Chemokine signaling in innate immunity of zebrafish embryos

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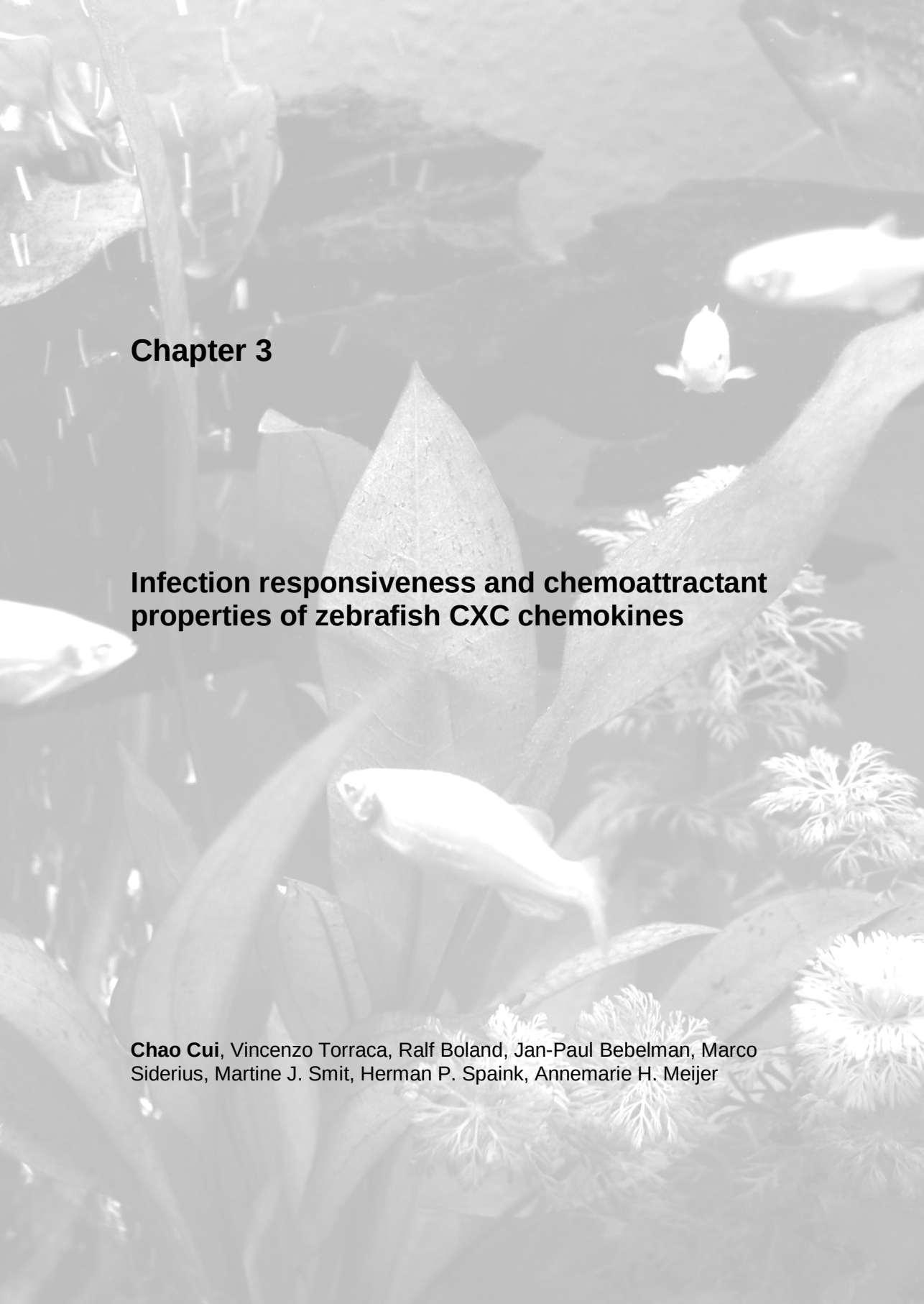

Chapter 3

Infection responsiveness and chemoattractant properties of zebrafish CXC chemokines

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Abstract

Chemokines are a group of small secreted cell-signaling proteins which play important roles in development and activation of the immune system. The CXC chemokine subfamily is known for its ability to promote trafficking of various types of leukocytes and to regulate angiogenesis and vascular remodeling. Zebrafish is an emerging model for studying chemokine signaling and chemokine-dependent cell migration. However, CXC chemokine functions in the zebrafish immune system still remain largely unknown. In the present study, we have analyzed available database resources for CXC chemokines in zebrafish and studied their phylogenetic relationships with human chemokines. We examined the expression of CXC chemokines in zebrafish embryos upon challenges with two different bacterial pathogens: *Salmonella typhimurium* and *Mycobacterium marinum*. Two infection-inducible chemokines were selected for protein purification using a *Pichia pastoris* expression system and subsequently used for *in vivo* leukocyte migration assays. These studies support zebrafish Il8 to exert chemoattractant properties for zebrafish embryonic neutrophils. Furthermore, the *in vivo* migration assays revealed a chemotattractant function on zebrafish embryonic macrophages for a chemokine similar to human CXCL11.

