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Phylogenetic analysis of vertebrate CXC chemokines reveals novel lineage specific groups in teleost fish

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ABSTRACT

In this study, we have identified 421 molecules across the vertebrate spectrum and propose a unified nomenclature for CXC chemokines in fish, amphibians and reptiles based on phylogenetic analysis. Expanding on earlier studies in teleost fish, lineage specific CXC chemokines that have no apparent homologues in mammals were confirmed. Furthermore, in addition to the two subgroups of the CXCL8 homologues known in teleost fish, a third group was identified (termed CXCL8_L3), as was a further subgroup of the fish CXC genes related to CXCL11. Expression of the CXC chemokines found in rainbow trout, *Oncorhynchus mykiss*, was studied in response to stimulation with inflammatory and antiviral cytokines, and bacterial. Tissue distribution analysis revealed distinct expression profiles for these trout CXC chemokines. Lastly three of the trout chemokines, including two novel fish specific CXC chemokines containing three pairs of cysteines, were produced as recombinant proteins and their effect on trout leucocyte migration studied. These molecules increased the relative expression of CD4 and MCSFR in migrated cells in an *in vitro* chemotaxis assay.

1. Introduction

Chemokines are a large family of soluble peptides directing cell migration in development and immune defence. They are small in size, typically 8–15 kDa, and in mammals are classified into four subfamilies, CC, CXC, CX_3C and XC, based on the distinctive patterns of the two N terminal cysteines (Alejo and Tafalla, 2011). The CC and CXC chemokine family are the two largest groups, containing multiple members, whilst the CX_3C and XC family consist of one or two members respectively. More recently a fish specific CX subfamily has been described in zebrafish (Nomiyama et al., 2008), with four members present.

Chemokines can be found across the whole vertebrate spectrum, from primitive fish to mammals, and their origin can even be found in amphioxus, an ancient chordate species that diverged approx. 520 million years ago (Sansom et al., 1992; Kuroda et al., 2003). In mammals, over 50 chemokines have been characterised to date, that interact with approximately 20 G protein-coupled receptors (Allen et al., 2007). Teleost fish appear to have an even larger repertoire of chemokines possibly due to an extra round of genome duplication, with 111 chemokines identified recently in the zebrafish genome, the majority of which (81 genes) belong to the CC family (Alejo and Tafalla, 2011; Nomiyama et al., 2008). Despite the conserved two pairs of cysteine residues seen in the CC and CXC family, the primary sequences of the chemokines are highly divergent, making it extremely difficult to determine the phylogenetic relationships among family members during evolution (Nomiyama et al., 2008; DeVries et al., 2006; Peatman and Liu, 2007; Laing and Secombes, 2004). Therefore, it has been problematic to establish a common nomenclature for some chemokines from early vertebrates simply based on the phylogenetic classification in mammals. In this study, we have undertaken extensive analysis of the phylogeny of the CXC chemokines using the sequences that have accumulated in the nucleotide databases from various vertebrate groups, to gain an in-depth understanding of their evolutionary origin with a particular focus on fish CXC chemokines.

In mammals, the CXC chemokine family consists of 17 members, divided into two main subgroups; those with an ELR sequence motif preceding the first cysteine and those without this motif (Pisabarro et al., 2006; Viola and Luster, 2008; Wang et al., 2005; Matloubian et al., 2000). The CXC chemokine ligands (CXCL)

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1–8 (except for CXCL4) and CXCL15 (lungkine) contain the ELR motif and are known to be essential for recruitment of neutrophils to an infection site in inflammatory responses and tissue injury. The genes encoding the ELR+ chemokines are closely clustered on chromosome 4 in humans or chromosome 5 in mice, and are believed to have expanded by gene duplication after the divergence of the bird and mammalian ancestral lineages, since fewer ELR+ chemokines are found in birds relative to mammals (Wang et al., 2005). Among the ELR- chemokines, CXCL9-11 are also tandemly placed in the genome and share considerable sequence homology. They act on activated T cells, memory T cells and NK cells via a common receptor CXCR3. In contrast, CXCL13 is a specific chemoattractant for B cells that express a high level of its cognate receptor CXCR5 (Burkle et al., 2007). CXCL12, also referred to as stromal cell factor, primarily directs cell migration during development (Vandercappellen et al., 2008; Bai et al., 2009). CXCL14, whose receptor has yet to be identified, is constitutively expressed in the mucosal tissues such as skin, gut and kidney, promoting growth and migration of both immune and non-immune cells including monocytes, dendritic cells, and fibroblasts (Augsten et al., 2009). Lastly, CXCL16 and CXCL17 are known only in humans and mice but show limited sequence homology with the other CXC family members (Pisabarro et al., 2006; Matloubian et al., 2000).

Recent studies demonstrate that several CXC chemokine genes exist in telelost fish. Homologues of the CXCL8 group, also referred to as interleukin (IL) 8, have been reported in several teleost species (Nomiyama et al., 2008; DeVries et al., 2006; van der Aa et al., 2010; Abdelkhalek et al., 2009; Corripio-Miyar et al., 2007; Huising et al., 2003; Laing et al., 2002), cartilaginous fish (Inoue et al., 2003) and an agnathan (lamprey) Najakshin et al., 1999. In zebrafish and common carp, an additional group, apparently displaying a closer phylogenetic relationship to mammalian IL-8s than the previously identified group, is also present (van der Aa et al., 2010; Abdelkhalek et al., 2009) and has been termed CXCL8_L2 (with the former called CXCL8_L1). Unlike their counterparts in higher vertebrates, the fish CXCL8 homologues mostly lack the ELR motif at the N terminus of the mature peptide, with the exception of gadoid CXCL8_L1 molecules, as seen in Atlantic cod and haddock (Corripio-Miyar et al., 2007; Seppola et al., 2008). Homologues have also been described for the CXCL9-11 group in carp, catfish, rainbow trout and zebrafish, whereas their phylogenetic relationship within the CXCL9-11 group has not been conclusively established (Nomiyama et al., 2008; Laing et al., 2002; Baoprasertkul et al., 2004; Chen et al., 2010; Savan et al., 2003). In contrast, the existence of CXCL12, 13 and 14 homologues in teleost fish is much more certain, as documented by several research groups (Baoprasertkul et al., 2004; Huising et al., 2004; Tian et al., 2010). Moreover, a novel group of CXC chemokines, termed CXCd, has recently been described exclusively in fish species (Wiens et al., 2006), and phylogenetic tree analysis of zebrafish and other fish genes suggests other novel groups are present (Nomiyama et al., 2008).

As in mammals, the CXC chemokines such as CXCL12 and CXCL14 that are involved in homeostasis, are constitutively expressed and not affected by infection in fish (Baoprasertkul et al., 2005), whilst others respond to infection and ligands of pattern recognition receptors (PRRs). Fish CXCL8 related molecules can be up-regulated at an early stage of infection and in monocytes/macrophages and granulocytes stimulated with inflammatory stimuli (van der Aa et al., 2010; Abdelkhalek et al., 2009; Laing et al., 2002). Differential responses of CXC chemokines to PRR ligands are reported. For example, the CXCL13 homologue was shown to be activated by LPS but not polyI:C (Kim et al., 2007) and a trout CXCL9-11 homologue was induced during viral infection and by polyI:C, a known ligand of Toll like receptor 3 (Chaves-Pozo et al., 2010; Montero et al., 2009). Some cytokines are also potent inducers of CXC chemokine expression, as seen with trout interferon gamma (IFN-γ) and tumor necrosis factor (TNF)-α that induce CXCL9-11 expression, which is clearly an IFN-γ inducible protein (γIP) (Laing et al., 2002; Chen et al., 2010; Zou et al., 2005). CXCL8_L1 and CXCL8_L2 have been produced as recombinant proteins and shown to primarily direct the migration of phagocytes and enhance their respiratory burst activity (RBA) (van der Aa et al., 2010; Harun et al., 2008; Montero et al., 2008). In contrast, recombinant carp CXCL9-11 (also called CXCb) has been shown to be chemoattractive for lymphocytes as well as phagocytes but does not prime RBA in the latter (van der Aa et al., 2010). All these data are largely in line with studies in mammals, suggesting many of the functions of the teleost CXC chemokines are relatively well conserved.

In this report, the EST databases of teleost fish and other vertebrate species were analysed extensively to gain an insight into the phylogeny of the CXC chemokines. This has led to the discovery and confirmation of several novel CXC chemokine subgroups in teleost fish. Using rainbow trout as a model, expression of all the CXC chemokines now known in this species was studied in fibroblasts, monocytes and primary cultures of head kidney leucocytes in response to proinflammatory and antiviral cytokines, and in fish after infection with bacterial pathogens. Constitutive expression in tissues of healthy fish was also studied. Lastly, three of the trout chemokines were produced in eukaryotic cells as recombinant proteins and examined for their effects on trout leucocyte migration.

2. Materials and methods

2.1. Data mining, gene cloning and sequence analysis

The annotated CXC chemokine sequences were retrieved from the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/nucleotide/) by key word search. The expressed sequenced tags (ESTs) were obtained by a Tblastn search using known CXC chemokine sequences and translated into proteins using the nucleotide translation tool (http://www.expasy.org/). Only full length protein sequences translated from the ESTs were selected for phylogenetic analysis.

Based on the obtained trout CXC sequences, primers were synthesised for PCR amplification of the full length cDNA sequences of rainbow trout (*Oncorhynchus mykiss*) CXC chemokines. For this, trout primary head kidney (HK) leucocytes (for cloning of CXCL8_L1, CXCL11_L1, CXCL12a, CXCL14 and CXCL_F5), a trout macrophage like cell line (RTS-11) (for cloning of CXCL12b, CXCL13, CXCL_F1 and CXCL_F4,) and a trout fibroblast like cell line (RTG-2) were stimulated for 4 h with 10 μg/ml phytohaemagglutinin (PHA) L (Sigma–Aldrich) or 100 ng/ml rIFN2 (Zou et al., 2007). Total RNA was then prepared from the stimulated cells using TRIzol reagent (Invitrogen) for PCR cloning.

All PCR products were ligated into pGEM T Easy vector (Promega) at 4 °C overnight or pcDNA3.3-TOPO® vector (Invitrogen) at room temperature for 5 min. The ligation reaction was transformed into competent *Escherichia coli* TAM cells (ActifMotif). Positive clones were screened by standard colony PCR and cultured at 37 °C overnight for plasmid preparation. Plasmid DNA was purified using a plasmid mini-preparation kit (Qiagen) and sequenced by Eurofins MWG Operon (http://www.eurofinsgenomics.eu).

For phylogenetic tree construction, the CXC chemokine sequences were retrieved from the NCBI nucleotide database and only full length molecules were included. Mouse CXCL15 was excluded since it has an unusually long C terminal region compared with other known CXC chemokine molecules. Multiple alignment of protein sequences was generated using the CLUSTAL W program

(http://www.ch.embnet.org/software/) with the default parameters (scoring matrix: Blosum; opening gap penalty: 10; end gap penalty: 10; extending gap penalty: 0.05; extension gap penalty: 0.05; separation gap penalty: 0.05) and used for construction of phylogenetic trees using the Neighbour-Joining method within the Mega4 programme (Tamura et al., 2007). The trees were boostrapped 10,000 times using the following parameters: pairwise deletion for gaps and missing data, poisson correction for amino acids, uniform rates for rates among sites.

The trout chemokine sequences were analysed for the presence of the signal peptide using the SignalP programme (version 3.0) (Emanuelsson et al., 2007). The theoretical molecular weight and net charge of the putative mature peptides were calculated using the tools listed on the Expasy website (http://www.expasy.ch/tools).

