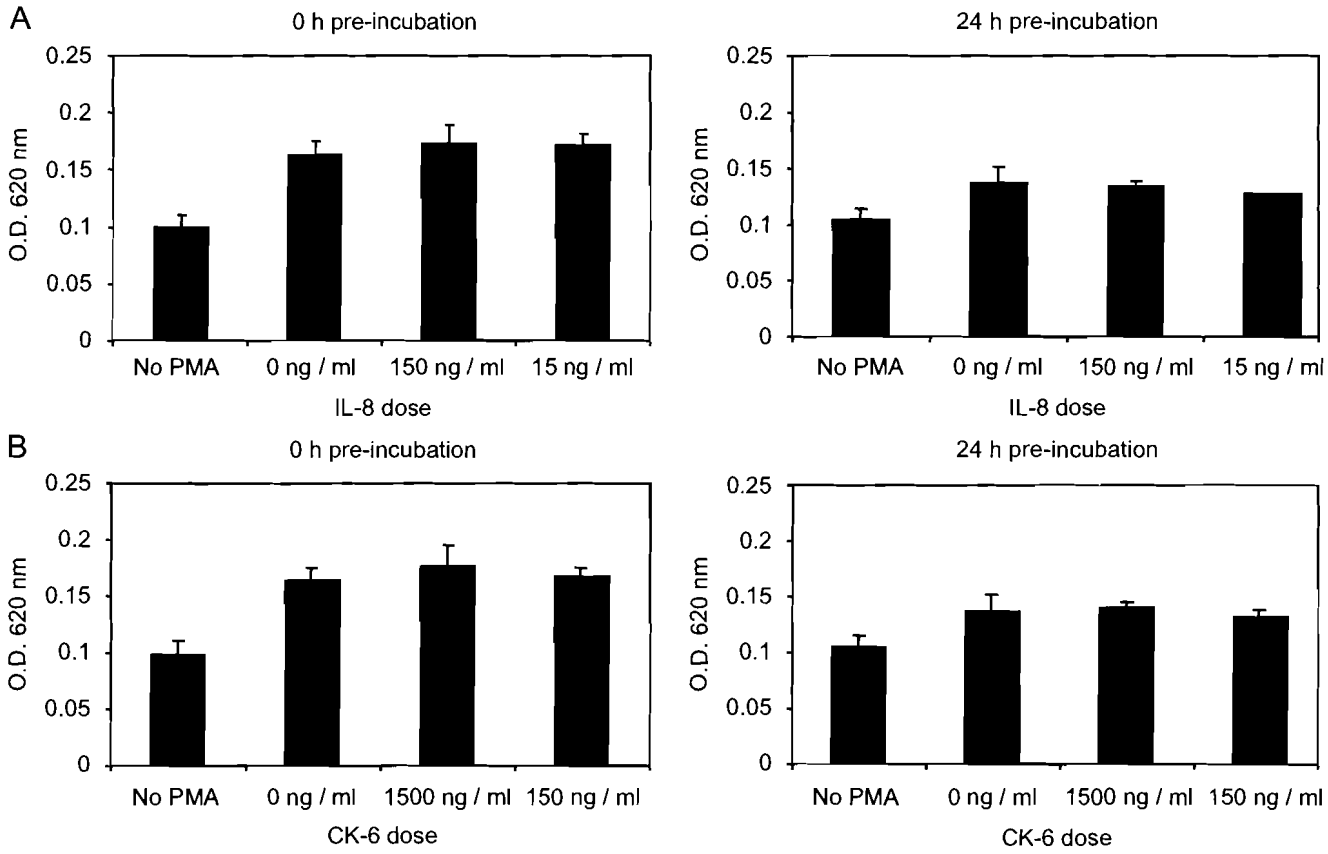

Figure 5 Effect of rainbow trout IL-8 and CK-6 on the respiratory burst of RTS11 cells. Results are shown as the mean reduction of NBT expressed as the O.D. at 620 nm. A negative control with NBT and no PMA (No PMA) was always included to verify that the respiratory burst had been triggered (O.D. at 620 nm significantly lower than that of cells treated with NBT and PMA). Different doses of IL-8 (A) or CK-6 (B) were added to the RTS11 either simultaneously to the addition of the PMA (0 h pre-incubation) or 24 h before the respiratory burst assay was conducted (24 h pre-incubation). The results obtained when chemokines were included were compared with the results obtained in wells treated with NBT, PMA and no chemokines. *Mean values significantly different from those obtained in the controls with PMA not treated with the chemokine ($p<0.05$).