Introduction

Chemokines are a family of small cell-signaling proteins (8–15 kDa) that direct the migration of cell types that express the corresponding receptors. They are best known for their functions in development and activation of the immune system (Esche *et al*., 2005; Kunkel *et al*., 1995). In the process of host defense, chemokines play important roles in guiding directional migration of immune cells to sites of inflammation and injury (Cyster, 1999; Campbell and Butcher, 2000). In addition to their functions in the immune system, certain chemokines and their receptors are also involved in processes such as angiogenesis (Arenberg *et al*., 1997; Keane *et al*., 1998), neurological development (Gordon *et al*., 2009; Belmadani *et al*., 2006), and germ cell migration (Doitsidou *et al*., 2002; Knaut *et al*., 2003; DeVries *et al*., 2006).

Chemokines are categorized into four families based on the positions of highly conserved cysteine residues: C, CC, CXC, and CX3C. The CXC chemokine family is known for its ability to promote trafficking of various types of leukocytes and to regulate angiogenesis and vascular remodeling (Folkman, 1997; Mackay, 2001; Balestrieri *et al*., 2008). The CXC subgroup can be further divided into ELR+ and ELR− chemokines, based on the presence or absence of the glutamic acid-leucine-arginine (ELR) sequence at the NH2 terminus. ELR+ CXC chemokines, like CXCL8 (interleukin-8, IL8), are involved in recruitment of neutrophils into inflamed tissues

(Constantin *et al*., 2000; Bizzarri *et al*., 2006), while the activation of T-cells is the best studied function of ELR− chemokines, such as CXCL9, CXCL10, and CXCL11, which signal via CXC chemokine receptor CXCR3 (Campbell *et al*., 2004; Gangur *et al*., 1998). Dysfunctioning of CXC chemokines and their receptors has been linked with various inflammatory and autoimmune disorders and malignancies (Hickey *et al*., 1999; Liu *et al*., 2005).

Zebrafish is an emerging model for studying chemokine signaling and chemokine-dependent cell migration (Raz and Mahabaleshwar, 2009). In recent years, many different chemokine and chemokine receptor sequences have been identified in teleost fish species, including the zebrafish (Alejo and Tafalla, 2011). Using expressed sequence tags (EST) and the zebrafish draft genome sequence, a total of 46 putative CC-chemokine genes were first identified in zebrafish (Peatman and Liu, 2007). Another analysis of the draft genome in the same year led to the identification of 24 putative chemokine receptor and 63 putative CC and CXC chemokine genes, many of which may be derived from local duplication events (DeVries *et al*., 2006). A more recent analysis of the EST and zebrafish genome databases detected over a hundred chemokine genes, including 25 CXC subgroup members and a novel subfamily CX (Nomiyama *et al*., 2008). It can be concluded from these studies that chemokines genes have undergone a strong diversification in zebrafish, in contrast to the chemokine receptors, which appear to be well conserved. A phylogenetic reconstruction showed that most zebrafish chemokines were generated after the divergence of fish and mammals and that clear phylogeny relationships between zebrafish and human can be observed only for CXCL12 and CXCL14 (Nomiyama *et al*., 2008).

Recently, the zebrafish orthologous gene of CXCL8/IL8 has been proposed based on synteny analysis (Oehlers *et al*., 2010). However, zebrafish Cxcl8/Il8 lacks the ELR sequence present in human IL8, and phylogeny analysis shows that several other zebrafish CXC chemokines have a similarly close relationship to human IL8 (Nomiyama *et al*., 2008; van der Aa *et al*., 2012). Supporting its role in the inflammatory response, *il8* transcription in zebrafish embryos was found to be induced after bacterial challenge, by lipopolysaccharide (LPS) treatment, and by exposure to the intestinal inflammation-inducing agent trinitrobenzene sulfonic acid (TNBS) (Stockhammer *et al*., 2009; Oehlers *et al*., 2010). In addition to *il8* expression, the *cxcl-c1c* gene was also responsive to bacterial challenge in zebrafish embryos (Stockhammer *et al*., 2009). It has been further suggested that some zebrafish CXC-chemokine genes (CXC-56, CXC-64, and CXC-66) may play a role in regulating Th1/Th2 responses following diverse stimuli (Chen *et al*., 2008). The common carp (*Cyprinus carpio* L), a large cyprinid fish that is closely related to zebrafish, has been used to study the function of some CXC chemokines by means of *in vitro* chemotaxis assays with head kidney leukocytes. In these assays, recombinant CXCL8-like chemokines (CXCa_L1 and CXCL8_L2) stimulated

chemotaxis of macrophages and granulocytes, and a CXCL9/10/11-like chemokine (CXCb) also stimulated lymphocyte infiltration, which supports their functional homology with mammalian CXCL8 and CXCL9/10/11, respectively (van der Aa *et al*., 2012). The CXCL8-like carp chemokines also induced *in vivo* leukocyte recruitment following intraperiteoneal injection. In summary, the available evidence suggests that CXC chemokines of zebrafish and carp function both in innate and adaptive immune responses similar as the human CXC family members. However, genomic duplication events in fish and the lack of functional data make it very difficult to predict functional homologies between the fish and human CXC chemokines.

In this study we further analyzed available publications and the new Zv9 database for CXC chemokine genes in zebrafish and studied their phylogenetic relationships. We then examined the expression of CXC chemokines in zebrafish embryos upon challenges with two different bacterial pathogens: *Salmonella typhimurium* and *Mycobacterium marinum*. We selected infection-inducible chemokines for protein purification using a *Pichia pastoris* expression system and performed *in vivo* leukocyte migration studies to establish the function of these chemokines during the inflammatory response. These studies revealed a chemotactic function on zebrafish embryonic macrophages for a chemokine similar to human CXCL11, which is putative ligand of the zebrafish Cxcr3.2 receptor, previously shown to be involved in macrophage recruitment to local bacterial infection sites (Zakrzewska *et al*., 2010; Chapter 2).