2.2. Constitutive expression of trout CXC chemokines in fish tissues, isolated HK leucocytes and cell lines

Fish tissues including brain, gills, intestine, head kidney, liver, skin, spleen and thymus were sampled from six healthy fish for RNA extraction and quantitative real time PCR analysis. Briefly, total RNA was extracted with the TRIzol reagent and reverse transcribed into cDNA using a first strand cDNA synthesis kit (Fermentas) according to the manufacturer's instructions. The synthesised cDNA was diluted with TE buffer (10 mM Tris, 1 mM EDTA, pH8.0) and kept at −20 °C for real time PCR analysis.

Primary HK leucocytes were isolated from freshly killed rainbow trout as described previously (Sharp et al., 1991). HK tissue was collected under sterile conditions and washed with Leibovitz medium (L-15, Invitrogen) containing 2% foetal calf serum (FCS, Invitrogen), 10 units/ml heparin (Sigma–Aldrich), 100 μg/ml penicillin and 100 units/ml streptomycin (P/S, Invitrogen), and gently pushed through a 100 μm nylon mesh (John Staniar Co., UK). The cells were loaded on a 51% percoll gradient (Sigma–Aldrich) and centrifuged at 400*g* at 4 °C for 30 min without the break. The cells at the interface between the upper aqueous phase and the percoll solution were collected and washed with L-15 medium containing 0.1% FCS, 10 units/ml heparin and P/S. The cells was then resuspended in L-15 medium containing 0.1% FCS and P/S, and cultured in L-15 medium containing 10% FCS and P/S at 20 °C in 25 cm^2 flasks at a concentration of 1×10^6 cells per flask.

RTS-11 and RTG-2 cells were maintained at 20 °C in L-15 medium containing 30% and 10% FCS respectively. The cells were passaged to fresh flasks at 80% confluence and cultured for two days prior to use. The isolated HK cells and cell lines were used initially for qPCR analysis to determine the constitutive expression of the trout CXC chemokine genes, as outlined below.

2.3. Modulation of trout CXC chemokine expression by IL-1β, and type I and II interferon

To investigate expression modulation of the identified CXC chemokines by proinflammatory and antiviral cytokines, trout RTG-2 and RTS-11 cells and primary HK leucocytes were stimulated at 20 °C for 24 h with recombinant IL-1β (rIL-1β) (Hong et al., 2004), rIFN2 (Zou et al., 2007) or rIFN-γ (Zou et al., 2005) at a dose of 20 ng/ml. The control cells were treated with an equal volume of the buffer solution (20 mM Tris–Cl, 100 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 20% glycerol, 0.1% NP40, 250 mM imidazole, pH 8.0) that was used for eluting the recombinant proteins during protein purification. Total RNA was extracted using the TRIzol reagent, treated with RNase free DNase I (Fermentas) and reverse transcribed into cDNA as described above for real time PCR analysis (see below).

2.4. Modulation of trout CXC chemokine expression in HK cells after bacterial and viral infection

Brivax I, a genetically attenuated strain of *Aeromonas salmonicida* was inoculated from glycerol stock onto tryptic soy agar (Sigma–Aldrich) containing 50 μg/ml kanamycin and grown at 20 °C for 2 days (Marsden et al., 1996). A single colony was inoculated into 50 ml tryptic soy broth (Sigma–Aldrich) and cultured under static conditions at 20 °C for 3 days. The bacterial cells were centrifuged at room temperature at 4500 rpm for 30 min and the cell pellet was washed with 50 ml sterile phosphate buffered saline (PBS). The bacteria were then resuspended in 10 ml of PBS and kept at 4 °C before use. To determine the colony forming units (CFU) of the bacterial suspension solution, a serial dilution of bacteria was prepared and plated onto TSA plates containing 50 μg/ml kanamycin. The plates were kept at 20 °C for 3 days and the colonies on the plates counted.

Twenty fish (10 each tank, ∼300 g) were maintained at 14 °C in the indoor freshwater aquarium at the Scottish Fish Immunology Research Centre, University of Aberdeen. Fish were anaesthetised with 2-phenoxyethanol (0.05%, v/v, Sigma–Aldrich) before intraperitoneal (i.p.) injection with 250 μl of 2×10^{10} cells/ml Brivax I or an equal volume of sterile PBS. At 24 and 72 h post-infection HK tissue was sampled from five fish of both groups. Total RNA was extracted from the HK samples using TRIzol reagent, according to the manufacturer's instructions, for qPCR analysis of gene expression.

2.5. Chemotactic activity of CXC chemokines

Expression analysis revealed that CXCL8_L1, CXCL11_L1, CXCL_F4 and CXCL_F5 were most responsive to cytokines and bacterial infection. Trout CXCL8_L1 was previously shown to enhance leucocyte migration *in vitro* (Harun et al., 2008). The other three chemokines were therefore chosen for further characterisation of their chemoattractant activity. For this, expression plasmids were generated for transient transfection into Chinese Hamster Ovary (CHO) cells to produce recombinant proteins for CXCL11_L1, CXCL_F4 and CXCL_F5 respectively. The CHO cells were maintained in RPMI-1640 medium containing 5% FCS and P/S at 37 °C. The cDNA fragments encoding the full length protein, including a signal peptide, were inserted into vector ptGFP1, a plasmid modified from pTurboGFP-N vector (Evrogen) (Chang et al., 2011). All expression plasmids were confirmed by sequencing. Transfection of plasmids into the CHO cells was performed with an Amaxa Nucleofactor II transfection system (Lonza) according to the manufacturer's instructions. The transfected cells were cultured at 37 °C in L-15 medium containing 5% FCS overnight, with the FCS then reduced to 0.1% and cells cultured for a further 3 days. The transfected cells were confirmed by viewing GFP positive cells (10–30%) under a fluorescence microscope. Conditioned media containing chemokines were then collected, with medium from CHO cells transfected with the ptGFP1 plasmid (without an insert) used as a control. In addition, the CHO cells were also harvested to confirm the presence of the CXC transcripts by RT-PCR. The conditioned culture media were centrifuged at 3000*g* for 10 min to remove cell debris and stored at −80 °C prior to use in the chemotaxis assay.

The chemotaxis assay was performed using rainbow trout HK primary leucocytes as described previously (Peddie et al., 2001). Briefly, 30 μl of test solution was added to the lower wells of a 48 well microchemotaxis chamber and a polyvinylpyrrolidone-free polycarbonate filter (Nucleopore Corporation Filtration Products, USA) with 3 μm diameter pores was then placed on top of the lower wells, followed by a rubber gasket and the upper chamber. Forty three microlitres of freshly isolated HK leucocytes (5×10^6 cells/ml) were added into the upper wells and the apparatus then

incubated at 20 °C for 90 min in a dark humidity chamber. The filter was removed and cells adhering to the upper surface were dislodged by gently washing of the filter in PBS and scraping of the upper surface undirectionally with a rubber blade several times. The cells attached to the lower surface were carefully washed off with PBS, pooled with those that had migrated into the lower chamber, and centrifuged at 400*g* at 4 °C for 10 min. The cell pellets were used for gene expression analysis by real time PCR.

2.6. Real time PCR analysis

The primers used for real time PCR analysis are listed in Table 1. The real time PCR was performed on a Light Cycler® 480 II (Roche) using the following programme: 1 cycle of 95 °C for 10 min; 45 cycles of 95 °C for 20 s, 60 °C for 20 s, 72 °C for 30 s and 83 °C for 5 s for fluorescent detection. A standard curve was established using a series of 10-fold dilutions of plasmid DNA containing target gene inserts or purified PCR products. The expression data of individual genes was normalised to that of the house keeping gene elongation factor 1 alpha (EF-1α). Fold change of gene expression level was obtained by comparing the normalised gene expression level of the treated group against the untreated group (defined as 1). A fold change greater or less than 1 was regarded as up or down regulation of gene expression.

2.7. Statistical analysis

Gene expression data were analysed by student t test and differences with $^{*}p < 0.05$ or $^{**}p < 0.01$ between the experimental and control group were considered statistically significant.

3. Results

3.1. CXCL12, CXCL13 and CXCL14 homologues are present in teleosts

To gain a broad view of the phylogeny of the vertebrate CXC chemokines, a total of 421 full length protein sequences of the CXC chemokines and vertebrates including teleosts, amphibians, reptiles, birds and mammals were retrieved from the nucleotide and protein databases (Supplementary Fig. 1). A phylogenetic tree was firstly constructed using the Neighbour-Joining method to evaluate the evolutionary relationships of all the CXC chemokine groups (Fig. 1A). Consistent with previous reports, clear homologues of CXCL12, CXCL13 and CXCL14 exist in teleosts, amphibians, reptiles and birds. In some teleost species such as carp (Accession Nos. AJ536027 and AJ627274), medaka (Accession Nos. NM_001104727 and AM850703) and rainbow trout (Accession Nos. HE578135 and HE578136), two variants of CXCL12 with significantly high sequence homology are present and are likely to have duplicated from a single progenitor. Two CXCL13 variants

Table 1
Primers used.

Gene	Primer	Sequence (5′–3′)	Application
CXCL8_L1	CXCL8b-F1	caagcttctgttaccagctagataacaacc	cDNA cloning, construction of expression plasmid
	CXCL8b-R1	agaattctcatggaatgatgacccatgga	
CXCL_F4	CXCF6-F1	caagcttactgcctcttgactcctcacc	
	CXCF6-R1	agaattctccagccgttgggttttccatgc	
CXCL_F5	CXCF7-F1	caagctt gaaatatgaactctgtcctgtc	
	CXCF7-R1	agaattc tcaccctaaggcagggattggtg	
CXCL11_L1	CXCL_F1-F1	caagctttccatcacttgactgagctgtcca	
	CXCL_F1-R1	agaattccaagttctcagtgggattggtg	
CXCL12a	CXCL12Aas-F1	caagcttgctctggactctacactgaacgga	
	CXCL12Aas-R1	agaattcgtagatcctcacttggcactgt	
CXCL12b	CXCL12Bas-F1	caccatggatgtcaaagtactg	
	CXCL12Bas-R1	gaagctt cctttactgagcacgtcttct	
CXCL13	CXCL13-F1	caagatgctcttcaagccacacta	
	CXCL13-R1	gaagctt ttactgtagctgttccactatcgt	
CXCL14	CXCL14-F1	caagcttcaagcgaaaccagtcgggaggt	
	CXCL14-R1	agaattcgaccaagtactgatttctgggag	
CXCL_F1a	CXCL_F3a-F1	caagcttaccttctgatacaacagccac	
	CXCL_F3a-R1	agaattcaaagataagccagcagaggggt	
CXCL_F1c	CXCL_F3c-F1	caagcttaacgtatatcaccatgaacacc	
	CXCL_F3c-R1	agaattctcagaattggtcactgggaag	
CXCL8_L1	CXCL8b-eF1	ctgaccattactgaggggat	Real time PCR analysis
	CXCL8b-eR1	ctcctgaccgctcttgctc	
CXCL_F4	CXCF6-eF1	tgcctactccttacagtgggta	
	CXCF6-eR1	tccgctggttttcttcca	
CXCL_F5	CXCF7-eF1	tgggagtttgtatacagctcaac	
	CXCF7-eR1	ctttcttccaacggggttcagg	
CXCL11_L1	CXCL_F1-eF1	tctgctcctggccaatgttgaag	
	CXCL11-eR1	tcccttctccgttcttcagagt	
CXCL12a	CXCL12Aas-eF1	_gacatacgttcctttcgcaca	
	CXCL12Aas-eR1	gctttcttcatcttgttgagggca	
CXCL12b	CXCL12Bas-eF1	catggatgtacctccctctacg	
	CXCL12Bas-eR1	cttcttcaccttgttaatggca	
CXCL13	CXCL13-eF1	caggtttcacaagttggaga	
	CXCL13-eR1	tcccttgttgttactttgtaac	
CXCL14	CXCL14-eF1	tccctacaagctgaagcctac	
	CXCL14-eR1	tgcttatccttccagatccg	
CXCL_F1a	CXCL_F3a-eF1	tcatctacatgatacaaggga	
	CXCL_F3a-eR1	tcaagtgctaggggtggtatg	
CXCL_F1c	CXCL_F3c-eF1	gctgcctttgccccaaagtg	
	CXCL_F3c-eR1	cctgcattcttttggttgatc	
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	EF1a-R	acagcgaaacgaccaagagg	