increased only with the highest CK-6 dose and there was no significant effect on TNF-α expression. Thus, the modulation of different genes by each chemokine may indicate a distinct function of both chemokines in the immune response.

Discussion

Very little information is available dealing with the role that fish chemokines have on inflammation and disease, regardless of the major role on the immune response that, eventually, is being elucidated in mammals. In the current study, we have evaluated the bioactivity of a recombinant CXC chemokine such as IL-8 and a CC chemokine, such as CK-6 on RTS11 monocyte-macrophage cells. This work reveals the potential of this cell line to be used in migration studies. Also, this study constitutes one of the very few examples in which the effects of IL-8, a CXC chemokine, on monocyte-like cells are described, contributing to turn down the paradigm of IL-8 as a neutrophil chemokine. More work should be done to determine whether rainbow trout primary monocytes are also attracted by IL-8, but until cellular markers become available for the clear distinction of blood cell types in fish, this remains a difficult task.

There has always been some controversy concerning whether cells from the monocyte–macrophage linage are attracted by IL-8. The two receptors responsible for the binding of IL-8 in mammals (CXCR1 and CXCR2) are known to be present in monocytes–macrophages [30,31]. Early reports showed no chemotactic activity of IL-8 for monocytes *in vitro* [32] and no increased infiltration after intradermal administration in rodents [33]. Comparable results have been obtained recently in rainbow trout, where, after the intraperitoneal injection of recombinant IL-8, neutrophils were predominantly attracted and no significant increase in monocyte infiltration was observed [22]. Moreover, migration of a monocyte cell line towards IL-8 has been never described.

In the current study, we demonstrate that rainbow trout IL-8 specifically attracted RTS11 cells, which can be identified as more immature monocytes, having no effect on mature macrophages. This result is in concordance to that obtained by Smythies et al. [8], where they observed that intestine macrophages did not migrate to IL-8 despite the expression of CXCR1 and CXCR2, whereas autologous blood monocytes expressing both receptors specifically