Materials and methods

Zebrafish husbandry

Zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (http://ZFIN.org). Embryos were raised at 28.5°C in egg water (60 µg/ml Instant Ocean sea salts). Pigmentation in embryos used for whole-mount *in situ* hybridization was inhibited using 1-phenyl-2-thiourea (PTU; Sigma). For the duration of bacterial injections embryos were kept under anaesthesia in egg water containing 0.02% buffered 3-aminobenzoic-acidethylester (tricaine; Sigma).

RNA extraction

Pools of 20 embryos were snap frozen in liquid nitrogen and stored in -80°C. Total RNA from the samples was extracted using TRIZOL followed by treatment with RNase-free DNase (Qiagen). RNA concentrations were measured on a nanodrop ND-2000 (Thermo scientific).

cDNA synthesis and quantitative real-time PCR

cDNA synthesis reactions were performed in a 20 µl mixture of 500 ng of RNA, 4 µl of 5×iScript reaction mix (Bio-Rad Laboratories), and 1 µl of iScript reverse transcriptase (Bio-Rad Laboratories). The mixtures were incubated at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. Quantitative Real-time PCR was performed using the Chromo4 Real-time PCR detection system (Bio-Rad Laboratories) according to the manufacturer's instructions. Each reaction was performed in a 25 µl mixture comprised of 1 µl of cDNA, 12.5 µl of 2× iQ SYBR Green Supermix (Bio-Rad Laboratories), and 10 pmol of each primer. All reactions were performed in technical duplicates. Peptidylprolyl isomerase A-like (*ppial*), which showed no changes over the infection time course series, was used for normalization. Results were analyzed using the $\Delta\Delta$Ct method. Primer sequences are indicated in Supplementary Table S1.

Construction of phylogenetic tree

For phylogenetic analysis, the zebrafish and human chemokine protein sequences were aligned using CLC Workbench (Aarhus). For the alignment, sequences corresponding to the signal peptides were deleted. Amino acid distance matrices were used to infer phylogenetic trees by the neighbor-joining method with 1000 iterations. NCBI reference sequences for human chemokines were: CXCL8 NP_000575, CXCL9 NP_002407, CXCL10 NP_001556, CXCL11 NP_005400, CXCL12 NP_000600, CXCL13 NP_006410, and CXCL14 NP_004878. Zebrafish chemokine sequences used for the tree construction are summarized in Table 1.

Bacterial strains and infection of zebrafish embryos

Salmonella enterica serovar Typhimurium *(S. typhimurium)* LPS mutant strain (Ra) SF1592 (van der Sar *et al*., 2003) and *Mycobacterium marinum* strain E11 (van der Sar *et al*., 2004) were used for the infection of zebrafish embryos. *S. typhimurium* Ra bacteria were freshly grown overnight on LB agar plates supplemented with 100 µg/ml carbenicillin, and resuspended in phosphate-buffered saline (PBS) prior to injection. *M. marinum* E11 bacteria were grown in Difco Middlebrook 7H9 broth (BD Biosciences) supplemented with 10% albumin-dextrose-catalase (BD Biosciences) and 0.05% Tween 80 (Sigma-Aldrich) and the appropriate antibiotics overnight at 28.5 °C. Bacteria were resuspended to the desired concentration in 2% Polyvinylpyrrolidone (PVP40) in PBS (w/v). Embryos were staged at 28 hours post fertilization (hpf) (Kimmel *et al*., 1995) and infected with 50 colony forming units (cfu) of DsRed-expressing *S. typhimurium* Ra or mCherry expressing *M. marinum* E11. Bacteria were injected into the blood island for qPCR analysis of chemokine expression during systemic infections, or were locally injected into the tail muscle

above the urogenital opening for detection of chemokine expression by whole-mount *in situ* hybridization. As a control an equal volume of PBS was likewise injected. Injections were controlled using a Leica M50 stereomicroscope together with a FemtoJet microinjector (Eppendorf) and a micromanipulator with pulled microcapillary pipettes.

Table 1. Overview of CXC chemokine genes in zebrafish[1]

Gene	ENSDARG ID	GenBank accession	No. of amino acids (excluding signal peptide)
cxcl-c24e	ENSDARG00000071499		133
cxcl14	ENSDARG00000056627		77
cxcl-c13b	ENSDARG00000076618		90
cxcl-c1c	ENSDARG00000075045		89
cxcl11	ENSDARG00000093779 ENSDARG00000094706		86
cxc66		LOC798966	101
cxc56		LOC567656	66
cxc64	ENSDARG00000095747		82
cxcl11l		NC_007116	75
cxcl-c5g	ENSDARG00000092423		68
cxcl12a	ENSDARG00000037116		76
cxcl12b	ENSDARG00000055100		74
cxcl-c13d		NC_007118	95
il8l2		EH441857	92
cxcl-c5a	ENSDARG00000075056		91
il8		NC_007112	76
cxcl-c5c	ENSDARG00000075163		101

[1]Chemokine genes *cxcl-c2e*, *cxcl-c13b*, *cxcl-c1c*, *cxcl-c5g*, *cxcl-c13d*, *cxcl-c5a*, and *cxcl-c5c* are named by the chromosome on which they are located according to Nomiyama *et al.*, 2008. Genes *cxc66*, cxc56, and *cxc64* are named according to Chen *et al.*, 2008. All other gene names are according to Ensembl or ZFIN (http://www.zfin.org).