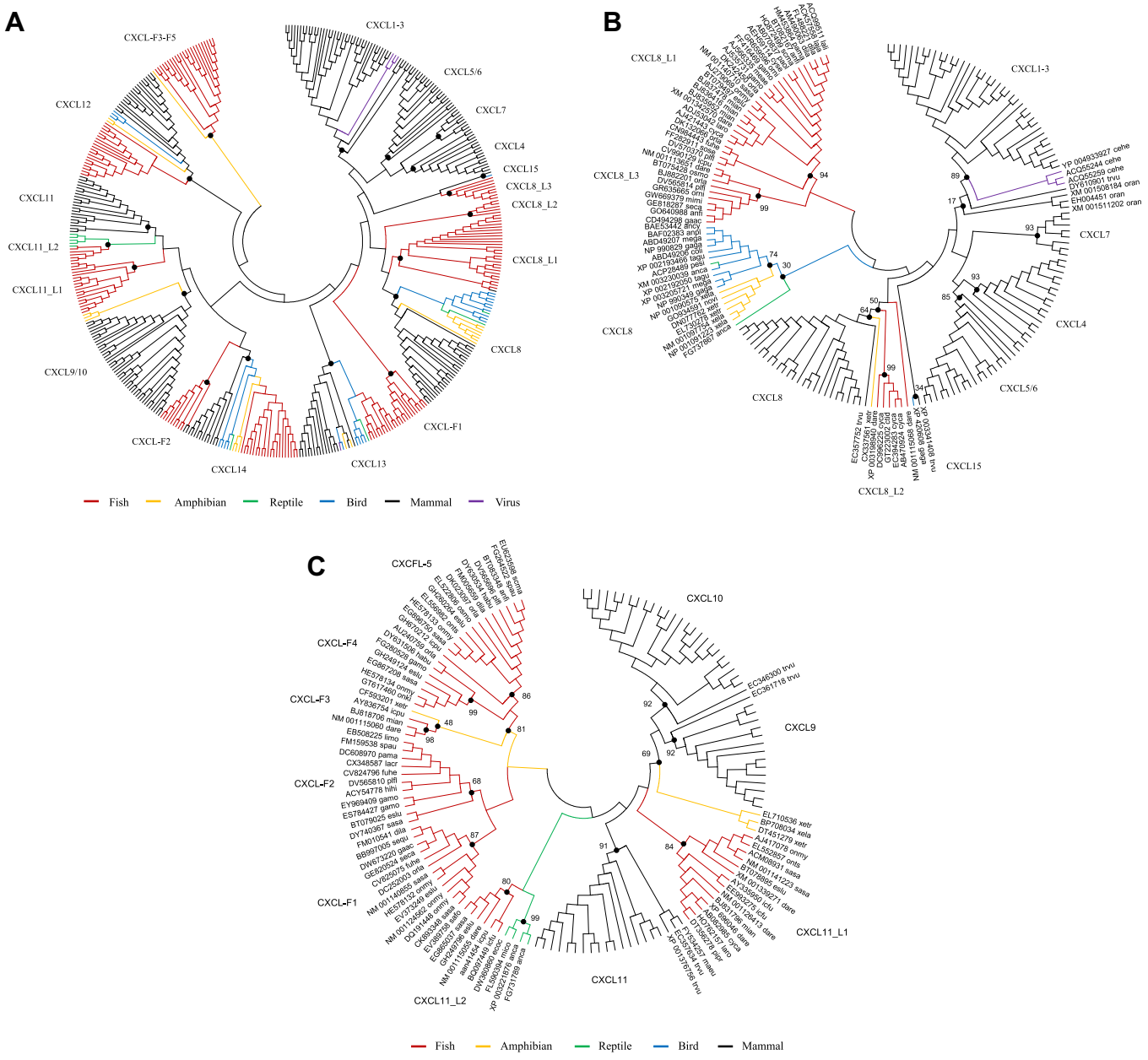

Fig. 1. Phylogenetic analysis of vertebrate CXC chemokines. Phylogenetic trees, containing all CXC chemokine family members (A) or related groups (B and C), were constructed using the Neighbour-Joining method within the Mega programme (version 4.0) using the full length CXC chemokine protein sequences. The tree was bootstrapped 10,000 times and unrooted. The nucleotide accession numbers of chemokines from non mammalian species and primitive mammals are shown. Solid black dots indicate the root of the phylogenetic group. The abbreviations for the non-mammalian species are: teleosts: anfi, *Anoplopoma fimbria*; caau, *Carassius auratus*; cocl, *Coregonus clupeaformis*; ctid, *Ctenopharyngodon idella*; cyca, *Cyprinus carpio*; dare, *Danio rerio*; dila, *Dicentrarchus labrax*; dima, *Dissostichus mawsoni*; eslu, *Esox lucius*; fuhe, *Fundulus heteroclitus*; gaac, *Gasterosteus aculeatus*; gamo, *Gadus morhua*; habu, *Haplochromis burtoni*; hihi, *Hippoglossus hippoglossus*; icfu, *Ictalurus furcatus*; icpu, *Ictalurus punctatus*; laca, *Lates calcarifer*; lacr, *Larimichthys crocea*; lali, *Latris lineate*; laro, *Labeo rohita*; limo, *Lithognathus mormyrus*; meae, *Melanogrammus aeglefinus*; mian, *Misgurnus anguillicaudatus*; mimi, *Miichthys miiuy*; orla, *Oryzias latipes*; onki, *Oncorhynchus kisutch*; onmy, *Oncorhynchus mykiss*; onts, *Oncorhynchus tshawytscha*; orni, *Oreochromis niloticus*; osmo, *Osmerus mordax*; pach, *Paralabidochromis chilotes*; pama, *Pagrus major*; paol, *Paralichthys olivaceus*; pefl, *Perca flavescens*; pipr, *Pimephales promela*; plfl, *Platichthys flesu*; potu, *Poeciliopsis turneri*; ptrs, *Ptyochromis* sp. -redtail sheller; safo, *Salvelinus fontinalis*; sasa, *Salmo salar*; scma, *Scophthalmus maximus*; seca, *Sebastes caurinu*; sequ, *Seriola quinqueradiatasose*, sich, *Siniperca chuatsi*; sose, *Solea senegalensi*; spau, *Sparus aurata*; taru, *Takifugu rubripes*; teni, *Tetraodon nigroviridis*. Amphibians: novi, *Notophthalmus viridescens*; xela, *Xenopus laevis*; xetr, *Xenopus tropicalis*. Reptiles: anca, *Anolis carolinensis*; ecoc, *Echis ocellatus*; mico, *Micrurus corallines*; pesi, *Pelodiscus sinensis*. Birds: ancy, *Anser cygnoides*; anpl, *Anas platyrhynchos*; coli, *Columba livia*; gaga, *Gallus gallus*, mega, *Meleagris gallopavo*; tagu, *Taeniopygia guttata*. Mammals: maeu, *Macropus eugenii*; oran, *Ornithorhynchus anatinus*; trvu, *Trichosurus vulpecula*. Virus: cehe, *Cercopithecine herpesvirus*.

(Accession Nos. GR671585 and GR675601) with 78.3% sequence similarity are also found in tilapia, as seen also in chicken (Accession Nos. FR874037 and FR874037) and zebrafinch (Accession Nos. XM_002193166 and XP_002193177) where duplicated genes are apparent. CXCL14 is encoded by a single copy gene in most vertebrates except for large yellow croaker (Accession Nos. DK108083 and CX348196) and green lizard (Accession Nos. FG687123 and FG724528). Among all the CXC chemokines, CXCL12 and CXCL14 are the most conserved molecules within vertebrates.

3.2. Three distinct subgroups of the CXCL1-8/15 subfamily exist in teleosts

Three distinct subgroups of CXC chemokines belonging to the CXCL1-8/15 group were apparent in teleosts as revealed by

phylogenetic analysis (Fig. 1A). These include the two groups previously reported, the so called CXCa or CXCL8_L1 group and CXCc or CXCL8_L2 (Table 3), and a third group that appears to be related to CXCL15s (van der Aa et al., 2010; Abdelkhalek et al., 2009; Corripio-Miyar et al., 2007; Laing et al., 2002) which was designated as CXCL8_L3 in the present study. To further define the phylogenetic relationships within the CXCL1-8/15 subfamily, a separate tree was constructed using molecules only from this subfamily (Fig. 1B), showed similar tree topology to that obtained using all CXC chemokine molecules (Fig. 1A and B) and significantly high bootstrap values of 94%, 99% and 99% for the CXCL8_L1-L3 groups respectively. Consistent with previous studies (van der Aa et al., 2010; Abdelkhalek et al., 2009) but in contrast with the tree with all CXC members present, in this second tree the teleost CXCL8_L2 proteins are more closely clustered with the mammalian CXCL8 molecules than the CXCL8_L1 group. However, the CXCL8 molecules from amphibians, reptiles and birds which branched with mammalian CXCL8s in the first tree are also more distant in this tree. Within the CXCL8_L2 group two members are present in zebrafish, as apparent in this second phylogenetic tree (Fig. 1B) where one of the zebrafish molecules (Accession No. NM_001115068) branches from the base of the CXCL8_L2 group. Both CXCL8_L2 and CXCL8_L3 are limited to relatively few species, with CXCL8_L2 found exclusively in cyprinids to date. In contrast, the CXCL8_L1 group was found in 19 teleost species in the present study. Furthermore, the ELR motif preceding the CXC motif near the N terminus, a conserved feature of the mammalian CXCL1-8/15 chemokines (except CXCL4) that act as chemoattractants for neutrophils, is seen in only CXCL8_L1 molecules of some fish species (such as Atlantic cod and haddock) but not in fish CXCL8_L2 (Supplementary Fig. 2A) and CXCL8_L3 (Supplementary Fig. 2B). However, it is common to see "LR" upstream of the CXC motif in CXCL8_L1, with a conservative substitution such as aspartic acid in place of glutamic acid. It is also worth noting that the ELR motif is present in the mature peptide of the amphibian and reptile CXC chemokines belonging to this phylogenetic group (data not shown).