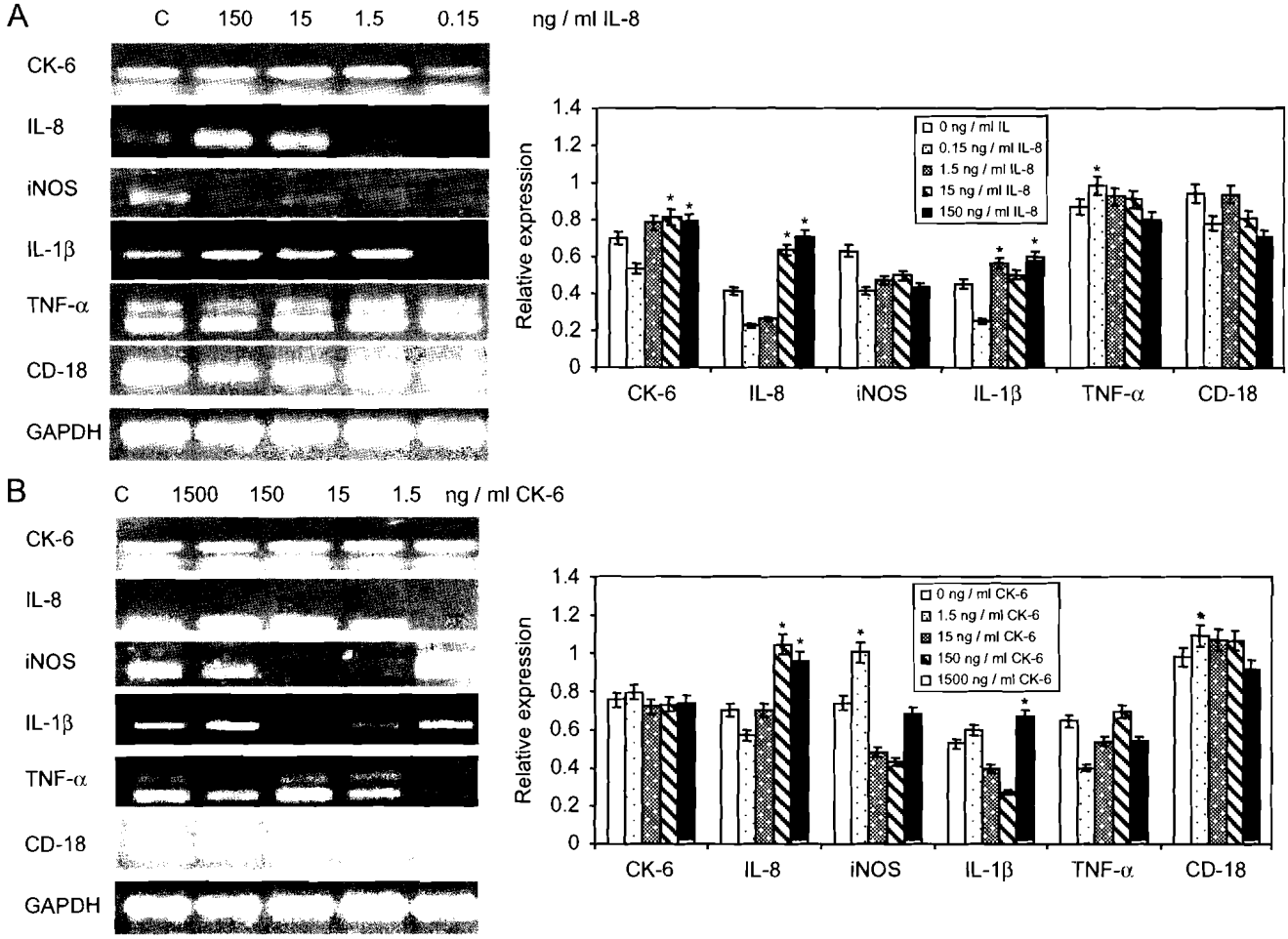

Figure 6 Effect of IL-8 and CK-6 on the levels of expression of different immune molecules in RTS11 cells. RTS11 cells in 24-well plates were exposed to different concentrations of both chemokines. Control wells not treated with chemokines were also included. After 24 h of incubation at 20 °C, RNA was extracted from the cells and the levels of expression of IL-8, CK-6, iNOS, IL-1β, TNF-α and CD18 were evaluated through semi-quantitative RT-PCR. Stained gel showing gene amplification in a representative experiment out of two different experiments performed. Corresponding GAPDH bands for these samples are shown below. Data are also presented as the mean expression relative to corresponding GAPDH expression. *Mean values significantly higher than those obtained in the controls not treated with the chemokine ($p < 0.05$).

migrated towards IL-8. In our RTS11 cultures, the two sub-populations found among the non-adherent cells could be clearly identified as immature monocytes (R1) and more mature macrophages (R2), as previously established in other macrophage fish cell lines [27,28]. The inability of R2 mature macrophages to migrate is a particular property of IL-8, since this sub-population of RTS11 cells specifically migrated towards CK-6. For this CC chemokine, although at first we detected the migration of both R1 and R2 sub-populations, we established that only the migration of the R2 sub-population was specific chemotaxis, since this was the only migration that was down-regulated by the desensitisation of the receptors (through the addition of chemokine in the upper compartment of the chemotaxis chambers). The reason for the absence of chemotaxis for immature monocytes of CK-6 is unknown. Trout CK-6 did not show a distinct phylogenetic relation to any human chemokine when a phylogenetic tree showing all vertebrate CC chemokines was performed [16], and formed a fish-only clade in which its sequence appeared alone with what had been named as flounder MIP1α [23], making it difficult to elucidate its biological activity. In our work, we have demonstrated its chemotactic capacity, as well as its effects on phagocytosis, and the levels of expression of IL-8, iNOS and CD-18, evidencing a role on the inflammatory process.