Whole-mount in situ *hybridization*

Pools of 20-40 embryos were fixed overnight in 4% paraformaldehyde in PBS at 4°C and whole-mount *in situ* hybridization using alkaline phosphatase detection with BM purple substrate (Roche) was performed according to Thisse *et al*. (2007). Genomic DNA was used to generate templates for *il8* and *cxcl-c1c* riboprobes synthesis by PCR using gene-specific primers sets including the binding site for T7 RNA polymerase in the reverse primer. Primer sequences are indicated in Supplementary Table S2. The coding sequences of *cxcl11* and *cxcl11l* were

obtained using the gene synthesis service of BaseClear (Leiden, The Netherlands) and cloned in pCS2+ vector, which was digested with BamHI to generate templates for probe synthesis. Digoxigenin-labeled riboprobes were synthesized using the labeling mixes from Roche and Ambion MEGAscript reagents for *in vitro* transcription.

Production of recombinant zebrafish chemokine proteins

The sequences of zebrafish chemokines were codon optimized for *Pichia pastoris* expression and obtained using the gene synthesis service of BaseClear (Leiden, The Netherlands). An HA-epitope tag was added at the 3' end. The sequences were cloned in the *Pichia pastoris* expression vector pICZαA (Invitrogen). Following transformation, *Pichia pastoris* clones were grown in small scale culture and screened by Western blot analysis to select high production clones for purification from a large culture. Recombinant zebrafish chemokines were purified by High-Performance Liquid Chromatography (HPLC) and stored at 4°C in 50 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 6.5) containing 1M NaCl.

Leukocyte migration studies

For testing chemoattractant properties on zebrafish embryonic macrophages, 1 nl of chemokine protein (0.5 µg/µl) purified from *Pichia pastoris* was delivered by micro-injection into the hindbrain ventricle at 28 hpf. Injected embryos were fixed for L-plastin immunofluorescence detection at 2.5 hours post injection (hpi). For neutrophil chemotaxis assays, chemokines were injected into the otic vesicle of embryos from the Tg(*mpx:egfp*)^{i114} line at 2 dpf. GFP-labeled neutrophils infiltrating the otic vesicle were counted at 2.5 hpi. As a control in macrophage or neutrophil chemotaxis assays an equal volume of the chemokine storage buffer was likewise injected.

Results

Phylogeny of CXC chemokine family members in human and zebrafish

It has been reported that zebrafish possess a considerably larger and more complicated chemokine system than mammals, as a result of local duplication events and a fast evolving rate of chemokine genes (DeVries *et al*., 2006, Nomiyama *et al*., 2008). Due to this complexity, the description of the zebrafish chemokine family is still incomplete and there are inconsistencies in the annotation of chemokine genes between subsequent versions of the zebrafish genome assembly. To provide an updated description and clarify the divergence of CXC chemokines between human and zebrafish, we searched the recent literature, the

NCBI database, and the most recent version of the zebrafish genome in the Ensembl database (Zv9) to accumulate information for all the members in this chemokine subfamily. As summarized in Table 1, a total of 17 CXC chemokine sequences could be retrieved from different sources. While only 10 of these could be mapped to the current Zv9 genome assembly, we considered all sequences for further analysis. Signal peptide sequences were predicted by the web-based SMART tool for most of the zebrafish chemokines (Supplementary Table S1). To analyze the evolutionary link between zebrafish and human chemokine genes, we constructed a phylogenetic tree based on the encoded amino acid sequences excluding the signal peptide sequences (Fig. 1). Several clusters of chemokines could be distinguished in the tree. Zebrafish and human chemokines grouped together with high bootstrap support in the CXCL12 and CXCL14 clusters. A moderate bootstrap support was observed for the relationship between human CXCL13 and zebrafish Cxcl-c24c. In addition, a CXCL8 cluster could be distinguished, which included human CXCL8 (IL8), the zebrafish chemokine that has been previously annotated as Il8 (Oehlers *et al.,* 2010), and three other zebrafish CXC chemokines. Two closely related zebrafish chemokines, Cxcl-c1c