3.3. Analysis of the genes related to mammalian CXCL9-11

Mammalian CXCL9, 10 and 11 are tandemly clustered in the human genome and are closely related chemokines upregulated by IFN-γ and viral infection. The corresponding locus harbouring these three chemokine genes is known to be absent in birds (Wang et al., 2005). Fig. 1A reveals that four branches including one for reptiles, one for amphibians and two for fish, are sandwiched between the CXCL9/10 and CXCL11 groups, indicating that the loss of this group of chemokines is confined to birds. Both trees (Fig. 1A and C) group the amphibian molecules to the base of the CXCL9/10 clades, suggesting they could be related to an ancestral gene that subsequently diverged into CXCL9 and CXCL10. The reptile group is shown to be more closely related to CXCL11 than CXCL9/10 in Fig. 1A but the low bootstrap value does not provide firm support of the grouping. In fact, Fig. 1C places the reptile group as an outgroup for CXCL9-11. A more confident grouping of the reptile sequences (Accession Nos., DW360860, FG731789, FL590394 and XP_003221876) with CXCL11s was revealed with a bootstrap value of 87% in a tree constructed using only these reptile sequences and mammalian CXCL9-11s (Supplementary Fig. 3a). Similarly, the amphibian CXCL9/10 molecules group with the CXCL9/10 branch with a bootstrap value of 87% in a tree containing only the Xenopus sequences (Accession Nos., BP708034, DT451279 and EL710536) and mammalian CXCL9-11s (Supplementary Fig. 3B). Therefore, these two groups were referred to as reptile CXCL11 and amphibian CXCL9/10 in the present study.

Our previous study identified a trout molecule which is induced by IFN-γ and which appeared to be a homologue belonging to this cluster of chemokines (Laing et al., 2002). This molecule is placed in a branch consisting of 13 more sequences from other teleost species (with the cyprinid molecules previously called CXCb) which tend to cluster with the CXCL11 branch (Fig. 1A). However, this grouping is weak and the branch displays a similar phylogenetic distance to CXCL9/10s and CXCL11s in a separate tree (Fig 1C). Interestingly, another fish group is consistently shown to group weakly with reptile CXCL11s in both trees presented, suggesting it is related to this family of chemokines. Hence, we propose to call this group CXCL11_L2 (Supplementary Fig. 4), and CXCL11_L1 for the more distantly related group because it was first reported. In zebrafish, CXCL11_L2 is encoded by a single copy gene which is located in chromosome 5 (Nomiyama et al., 2008).

3.4. Additional lineage specific CXC groups are present in teleosts

Chemokines are believed to have undergone extensive expansion within teleost fish and specific CXC chemokines that are not found in other vertebrate orders have been reported (Nomiyama et al., 2008; DeVries et al., 2006; Huising et al., 2003; Wiens et al., 2006). For example, trout CXCd1 and CXCd2 are fish specific chemokines that are not related to any known vertebrate CXC chemokine group (Wiens et al., 2006). As can be seen in Fig. 1C, these molecules form a distinct fish specific clade which we propose to call CXCL_F1. Thus we refer to these two molecules as CXCL_F1a and CXCL_F1b here (Table 3). In the present study, a third related molecule has been found in trout (Fig. 1C), sharing moderate sequence similarity with CXCL_F1a (56.5%) and CXCL_F1b (57.4%), which we call CXCL_F1c. Four other fish clades are also apparent from the tree in Fig. 1C, which we call CXCL_F2-F5, although CXCL_F3 has an amphibian member. The CXCL_F2 group is a novel phylogenetic group which has not be described previously in fish and is found in 10 teleost species including salmonids and gadoids. Like the CXCL_F1 group, it has no apparent homologues in the zebrafish genome (Nomiyama et al., 2008). The CXCL_F3-5 groups are unique in terms of cysteine number, with an additional pair of cysteines seen in the C terminal region of their mature peptides compared to other known CXC chemokines (Supplementary Fig. 4). The CXCL_F3 group consists of three molecules, all from cyprinid species including catfish, loach and zebrafish (previously referred to CXCL-chr1c) (Nomiyama et al., 2008). In contrast, CXCL_F4s and CXCL_F5s are present in varied teleost families such as salmonids, gadoids and cyprinids.

3.5. CXC chemokines are differentially expressed in fish tissues, fibroblasts, monocytes/macrophages and primary HK leucocytes in rainbow trout

To gain further information on the role of the CXC chemokines in teleost fish, especially the novel groups that do not have apparent homologues in mammals, their expression in the rainbow trout tissues and cells was studied. To our knowledge, only four CXC chemokines were previously reported in trout, namely CXCL8_L1 (Laing et al., 2002), CXCL11_L1 (γIP) Laing et al., 2002; O'Farrell et al., 2002, CXCL_F1a (CXCd1) and CXCL_F1b (CXCd2) Wiens et al., 2006. The new trout chemokine sequences were deposited in the NCBI nucleotide database under accession numbers: HE578132–HE578138.

Trout CXC chemokines contain predicted signal peptides of 20–27 amino acids (aa), suggesting they are secreted peptides (Fig. 2). The putative mature peptides are small in size, ranging from 75 aa to 109 aa, and all are positively charged (Table 2). Eight peptides including CXCL12a, CXCL12b, CXCL14, CXCL11_L1, CXCL_F1a, CXCL_F1b, CXCL_F1c and CXCL_F5 have a net charge of at least +10, with CXCL_F5 the highest charge at +20.5. In fact, the extended C terminal region of the trout CXCL_F5 contains multiple

Table 2
Sequence information and features of trout CXC chemokines.

Trout CXC ligand	Accession No.	Signal peptide	Mature peptide			
			Length	MW	No of cysteines	Charge
CXCL8_L1	AJ279069	22	75	7932	4	+4.0
CXCL11_L1	AJ417078	20	95	10,789	5	+10.5
CXCL12a	HE578135	23	75	8939	4	+11.3
CXCL12b	HE578136	23	76	9034	4	+13.0
CXCL13	HE578137	22	97	10,696	4	+7.3
CXCL14	HE578138	23	77	9518	4	+13.5
CXCL_F1a	DQ191448	22	90	10,366	4	+12.8
CXCL_F1b	NM_001124562	21	90	10,164	4	+10.5
CXCL_F1c	HE578132	22	93	10,480	4	+14.5
CXCL_F4	HE578134	25	84	9747	6	+4.5
CXCL_F5	HE578133	27	109	12,465	6	+20.5

basic aa residues (eg arginine and lysine). The four cysteine residues are well aligned, with the first two cysteine residues spaced by a single residue. Interestingly, 9 of the 11 trout molecules possess a cationic arginine residue that precedes the CXC signature motif (Fig. 2), with a lysine present in this position in CXCL14.

Expression of the trout CXC chemokines (except CXCL_F1b which is quite similar to CXCL_F1a – 84–87% aa identity) was examined in a fibroblast (RTG-2) and monocyte/macrophage cell line (RTS-11) and in primary HK leucocytes (Fig. 3). In RTG-2 cells, relatively high levels of CXCL8_L1, CXCL_F1a and CXCL_F4 transcripts were detected, with the highest level recorded for CXCL_F4 (Fig. 3A). In RTS-11 cells, CXCL_F5 was the most highly expressed chemokine, with moderate levels of CXCL8_L1, CXCL12a and CXCL13 transcripts also detected (Fig. 3B). The primary HK leucocytes freshly isolated from healthy fish displayed relatively high constitutive expression for CXCL8_L1, CXCL11_L1, CXCL_F4 and CXCL_F5, with lower levels of CXCL12a and b (Fig. 3C). In summary, CXCL8_L1 was constitutively expressed in all three cell types and the transcript levels of CXCL14 and CXCL_F1c were always very low. In addition, CXCL_F4 and CXCL_F5, which have three pairs of cysteine residues in the mature peptides, exhibited distinct expression patterns in RTG-2 and RTS-11 cells, suggesting they may be produced by specific cell types.

Analysis of constitutive expression in different tissues revealed distinct patterns of expression profiles for the CXC chemokines (Fig. 4). Among the non-teleost specific CXC chemokines, CXCL12 and CXCL14 were highly expressed in some tissues. For example, high levels of CXCL12a transcripts were detected in HK and spleen whilst comparable level of CXCL12b was seen only in HK. Interestingly, brain and gills but not other tissues contained the highest levels of CXCL14. For the teleost specific CXC chemokines, marked expression of CXCL_F4 was noted in both systemic (HK and spleen) and mucosal (gills and skin) lymphoid tissues (Fig. 4B). Gills exhibited the highest level of CXCL_F1a and CXCL_F5 compared with other tissues. In general, CXCL_F1C and CXCL13 were expressed at low levels in all tissues. Of the tissues analysed, liver and thymus generally had the lowest expression levels of the different chemokines studied.

3.6. Proinflammatory and antiviral cytokines have distinct effects on CXC chemokine expression in fibroblasts, macrophages and HK leucocytes

It has been well documented that the expression of chemokines is regulated by cytokines. In this study, trout cytokines known to trigger different immune responses were selected for evaluating their impact on the gene expression of the chemokines identified. These included IL-1β as a key proinflammatory cytokine, type I IFN as a typical antiviral cytokine, and IFN gamma (IFN-γ) that activates macrophages and regulates cell mediated immunity. RTG-2, RTS-11 and primary HK leucocytes were stimulated with these cytokines for 24 h at a dose of 20 ng/ml, and chemokines expression analysed by qPCR. Our previous studies have shown that these cytokines are biologically active at the concentration used (Zou et al., 2005, 2007; Hong et al., 2004).

Stimulation of RTG-2 cells with rIFN2 resulted in induction of CXCL11_L1 and CXCL_F4, but with a less than 10-fold increase of the transcripts (Fig. 4). A downregulation of CXCL12a was also observed in cells treated with rIFN2. rIL-1β induced gene expression of CXCL8_L1, CXCL11_L1, CXCL12a, CXCL_F4, and CXCL_F5 (Fig. 5A). An increase of 395- and 254-fold was recorded for CXCL8_L1 and CXCL11_L1 respectively. In contrast, expression of other CXC chemokine genes was not affected by rIL-1β treatment. In agreement with our previous study, rIFN-γ dramatically induced CXCL11_L1 expression, with a remarkable increase of 1571-fold. Expression of CXCL_F4, CXCL_F5, and CXCL12b was also upregulated by rIFN-γ, but to a more moderate extent. Similar to rIFN2, rIFN-γ had an inhibitory effect on CXCL12a expression. Taken together, these data indicate that CXCL11_L1 was upregulated by all three cytokines in RTG-2 cells whilst CXCL12a was down-regulated by the antiviral cytokines rIFN2 and rIFN-γ.

Modulation of the CXC chemokine expression was also studied in RTS-11 cells (Fig. 5B). Trout CXCL8_L1, a putative proinflammatory chemokine, was significantly induced by rIL-1β. A 21-fold increase was also seen for CXCL_F4 transcripts. For CXCL_F5, CXCL11_L1 and CXCL13, a moderate increase (typically less than

Table 3
The nomenclature of fish CXC chemokines proposed in this study.

Chemokine	Fish species		Reference
	Salmonids	Cyprinids	
CXCL8_L1	IL-8, CXCL8	IL-8, CXCL8, CXCa, CXCa_L1, CXCL_chr1a, CXCL_chr1b	Nomiyama et al. (2008), van der Aa et al. (2010), Abdelkhalek et al. (2009), Laing et al. (2002) and Castro et al. (2008)
CXCL8_L2		IL-8, CXCLc, CXCc_L2, CXCd, CXCL_chr13c	Nomiyama et al. (2008), van der Aa et al. (2010), Abdelkhalek et al. (2009) and Castro et al. (2008)
CXCL8_L3		CXCL_chr13d	Nomiyama et al. (2008)
CXCL11_L1	γIP, Vig-7/8, CXCL10	CXCL10, CXCb, CXC64, CXCL-chr5b, CXCL-chr5d, CXCL-chr5f	Nomiyama et al. (2008), Huising et al. (2003), Laing et al. (2002), Chen et al. (2010), Savan et al. (2003) and O'Farrell et al. (2002)
CXCL11_L2		CXCc, CXCL-chr5c	Nomiyama et al. (2008) and Huising et al. (2003)
CXCL12	SDF	SDF, CXCL12a , CXCL12b, CXCL12bl,	Nomiyama et al. (2008) and Baoprasertkul et al. (2004)
CXCL13		CXCL_chr13a	Nomiyama et al. (2008)
CXCL14		Scyba,CXCL-chr14	Nomiyama et al. (2008)
CXCL_F1	CXCd		Wiens et al. (2006)
CXCL_F3		CXCL2 like, CXCL-chr1c	Nomiyama et al. (2008) and Baoprasertkul et al. (2005)

5-fold) was observed. Other CXC chemokines did not respond to rIL-1β stimulation. In contrast to rIL-1β, rIFN2 treatment resulted in a significant induction of CXCL11_L1 expression (383-fold) and a weak induction of CXCL8_L1, CXCL_F4 and CXCL_F5 expression. Apparently, rIFN-γ stimulation triggered a pan CXC chemokine response, with the exception of CXCL14 that was not affected. The most responsive gene, CXCL11_L1, displayed an 811-fold increase, followed by CXCL_F5 (66-fold increase). Unlike rIL-1β and rIFN2 that up-regulated CXC expression, rIFN-γ down-regulated CXCL12a and CXCL_F1a expression.