We also studied the effect that these two chemokines have on the phagocytic capacity of RTS11 cells. In this case, only the R2 sub-population was capable of phagocytosing yeast particles, as determined in our flow cytometry assay. The absence of phagocytosis of the immature sub-population was also corroborated by direct visualisation of the RTS11 cells 30 min after incubation with the yeast particles under the optic microscope (data not shown). For the R2 phagocytic sub-population, both chemokines produced an inhibition of this phagocytic capacity upon contact, although this inhibition was only significant in the case of IL-8. These negative effects were then no longer significant when the phagocytic assay was conducted 24 h after the pre-incubation with the chemokines. This inhibitory effect found on phagocytosis was completely unexpected. We found no data

on the effect that IL-8 might have on monocyte–macrophage phagocytosis due to the scarce number of papers in which the effects of IL-8 were examined on these cell types. However, IL-8 has shown to stimulate the phagocytosis of neutrophils [34], although after the phagocytosis the chemotactic responses are impaired [35]. On the contrary, what usually occurs in those cell types attracted by chemokines [22], no significant effect of either chemokine was observed, however, on the respiratory burst. This could be due to the fact that this immune function has been considerably affected throughout the establishment of the cell culture, since although the respiratory burst had been effectively triggered, the respiratory burst obtained was much lower than the respiratory burst capacity usually obtained in primary monocytes–macrophages. Despite this, our work constitutes the first report of the respiratory burst activity in RTS11 cells; however, in case future studies reveal that primary monocytes are attracted by IL-8 as RTS-11 monocytes, the effect of IL-8 on this function should be studied again as it would be most probably affected.

CK-6 has been shown to act in the R2 macrophage sub-population, both in the chemotaxis and in the phagocytosis experiments. By contrast, in the case of the IL-8, the chemotaxis assays revealed an effect on the R1 sub-population, and the phagocytosis assays revealed an effect on the R2 sub-population. These results suggest that both sub-populations express the receptor(s) responsible for the binding of IL-8, but that the signal transduction pathways that drive phagocytosis are independent of the pathways that drive chemotaxis, as suggested previously [8]. In our RTS11 cultures, the IL-8 receptor that was identified in rainbow trout to be homologous to the IL-8 receptors found in mammals [36] is expressed (data not shown). Whether only this receptor is responsible for the binding of IL-8, or if there are two receptors for IL-8 [30] remains unknown in fish. Moreover, no data are available concerning the receptor responsible for the signal transduction of CK-6. Studies performed with the chemokine receptors are essential in the elucidation of the chemokine roles.

Both chemokines were capable of modulating the levels of expression of different genes related to the immune response, revealing a different function of both chemokines within the immune response. IL-8 produced a strong induction of pro-inflammatory genes, but no effect on iNOS and the integrin CD18, known to take part in the mammalian leukocyte binding to denatured protein matrices [26]. By contrast, CK-6 had almost no effect on pro-inflammatory genes but a strong effect on CD18 and iNOS, revealing a role in macrophage mobilisation and activation. Since the expression of the CD18 integrin could be associated with chemotaxis and IL-8 exerts its chemotactic effects on the R1 sub-population (13.8% of the RTS11 cultures), the lack of effect on CD18 could be attributed to the up-regulation in this attracted sub-population. In any case, IL-8 induces CK-6, as previously demonstrated in our group [37], and CK-6 induces IL-8. Thus, the data suggest that all effects are gathered to collaborate in the immune response *in vivo*.

In conclusion, we have established the RTS11 monocyte–macrophage cell line as a useful tool to study chemotaxis in rainbow trout, revealing the effects of both a CC chemokine such as CK-6 and a CXC chemokine as IL-8. This constitutes one of the very few studies in which the chemotactic capacity of IL-8 towards a monocyte-like cell is demonstrated. We have demonstrated that IL-8 attracts monocyte-like cells and inhibits the phagocytic capacity of macrophage-like cells, whereas CK-6 attracts macrophage-like cells and slightly inhibits the phagocytic capacity in this sub-population.

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