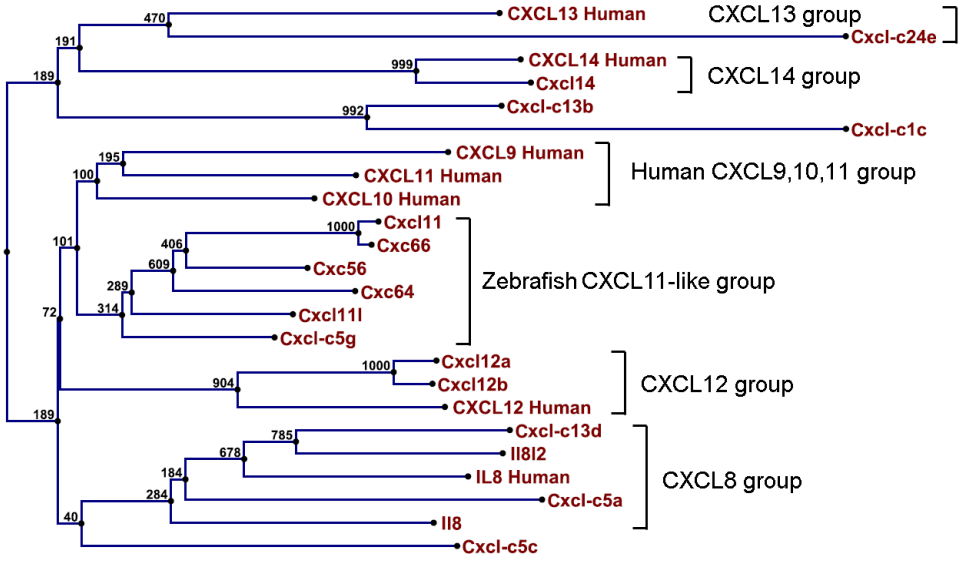

Figure 1. Phylogenetic analysis of zebrafish and human CXC chemokines. The phylogenetic tree was constructed from the amino acid sequences of zebrafish and human CXC chemokines by the Neighbour Joining method. The signaling peptide sequences were excluded from the total protein sequences for this analysis. Five subfamilies of chemokines are indicated with brackets, namely the human CXCL9, 10, 11 group, the zebrafish Cxcl11-like group, and three groups (CXCL14, CXCL13, CXCL12, CXCL8) comprising both human and zebrafish chemokines. Numbers at the branch nodes represent the confidence value of interior-branch test from 1000 reiterations.

and Cxcl-c13b, and another family member, Cxcl-c5c, fell outside any human-zebrafish clusters. Furthermore, no obvious orthology relationships with zebrafish chemokines could be predicted for CXCL9, 10, and 11, which are the three ligands of receptor CXCR3 in human. However, a cluster of six zebrafish chemokines, including Cxcl11, Cxcl11l, Cxc56, Cxc64, Cxc66, and Cxcl-c5g, is relatively close in the tree, which makes them putative candidate ligands for the zebrafish CXCR3 homolog (Cxcr3.2), which we previously found to be required for macrophage migration to bacterial infection sites in zebrafish embryos (Zakrzewska *et al.,* 2010; Chapter 2).

Inducible expression of zebrafish CXC chemokines upon systemic infection with S. typhimurium *and* M. marinum

To determine which of the 17 identified zebrafish CXC chemokines may be involved in the host immune response of zebrafish embryos, we performed systemic bacterial infections with the *S. typhimurium* Ra strain or *M. marinum* E11 strain. Zebrafish embryos were injected with fluorescent-labeled bacteria or PBS into the blood island at 28 hours post fertilization (hpf). Based on earlier studies of the transcriptional response of zebrafish innate immune genes to these infections (Stockhammer *et al.*, 2009; van der Sar *et al*., 2009), the embryos were sacrificed at 3 and 8 hours post infection (hpi) for total RNA isolation. An additional time point of 5 days post infection (dpi) was included for the infection with *M. marinum*, which persists in granuloma-like immune cell aggregates during zebrafish larval development (Davis *et al.*, 2002), in contrast to *S. typhimurium* Ra infection, which is non-pathogenic and cleared by 5 dpi (van der Sar *et al.,* 2003). Quantitative RT-PCR (qRT-PCR) was performed using specific primers for all the 17 CXC chemokine genes to reveal their expression levels upon bacterial challenge. The expression levels were normalized to *ppial* (NM_199957), which showed no change over the infection time course. Expression levels in the infected groups were determined relative to the PBS-injected control group (Fig 2). Among 17 CXC chemokines, 5 genes (*cxcl11l, cxcl-c13b, cxcl-c1c, il8,* and *il8l2*) showed clear induction (fold change >2) upon *S. typhimurium* Ra infection at 3 hpi, of which *cxcl11l* and *il8* continued to show increased expression by 8 hpi (Fig.2 A, B). In the case of *M. marinum* infection, *cxc66, cxcl11l, cxcl-c5g,* cxcl-*c1c,* and *il8* were over 2-fold induced at 3hpi, and among these *cxcl11l*, *cxcl-c1c*, and *il8* showed highest induction at 8 hpi (Fig. 2A, B). At the later stage of *M. marinum* infection (5 dpi), three CXC chemokine genes, *cxcl11, cxcl11l,* and *cxcl-c1c*, showed over 15-fold induction levels, while several other chemokines genes (cxc56, cxcl-c13b, cxcl-c5a, cxcl-L2, cxcl-c13d and il8) showed inductions in the range of 2-5-fold (Fig. 2C). In conclusion, several members of the zebrafish CXC chemokine family were induced at different stages of bacterial infection, suggesting that these play a role in host defense of the zebrafish embryo.