The HK in fish is a lymphoid tissue equivalent to mammalian bone marrow and is important for leucocyte haematopoiesis. In HK leucocytes, rIFN2 had a stimulatory effect on CXCL11_L1 expression but not on other chemokines (Fig. 5C). rIL-1β enhanced gene expression of a wider range of genes, including CXCL8_L1, CXC12a, CXC13, CXCL_F4 and CXCL_F5. However, rIFN-γ was the most potent inducer in HK cells, with 7 out of the 10 genes studied up-regulated (CXCL8_L1, CXCL11_L1, CXCL12a, CXCL12b, CXCL13, CXCL_F4 and CXCL_F5), with the largest effect on CXCL11_L1 (95-fold increase) (Fig. 6). Overall, rIL-1β, rIFN2 and rIFN-γ had distinct effects on CXC chemokine gene expression in RTG-2, RTS-11 and HK cells, with the largest effects of cytokine treatment seen in RTG-2 and RTS-11 cells.

3.7. CXC chemokines are modulated in vivo during bacterial infection

A genetically attenuated strain of *A. salmonicida* (Brivax-I) was used to study chemokine expression during bacterial infection in rainbow trout (Marsden et al., 1996). Fish were injected i.p. with Brivax-I or PBS, and HK tissues sampled at days 1 and 3 for qPCR analysis. Fish responded well to bacterial exposure, as shown by an elevated transcript level of IL-1β at both days 1 and 3 (Fig. 6). In terms of chemokine expression, CXCL8_L1, CXCL11_L1, CXCL_F4, and CXCL_F5 were all induced significantly at day 1 and CXCL11_L1 and CXCL_F5 also at day 3 although more weakly, in line with induced IL-1 expression. Conversely, expression of CXCL12b and CXCL_F1a was decreased at day 1 but returned to control fish levels at day 3.

3.8. CXCL11_L1, CXCL_F4 and CXCL_F5 are involved in regulating migration of CD4+ cells and macrophages

The expression data showed that CXCL8_L1, CXCL11_L1, CXCL_F4 and CXCL_F5 were the most responsive chemokines to proinflammatory and antiviral cytokines. It has been shown previously that recombinant trout CXCL8_L1 enhances migration of neutrophils and macrophages (Montero et al., 2009; Harun et al., 2008),

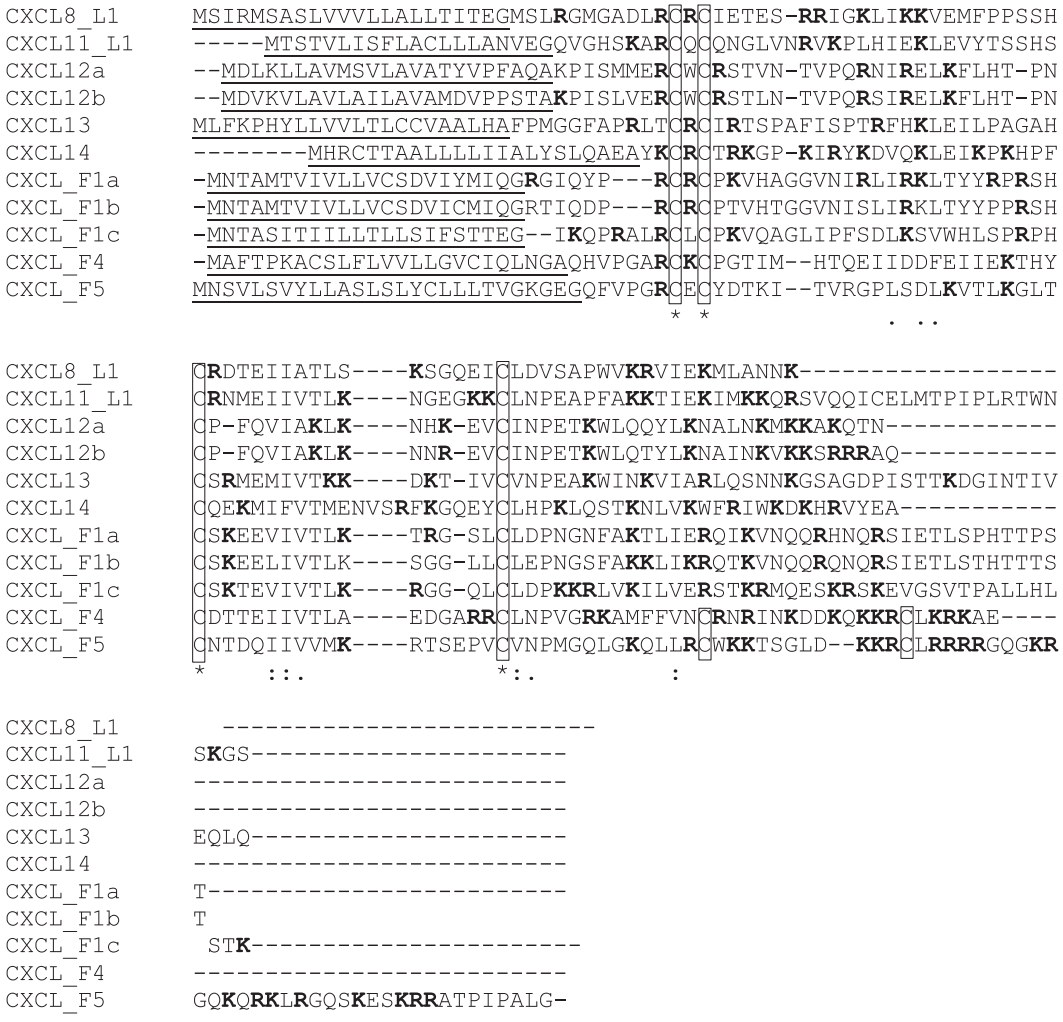

Fig. 2. Identification and sequence analysis of CXC chemokines in rainbow trout. A. Multiple alignment of the full length protein sequences. Identical amino acids among all sequences are indicated by asterisks whereas those with high or low similarity are indicated by ':' and '.' respectively. Putative signal peptides are underlined and conserved cysteine residues boxed. Cationic residues in the mature peptide (arginine and lysine) are highlighted in bold.

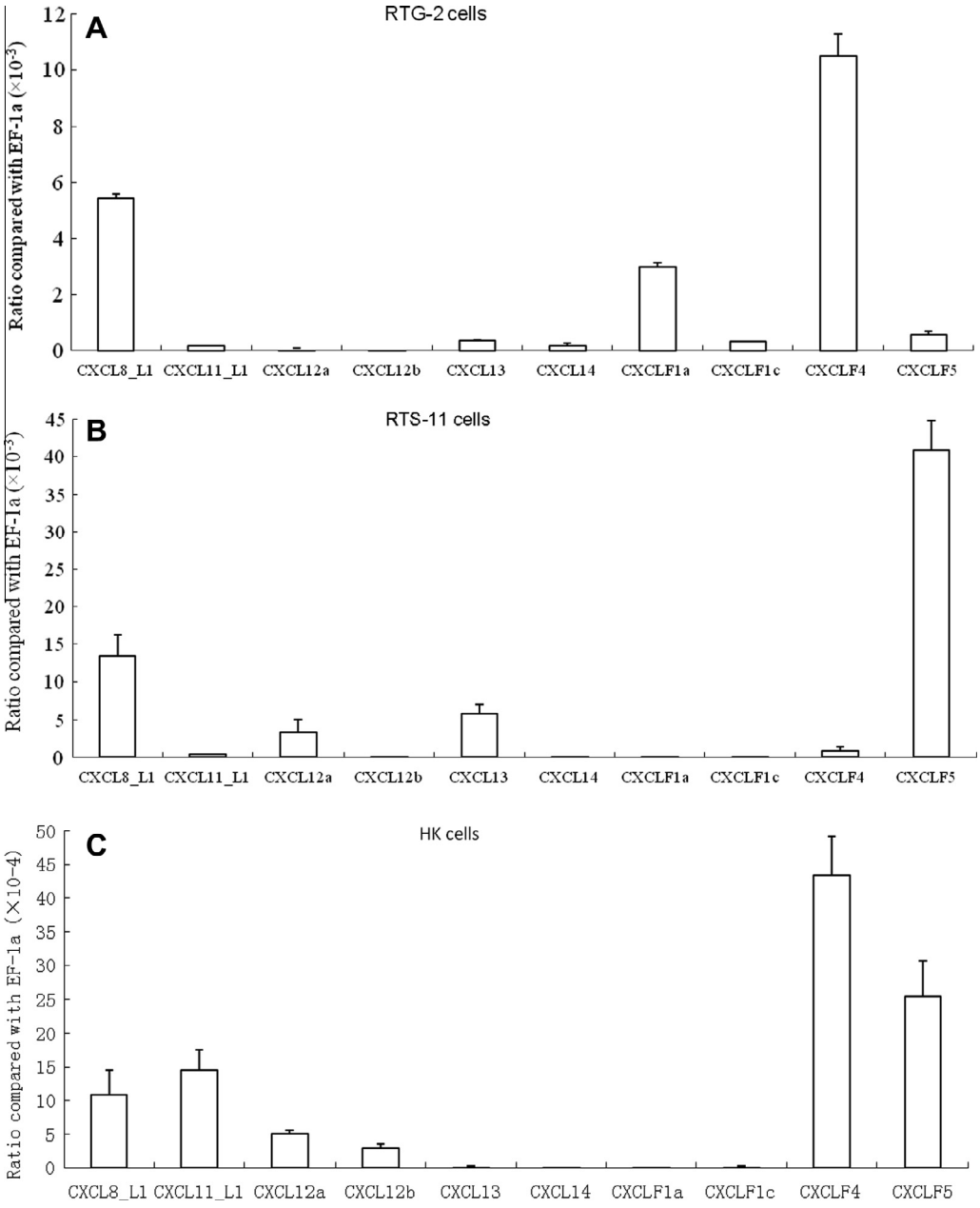

Fig. 3. Constitutive expression of CXC chemokines in RTG-2 (fibroblasts) (A), RTS-11 (monocytes/macrophages) (B), and primary head kidney (HK) leucocytes (C). Total RNA was extracted from RTG-2 and RTS-11 cells or HK tissue, and analysed for gene expression by real time PCR. Expression levels of chemokine genes were normalised to the house keeping gene elongation factor 1 alpha (EF-1α). The data are presented as means of three independent flasks of RTG-2 and RTS-11 cells or tissue from three fish.

but the chemoattractant activity of CXCL11_L1, CXCL_F4 and CXCL_F5 has not been investigated before in trout, or indeed any fish species in the case of CXCL_F4 and CXCL_F5. Thus, in this study, these three trout CXC chemokines were produced as recombinant proteins in CHO cells transfected with the corresponding expression plasmids and assessed for their effect on migration of primary HK leucocytes. Expression of the leucocyte surface markers for macrophages (MCSFR), T cells (CD4 and CD8) and B cells (IgM heavy chain, IgMH) was analysed in migrated and unmigrated cells, as an indicator of the leucocyte types affected by the recombinant chemokines, and in control experiments with conditioned media from cells transfected with an empty plasmid IgMH was the dominant transcript present (78–82%), with CD4 and CD8 being relatively equal in proportion within the remaining transcripts (7–8% for CD4 and 11–14% for CD8) (Fig. 7). After confirmation of the trout chemokines transcripts in CHO cells transfected with CXCL11_L1, CXCL_F4 or CXCL_F5, the conditioned media were used for the chemotaxis assay. All three chemokines had similar effects on gene expression in the migrated HK leucocytes, resulting in a significantly higher expression of CD4 and MCSFR in migrated cells (lower chamber) compared with the non-migrated cells (upper chamber) (Fig. 7). Conversely, reduced levels of CD8 were seen in the migrated cells vs the non-migrated cells. In addition, with the CXCL11_L1 conditioned media, an increase in relative IgM expression was seen in the migrated cells, which was not seen with the CXCL_F4 and CXCL_F5 conditioned media. These data suggest that CXCL11_L1, CXCL_F4 and CXCL_F5 may play a role in co-ordinating the trafficking of macrophages and CD4+ cells in fish.