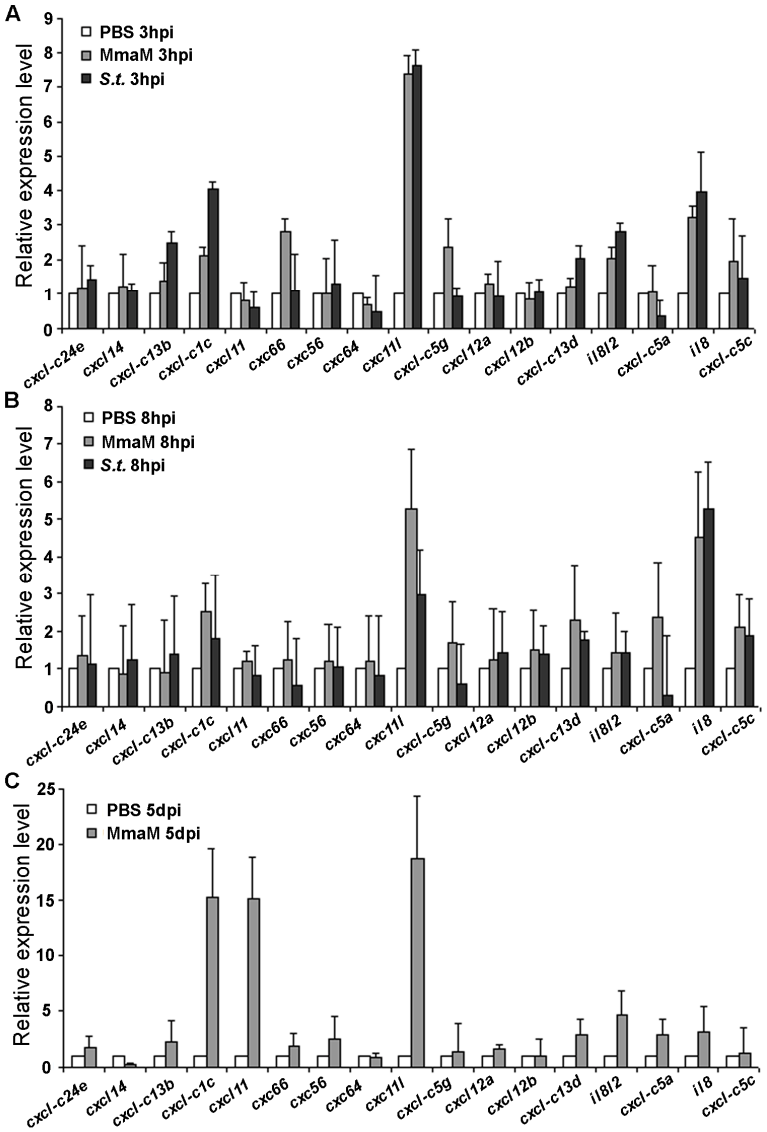

Figure 2. Expression of zebrafish *cxc* chemokine genes in response to bacterial infection. Seventeen zebrafish CXC chemokine genes were analyzed by qRT-PCR analysis on total RNA samples from embryos infected by blood island injection of 150 CFU of the *S. typhimurium* Ra strain (*S.t.*) or 150 CFU of the *M. marinum* M strain (MmaM). Samples were taken at 3 hpi (A) and 8 hpi (B), and for MmaM infection additionally at 5 dpi (C). Mock injections with PBS were used as a control. qRT-PCR results were normalized to peptidylprolyl isomerase A-like (*ppial*), and data are presented as relative induction of the infected groups compared with the relevant mock-injected control groups. Values are the means ± SEM of two independent experiments.

Spatially restricted expression of zebrafish CXC chemokines upon local infections with S. typhimurium *and* M. marinum

Four of the CXC chemokines, *cxcl-c1c, il8, cxcl11*, and *cxcl11l*, which were induced by infections, were selected for further analysis by whole-mount *in situ* hybridization. To this end, we induced local bacterial challenge in the zebrafish embryos by injecting the *S. typhimurium* Ra strain or *M. marinum* E11 strain into the tail muscle above the urogenital opening at 28 hpf. Whole-mount *in situ* hybridization at 3 hpi showed that mRNAs of *cxcl-c1c* and *il8* accumulated in a localized area around the *S. typhimurium* and *M. marinum* infection sites, while they were not induced by mock injection with PBS buffer (Fig. 3). *In situ* hybridizations of *cxcl11* and *cxcl11l* were not successful. The localized expression around infection sites is consistent with a possible function of the Cxcl-c1c and Il8 in forming a gradient for leukocyte chemotaxis during the bacteria-induced host immune response.

Chemoattractant activities of zebrafish CXC chemokines on zebrafish embryonic leukocytes

To date, no functional studies of the specific roles of zebrafish CXC chemokines in immune cell migration have been reported. To investigate the predicted function of CXC chemokines as chemoattractants for leukocyte migration in zebrafish embryos, we produced recombinant proteins using a *Pichia pastoris* expression system. As first candidates for purification, we selected zebrafish Il8, which we expected to exert chemoattractant properties for neutrophils, and Cxcl11, a *M. marinum*-infection inducible chemokine. This chemokine is one of the group of six chemokines similar to the human CXCL9/10/11 group and therefore represents a putative ligand for receptor Cxcr3.2, for which we previously demonstrated a function in zebrafish embryonic macrophage migration during bacterial infection (Zakrzewska *et al.*, 2010; Chapter 2). As an assay for neutrophil migration we injected the purified proteins into the otic vesicle of 2 dpf zebrafish embryos. Injection of the purified Il8 protein resulted in a significantly increased accumulation of Mpx-positive neutrophils in the otic vesicle at 2.5 hpi as compared to injection of the Cxcl11 protein or mock injection with buffer only (Fig. 4). To assay macrophage migration, we injected the proteins into the hindbrain at 28 hpf. We used the pan-leukocytic L-plastin marker to detect macrophages, since neutrophils do not actively migrate into the hindbrain ventricle at this developmental stage (data not shown) and no other leukocytes have yet developed. Hindbrain injection of Cxcl11 resulted in a significantly increased infiltration of the hindbrain ventricle with macrophages at 2.5 hpi as compared to the mock-injected group (Fig. 5A-C). In contrast, injection with Il8 had no effect on macrophage migration (Fig. 5C). In conclusion, we have demonstrated chemoattractant functions for two infection-

inducible zebrafish CXC chemokines, showing the specificity of Il8 for neutrophils and of Cxcl11 for macrophages in zebrafish embryos. Furthermore, the distinctive role of Cxcl11 in macrophage chemoattraction suggests that this particular chemokine might function as a ligand for the embryonic macrophage-specific Cxcr3.2 chemokine receptor.