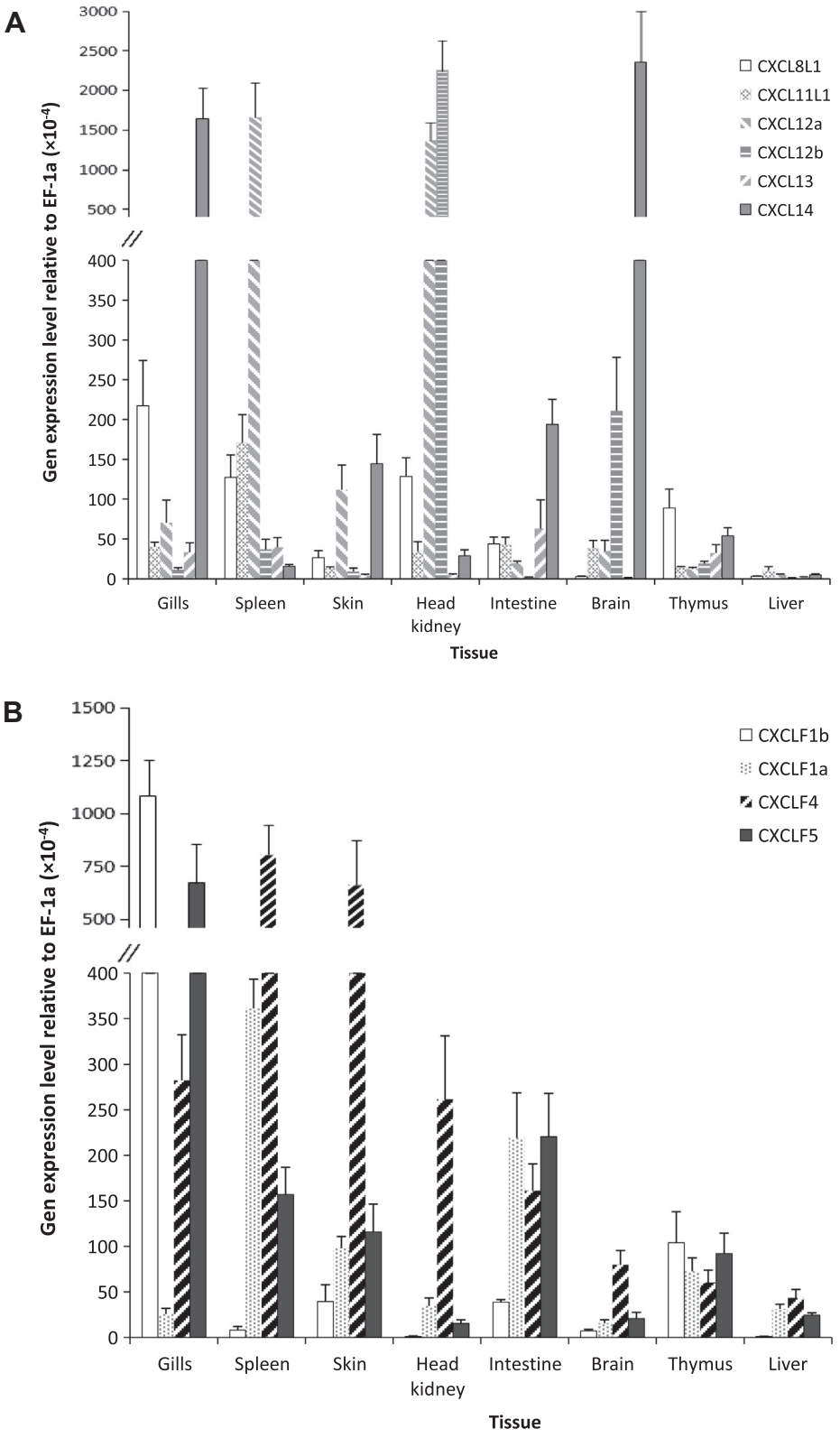

Fig. 4. Tissue distribution of CXC chemokines in healthy fish. Tissues including brain, gills, head kidney, intestine, liver, skin, spleen and thymus were collected from six trout for real time PCR analysis of common vertebrate (A) and teleost specific (B) CXC chemokines.

4. Discussion

Defining the phylogenetic relationships of CXC chemokines between lower and higher vertebrates has been problematic. However, as increasing numbers of chemokine genes are discovered and sequenced from early vertebrates, more refined analysis is possible. In this study, 421 full length protein sequences of the CXC chemokines were retrieved from the nucleotide and protein

databases of all vertebrate classes including teleost fish, amphibians, reptiles, birds and mammals, and their phylogenetic relationships analysed. In teleosts, 164 sequences of CXC chemokines were obtained from 48 fish species. These included the previously reported molecules with relatedness to CXCL8-14 and various novel lineage specific groups that do not have apparent homologues in mammals. In addition, teleost fish were found to possess a group that is related to avian CXCL15, which we term CXCL8_L3 in accordance with the recent nomenclature used for the two other CXCL8 related groups (CXCL8_L1 and CXCL8_L2) characterised in fish (van der Aa et al., 2010). A new group of CXC molecules related to CXCL9-11 was also discovered, which we term CXCL11_L2 using this same nomenclature, with CXCL11_L1 used for a known but more distantly related (to CXCL11) group of fish CXC chemokines. Furthermore, five novel groups of CXC chemokines, three containing three pairs of cysteines, were identified in teleost fish in a clear way for the first time. Based on the phylogenetic analysis, a new nomenclature for these fish CXC chemokines that do not have apparent homologues is proposed (ie CXCL_F1-5). We next analysed gene expression of most of these genes in rainbow trout *O. mykiss*, using cultured cells stimulated with proinflammatory and antiviral cytokines or fish exposed to bacterial infections. Three trout CXC chemokines (CXCL11_L1, CXCL_F4 and CXCL_F5) which were the most responsive genes in these expression studies, were made as recombinant proteins and shown to preferentially affect the migration of cells expressing CD4 and MCSFR in an *in vitro* chemotaxis assay.

The present analysis confirms and extends previous studies demonstrating that the CXC chemokines have expanded extensively in teleost fish (Nomiyama et al., 2008; DeVries et al., 2006; Wiens et al., 2006). Thirteen phylogenetic groups of CXC chemokines, namely CXCL8_L1-3, CXCL11_L1-2, CXCL12-14 and CXCL_F1-5, were identified in teleost fish, compared to six found in amphibians, four in reptiles and five in birds (Fig. 1A). It is apparent that the CXCL1-7 cluster is confined to mammals and appears to have expanded from a common progenitor shared by CXCL8/15 more recently in evolution. Whilst birds lack CXCL9-11, it is evident from the present study that gene(s) related to CXCL9-11 are present in fish, amphibians and reptiles (Fig. 1). Close clustering of CXCL9 and CXCL10 in the phylogenetic trees suggest they have arisen from a gene duplication event during tetrapod evolution.

Compared to mammals where the CXCL8 group consists of more than eight members, it appears that in fact three related groups, CXCL8_L1, _L2 and _L3, are present in teleosts. Recent studies have shown that teleost CXCL8_L2 appears to be more closely related to mammalian CXCL8's than teleost CXCL8_L1's, which were initially thought to be true CXCL8 homologues (van der Aa et al., 2010; Abdelkhalek et al., 2009; Laing et al., 2002). However, to date CXCL8_L2's have only been found in cyprinids. This is in contrast to the CXCL8_L1's which appear to be pan teleost chemokines, found in 19 species so far. Although the bootstrap value between fish CXCL8_L2 and mammalian CXCL8 is relatively low in Fig. 1B, this tree topology supports the notion that fish CXCL8_L2's are phylogenetically closer to mammalian CXCL8's than fish CXCL8_L1's. However, in Fig. 1A, where amphibian, reptile and bird CXCL8's branch with mammalian CXCL8, the fish CXCL8_L1 group is closer, and probably reflects a common ancestry of both genes. The third group, CXCL8_L3, appears to have relatedness to chicken CXCL15 which is an ELR$^+$ CXC chemokine, and is identified for the first time in fish. However, CXCL8_L3 lacks the ELR+ motif. The genes encoding CXCL9-11 are tandemly clustered in the human genome and are absent in birds. The present study shows that amphibians and reptiles possess a single group of homologues of this group of chemokines. Curiously, the amphibian group is more closely related to CXCL9/10 than CXCL11 and conversely the reptile group appears to be a homologue of CXCL11. In fish, two groups (CXCL11_L1 and CXCL11_L2) with relatedness to CXCL9-11s exist, one of which (CXCL11_L1) was described as a gamma interferon induced protein (γIP) in rainbow trout in our previous study (Laing et al., 2002). While the phylogenetic relationship of fish CXCL11_L1 within the CXCL9-11 cluster remains ambiguous, fish CXCL11_L2 molecules group with reptile CXCL11s as shown by the consistent grouping in the two trees generated (Fig. 1A and C). In zebrafish, where multiple chemokine molecules related to the CXCL11_L1 group exist (e.g. Accession Nos. NM_001126413, XM_001339271 and XP_696046) (Nomiyama et al., 2008; Chen et al., 2010) only one is within the CXCL11_L2 group (Accession No., NM_001115055). Our analysis contrasts with the tree of Nomiyama et al. (2008) where this gene has no clear relationship to the CXCL11 clade. The genes encoding these chemokines in zebrafish are clustered in chromosome 5, suggesting they may have evolved from a common ancestral gene.

We are not the first to report the existence of fish specific CXC chemokines (Nomiyama et al., 2008; Wiens et al., 2006). For example, the trout CXCd group (referred to as CXCL_F1 in the present study) does not correlate to known chemokine groups in other vertebrates (Wiens et al., 2006), and is contained in a complex "Fish" group of CXC genes in the tree of Nomiyama et al. (2008). A related gene is reported in this latter tree (omCXCL-C), and may represent the third member of this clade identified in trout in our study (CXCL_F1c). Analysis of zebrafish CXC chemokines did not reveal any members of the CXCL_F1 group despite the large number of these genes present in this species. Four additional fish clades were revealed by our phylogenetic tree analysis, named CXCL_F2-CXCL_F5. Whilst there were many fish representatives in most of these groups, the exception was CXCL_F3 which appears to be another cyprinid only group although it also contains a Xenopus member, and is thus potentially a molecule that has been lost during tetrapod evolution. A catfish molecule was also in this group and in the CXCL_F5 family. Interestingly the CXC chemokines in the CXCL_F3-5 group are phylogenetically related with a high grouping bootstrap of 81% and are also unique in containing six cysteines in the mature peptides rather than four (see Fig. 2). One of their striking features is that they contain multiple cationic residues, such as arginine and lysine.

It is not clear why fish possess more chemokines than mammals. Studies in mammals have shown that many chemokines have redundant functions and share common receptors for signalling. In contrast to the ligands, fewer chemokine receptors exist (Viola and Luster, 2008). For example, mammalian CXCL1-8 interact with CXCR1 and 2, whilst CXCL9-11 bind to CXCR3. The homologous sequences for some of the CXC chemokine receptors have been reported in teleost fish and are known to be structurally conserved, consisting of seven transmembrane domains, a feature typical of the G-protein coupled receptor family (Alejo and Tafalla, 2011). Whilst comparative analysis may provide some clues as to the pairing of the ligands and receptors for the evolutionarily conserved CXC chemokines such as CXCL12 and CXCL14, it is more difficult to use such an approach to predict the receptors for the fish lineage CXC chemokines. Therefore, future experiments are needed to characterise the interaction between the lineage specific chemokines and their putative receptors.

Expression of CXCL8_L1 and CXCL8_L2 has been studied in several teleost species. As one of the earliest chemokines discovered in fish, CXCL8_L1 has been more extensively studied and is consistently shown to be up-regulated by the ligands of pattern recognition receptors (PRR) such as LPS, PHA, ergosan and zymosan (Alejo and Tafalla, 2011; van der Aa et al., 2010; Laing et al., 2002; Peddie et al., 2002; Sangrador-Vegas et al., 2002). IL-1β, one of the main cytokines promoting inflammation, significantly induced CXCL8_L1 expression in this study, in agreement with the findings that CXCL8 in mammals is up-regulated by classic

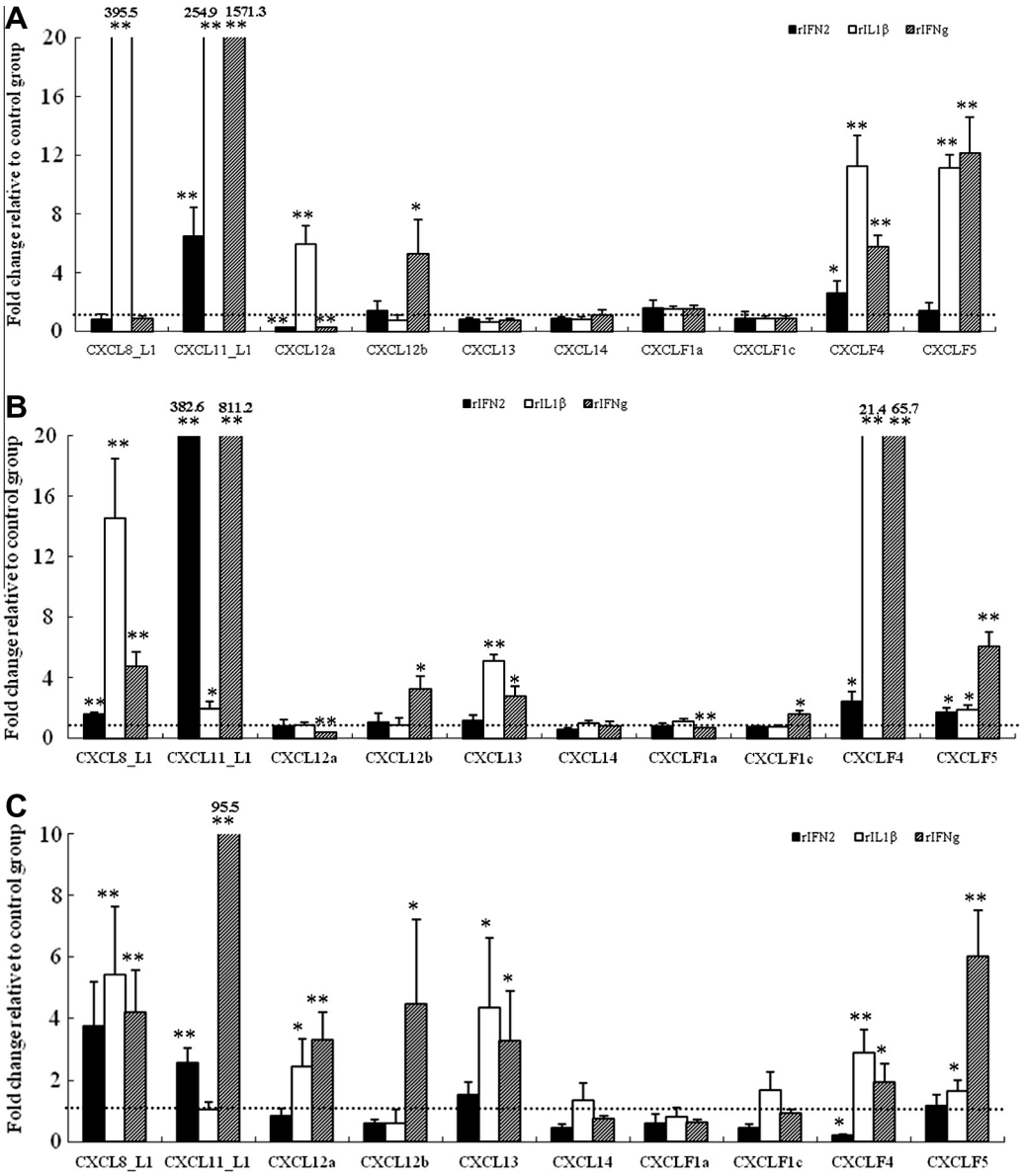

Fig. 5. Effects of rIL-1β, rIFN2 and rIFN-γ on expression of CXC chemokines in RTG-2 cells (5A), RTS-11 cells (5B) and primary head kidney (HK) leucocytes (C). The cells were stimulated for 24 h with 20 ng/ml of each recombinant cytokine and analysed for gene expression by real time PCR. Gene expression levels of chemokines were normalised to that of EF-1α and compared between the cytokine treated groups and the control group (control = 1) to obtain the relative fold changes. The data are presented as means (±SD) of three independent flasks of cells, with $^{*}p < 0.05$ or $^{**}p < 0.01$ considered significant. The primary HK leucocytes were isolated from three individual fish.

proinflammatory cytokines such as IL-1β, TNF-α and IL-6 (Sangrador-Vegas et al., 2002; Harada et al., 1994). Interestingly, trout CXCL8_L1 was also induced by IFN-γ in macrophage like RTS-11 cells but not in fibroblast like RTG-2 cells, suggesting CXCL8_L1 expression is regulated in a cell type specific manner. Type I IFN is a poor inducer of CXCL8 expression and had a marginal effect on CXCL8_L1 expression in RTG-2 cells in this study. This is consistent with the previous observation that polyI:C, a type I IFN inducer, also exhibited a weak stimulatory effect on CXCL8_L1 expression in trout (Laing et al., 2002). In contrast to CXCL8_L1, information on the gene expression of CXCL8_L2 is more limited but recent studies suggest that it is expressed and modulated by PRR ligands in a similar way to that of CXCL8_L1 (van der Aa et al., 2010; Abdelkhalek et al., 2009) and has similar biological effects (van der Aa et al., 2012).

Fish appear to have two groups of CXC genes with relatedness to the mammalian CXCL9-11 group. As in mammals, CXCL11_L1, also referred to as γIP, was indeed activated by trout rIFN-γ in both RTG-2 and RTS-11 cells in this study, in agreement with our past reports (Castro et al., 2008) and studies in carp using HK leucocytes (van der Aa et al., 2012). Although the exact signalling pathway leading to gene activation is not fully elucidated in fish, an IFN-γ activated sequence (GAS) element is present in the promoter region of trout CXCL11_L1 (Castro et al., 2008) together with an IFN-stimulated regulatory element (ISRE). Curiously, however, studies of the promoter of another trout rIFN-γ responsive gene (low molecular mass peptide 2, LMP2) suggest that responsiveness is not mediated by the GAS element alone, and that deletion of an ISRE ablates rIFN-γ responsiveness. A comprehensive analysis of the CXCL11_L1 genes present in zebrafish did not report GAS elements, although ISRE, IRF and STAT sites were universally present, with a NF-kB site also present in most cases (van der Aa et al., 2012). These data suggest that the CXCL11_L1 gene promoter(s) is regulated by other elements in addition to the GAS motifs in fish,

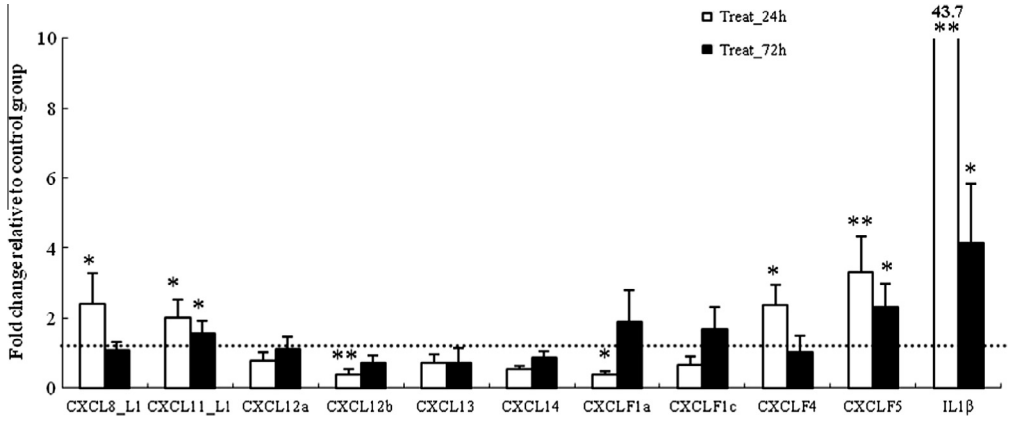

Fig. 6. CXC chemokine expression in response to bacterial infection. Trout were intraperitoneally injected with 250 μl of 2×10^{10} cells/ml *A. salmonicida* (Brivax I) or an equal volume of PBS. Head kidney (HK) tissue was sampled from five fish in each group at 24 and 72 h post infection and analysed for gene expression. Gene expression levels of chemokines were determined by real time PCR, and data normalised to that of EF-1α in both experiments and compared between the infected and control group (control = 1). Data are presented as fold changes relative to the controls (±SD), with $^{*}p < 0.05$ or $^{**}p < 0.01$ considered significant. Interleukin-1β (IL-1β) was used as a positive gene marker, since it is known to increase following infection.