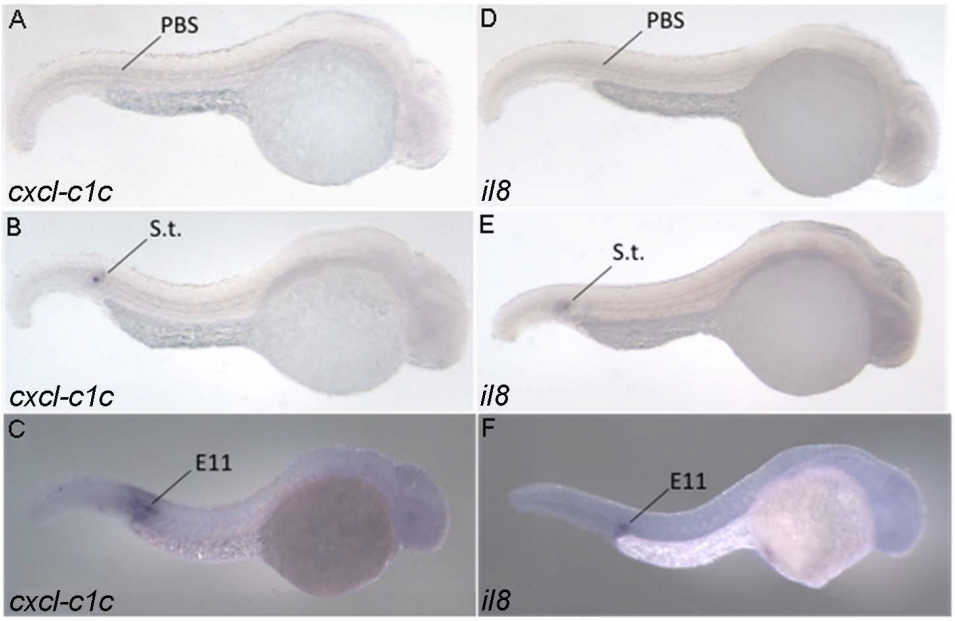

Figure 3. Analysis of spatial expression patterns of infection-induced CXC chemokine genes by whole-mount *in situ* hybridization. Expression patterns of *cxcl-c1c* (A-C) and *il8* (D-F) were analyzed using digoxigenin-labeled RNA probes. Embryos were injected at 28 hpf with approximately 30 CFU of *S. typhimurium* Ra (B, E) or *M. marinum* E11 strain (C, F) into the somite muscle tissue of the tail above the urogenital opening as indicated in the figure, or mock-injected with PBS (A, D) at the same location. Embryos were fixed for *in situ* hybridization at 3 hpi. All embryos are orientated anterior to the right and dorsal to the top. S.t. *S. typhimurium* Ra strain; E11, *M. marinum* E11 strain.

Discussion

Chemokines play important roles in guiding directional migration of immune cells to sites of inflammation and injury (Cyster, 1999; Campbell and Butcher, 2000). Despite increasing use of the zebrafish for immunological studies, CXC chemokine functions in the immune system of this model organism are still largely unknown. In this study we have investigated the role of CXC chemokines in bacterial-induced

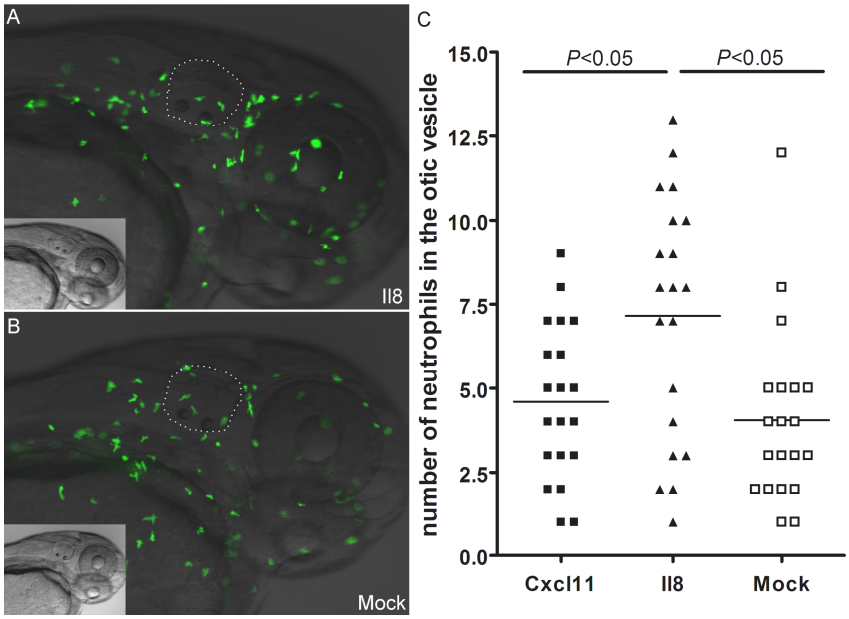

Figure 4. Neutrophil migration into the otic vesicle following injection of purified Il8 chemokine protein. (A, B) Representative fluorescence images of *mpx:EGFP* expressing neutrophils in the otic vesicle of 2 dpf embryos injected with Il8 (A) or mock injected with buffer solution (B). Embryos were fixed and imaged at 2.5 hpi. The otic vesicle is outlined with a dotted line. It should be noted that not all neutrophils in the otic vesicle are visible at the focal plane of the images. (C) Quantification of neutrophil migration induced by zebrafish CXC chemokines. *Mpx:EGFP* expressing neutrophils in the otic vesicle were counted manually under the stereo-fluorescence microscope on embryos fixed at 2.5 hpi. Il8 injection significantly increased neutrophil migration as compared to injection of Cxcl11 or mock injection with buffer solution. Each data point represents an individual embryo and lines indicate the mean value. *P* values indicate the level of statistical difference by one-way ANOVA.