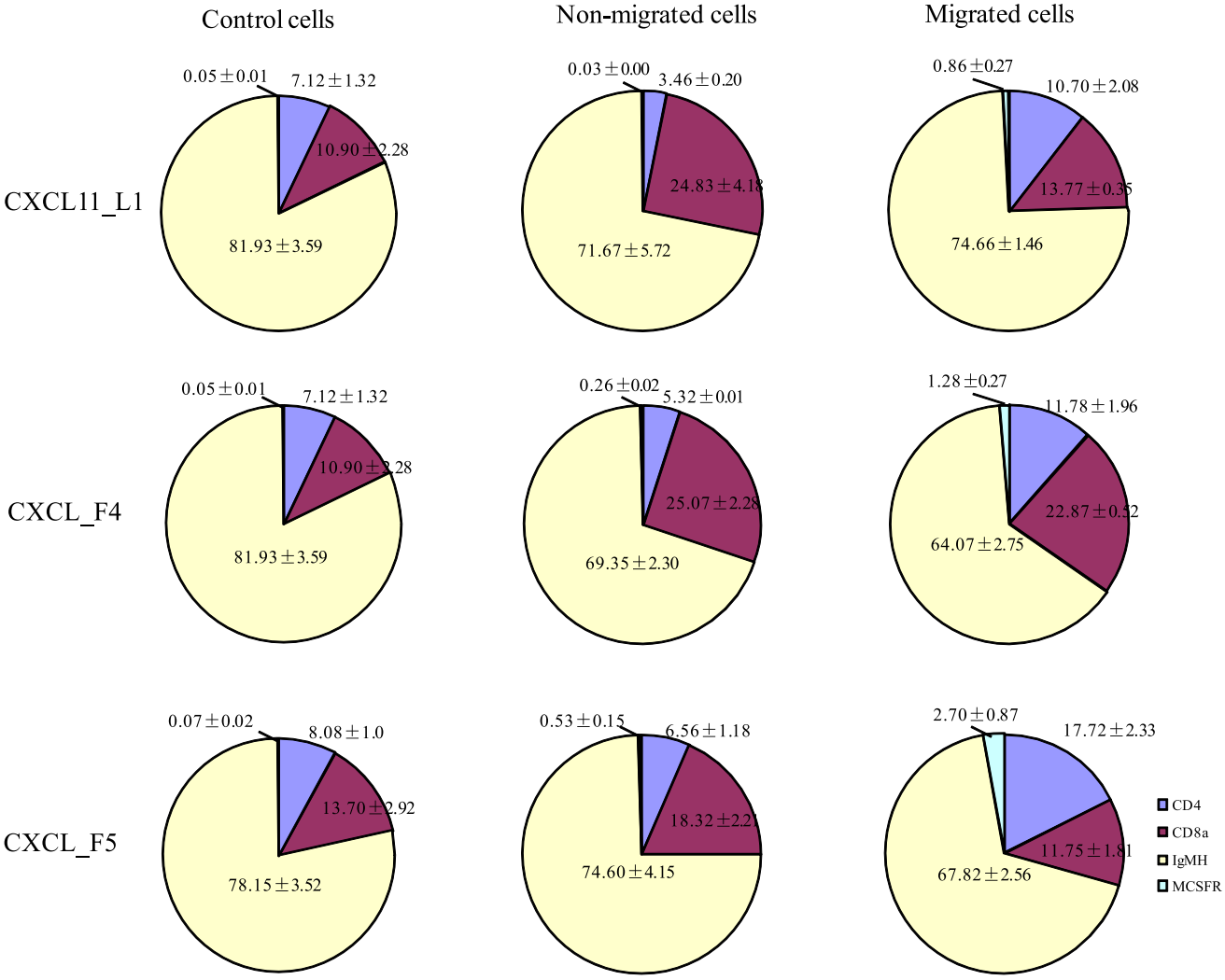

Fig. 7. Chemoattractant activity of CXCL11_L1, CXCL_F4 and CXCL_F5. Head kidney cells were freshly prepared from healthy fish for the chemotaxis assay. Conditioned media collected from CHO cells transfected with chemokine expression plasmids, or from controls transfected with empty plasmid, were used for chemotaxis assay as described in Section 2. Migrated and non-migrated cells were collected for expression analysis of leucocyte lineage markers including MCSFR, CD4, CD8 and IgMH. Gene expression levels were normalised to the house keeping gene elongation factor 1 alpha (EF-1α) and the relative percentage of each transcript calculated against the sum of the four marker genes. Three independent chemotaxis experiments were performed using cells collected from three individual fish and data are presented as percentage (±SD). Note cells from the same three individuals were used for analysing CXCL11_L1 and CXCL_F4 activity.

and this notion is supported by the finding in the present study that induction patterns of trout CXCL11_L1 expression by IL-1 and type I IFN are significantly different in fibroblasts and macrophages (Fig. 4). Interestingly the promoter of the zebrafish CXCL11_L2 gene (CXC-chr5c) was also analysed in the above study, and found to contain all of the above elements, suggesting it will be regulated in a similar manner to CXCL11_L1.

CXCL12, a stromal cell derived factor, promotes angiogenesis and also serves as a chemoattractant for T and B cells. It plays an important role in co-ordinating migration of primordial germ cells through CXCR4, demonstrating its critical role in organ development (Boldajipour et al., 2008). Fish possess two CXCL12 isoforms, one of which (CXCL12a) was shown to be dominantly expressed in lymphoid tissues (Nomiyama et al., 2008; DeVries et al., 2006; Huising et al., 2004). However, in catfish, ubiquitous expression in tissues was noted and in early stage embryos (Baoprasertkul et al., 2005). Both CXCL12a and CXCL12b were found to be highly expressed in head kidney and spleen of trout in the present study (Fig. 4), in agreement with previous studies. However, the two CXCL12 genes identified were expressed at a very low level in fibroblasts but at a relatively higher level in monocytes/macrophages and primary HK leucocytes (Fig. 3). Induced expression patterns of CXCL12a and 12b differed significantly depending on the cell lines and cytokines used, suggesting the two genes are regulated differentially. In some cases, down-regulation was apparent. However, caution must be taken in interpreting these results due to the low level of expression in unstimulated RTG-2 cells.

The primary role of CXCL13 in B and T cell migration has been documented in mammals (Viola and Luster, 2008; Burkle et al., 2007). The receptor interacting with CXCL13 is known to be dominantly expressed on the surface of B cells and transiently on $CD4^+$ T cells (Viola and Luster, 2008). In teleost fish, CXCL13 is expressed mostly in lymphoid organs including HK, spleen and peripheral blood leucocytes, and is induced by LPS in primary leucocytes (Kim et al., 2007). In agreement with this, trout CXCL13 was found to be induced significantly in RTS-11 cells in response to IL-1 and IFN-γ, suggesting it may play a role in recruiting immune cells during inflammation. Conversely, extremely low expression was detected in fibroblasts (RTG-2 cells) and tissues and there was no stimulatory effect of cytokines in RTG-2 cells. The fact that type I IFN did not modulate the CXCL13 transcript level in any of the cell types studied agrees with the finding that polyI:C was unable to affect CXCL13 expression in flounder leucocytes (Kim et al., 2007). Such data suggest that CXCL12 may be required for maintenance of homeostatic status of lymphoid organs in fish.

As with CXCL12, trout CXCL14 was not affected by cytokine stimulation in fibroblasts, macrophages and HK leucocytes. In vivo studies have shown that CXCL14 is expressed constitutively in a wide range of immune and nonimmune tissues and it has been suggested to be involved in immune defence, endocrine regulation and tissue development (Huising et al., 2004; Baoprasertkul et al., 2005). In fact, the present study showed a high level of CXCL14 expression in gills and brain (Fig. 4A). Interestingly, in humans, CXCL14 is highly expressed in mucosal tissues and was recently shown to directly kill bacterial pathogens, to some extent mimicking the actions of defensins (Augsten et al., 2009). Since CXCL14 is one of the most conserved chemokines, and has a high positive charge (Table 2), it would be of great interest to find out whether the fish CXCL14 molecules also play a role in mucosal defence against infection.

The trout CXC chemokines were also studied in vivo during bacterial infection. As in mammals, CXC chemokines responded differentially to bacterial infection in fish. Induction of CXCL8_L1 by bacterial pathogens has been well documented in several fish species (Alejo and Tafalla, 2011; Chen et al., 2005; Sigh et al., 2004; Sepulcre et al., 2007). Transient accumulation of trout CXCL_F1a (CXCd1) transcripts was also apparent after infection with *Yersinia ruckeri*, a pathogenic Gram negative bacterium, and appeared to correlate with bacterial loads in infected fish (Wiens et al., 2006). CXCL11_L1 was dramatically upregulated during infection with an intracellular bacterial pathogen, *Edwardsiella ictaluri*, in catfish (Baoprasertkul et al., 2004). However, the homeostatic CXC chemokines such as CXCL12 and CXCL14 are not affected in these fish species by bacterial infection (Baoprasertkul et al., 2005). In the present study, injection of a high dose of a genetically attenuated strain of *A. salmonicida* (Brivax I) resulted in an increase of expression of CXCL8_L1, CXCL11_L1, CXCL_F4 and CXCL_F5 in trout HK, suggesting that they are likely involved in regulation of inflammation during the early stages of infection. Indeed, this response was acute, with induction peaking at day 1 post infection. Interestingly, two CXC chemokines, CXCL12a and CXCL_F1a (CXCd1), were down-regulated at day 1 after bacterial injection, the latter in contrast to the study of Wiens et al. (Wiens et al., 2006) with *Y. ruckeri*. It is unclear whether this difference is due to the tissues sampled for analysis, the bacterial species used or dose of bacteria injected. In this study, fish were expected to survive infection and it may be interesting to assess the CXC chemokine expression in response to a pathogenic strain of *A. salmonicida*.

The functions of fish CXC chemokines are poorly understood. The best characterised chemokines to date are CXCL8_L1, CXCL8_L2 and CXCL13 (van der Aa et al., 2010; Tian et al., 2010; Harun et al., 2008; Montero et al., 2008). Recombinant trout CXCL8_L1 produced in bacteria or transfected cells has been shown to enhance leucocyte migration *in vitro* and induce expression of other chemokines (Harun et al., 2008; Sanchez et al., 2007). In a more recent study, it has been demonstrated that carp CXCL8_L1 and CXCL8_L2 are equally active in directing phagocyte migration (van der Aa et al., 2010). Given the fact that CXCL11_L1, CXCL_F4 and CXCL_F5 were significantly up-regulated *in vitro* by cytokines and in vivo during bacterial infection, they were further analysed for their role(s) in regulating fish leucocyte migration in trout. In mammals CXCL9, CXCL10 and CXCL11 bind to a single receptor (CXCR3) which is predominantly expressed on the surface of activated and memory T cells, and are believed to have redundant functions in directing T cells and NK cells to designated sites. Trout CXCL11_L1 appears to exhibit similar effects, in promoting migration of cells expressing the CD4 molecule. In addition, for the first time we show that the six cysteine containing CXC (fish) groups, CXCL_F4 and CXCL_F5, were also able to act primarily on cells expressing the CD4 molecule. It must be pointed out that since gene transcript levels of the cell lineage markers rather than the cell numbers were measured in the migrated and non-migrated cell fractions recovered in the chemotaxis assay, a direct impact of the chemokines on gene expression cannot be ruled out. Nevertheless, the finding that they are all inducible in response to IFN-γ is rather interesting. In addition to cells expressing the CD4 molecule, macrophages could also be targeted by CXCL11_L1, CXCL_F4 and CXCL_F5 since elevated transcript levels of MCSFR were seen in the migrated cell fraction. In fish, a putative receptor (CXCR3) for CXCL11_L1 has recently been identified and shown to be expressed in mononuclear phagocytes and macrophages (Aghaallaei et al., 2010; Wang et al., 2008). However, direct interaction between putative fish CXCR3 and CXCL11_L1 has yet to be determined. Furthermore, it will be important to determine the receptors for fish CXCL_F4 and CXCL_F5 and the cells that express them in future studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2013.05.006.

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