inflammatory responses in zebrafish embryos. We have analyzed available database resources for CXC chemokine genes in zebrafish and studied their phylogenetic relationships with human chemokines. In agreement with previous studies, our analysis shows a large divergence of the CXC subfamily. In particular, duplications appear to have occurred for zebrafish chemokines that are related to human CXCL8 or to the human CXCL9/10/11 group, while zebrafish chemokines homologous to human CXCL12 and CXCL14 are the most strongly conserved. In order to establish the function of zebrafish chemokines during inflammatory responses, we analyzed their expression upon systemic challenges with two different bacterial pathogens: *S. typhimurium* and *M. marinum*. Chemokine genes commonly induced by both infections at different time points included *il8*, *cxcl-c1c*,

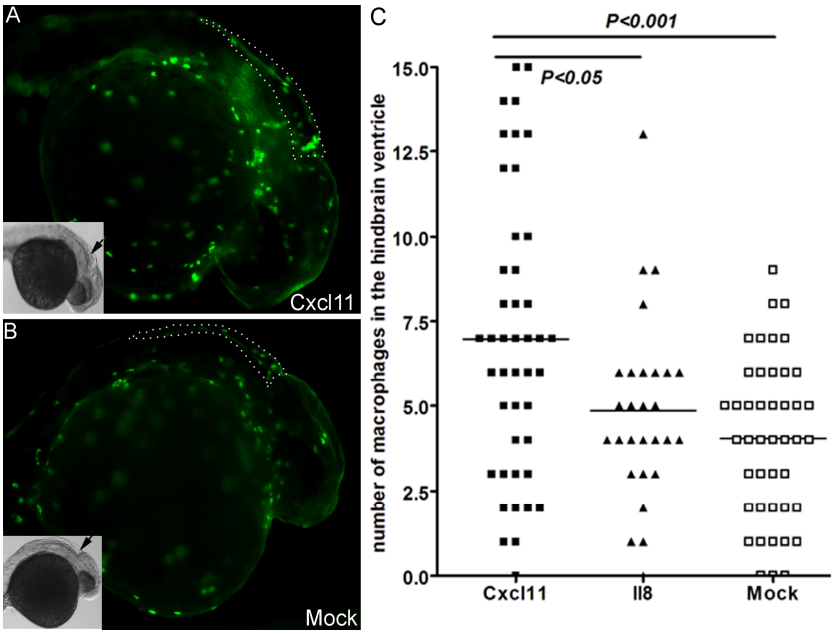

Figure 5. Macrophage migration into the hindbrain ventricle following injection of purified Cxcl11 chemokine protein. (A, B) Representative fluorescence images of L-plastin immuno staining to visualize macrophages numbers in the hindbrain of embryos injected at 28 hpf with Cxcl11 (A) or mock injected with buffer solution (B). Embryos were fixed at 2.5 hpi for L-plastin immuno staining. The hindbrain ventricle is outlined with a dotted line and indicated with an arrow in the bright field inset images. It should be noted that not all L-plastin-labeled immune cells in the hindbrain ventricle are visible at the focal plane of the images. (C) Quantification of macrophage migration induced by zebrafish CXC chemokines. L-plastin-labelled cells in the hindbrain were counted manually under the stereo-fluorescence microscope on embryos fixed at 2.5 hpi. Cxcl11 injection significantly increased macrophage migration as compared to injection of Il8 or mock injection with buffer solution. Each data point represents an individual embryo and lines indicate the mean value. *P* values indicate the level of statistical difference by one-way ANOVA.

and *cxcl11l*. Furthermore, the *cxcl11* gene was strongly induced at the later stage of *M. marinum* infection. A *Pichia pastoris* expression system was successfully employed to purify Il8 and Cxcl11 proteins and *in vivo* leukocyte migration assays demonstrated their chemoattractant properties for neutrophils and macrophages, respectively.

In our phylogenetic analysis, a CXCL8 cluster could be distinguished that included human CXCL8 (IL8), the zebrafish chemokine that has been previously annotated as Il8 (Oehlers *et al.,* 2010), and three other zebrafish CXC chemokines (Cxcl-c13d, Il8l2, and Cxcl-c5a). In line with another recent phylogeny study (van der Aa *et al.*,

2012), two of the three other CXCL8-related chemokines from zebrafish (Cxcl-c13d and Il8l2) are closer to human IL8 in the phylogenetic tree than the zebrafish gene that has been named *il8* based on synteny analysis (Oehlers *et al*., 2010). Among the four zebrafish chemokine genes in the CXCL8 cluster, we found the gene named *il8* to be the strongest inducible by *S. typhimurium* and *M. marinum* infections. Local injection of these two bacterial pathogens into muscle tissue of zebrafish embryos resulted in inducible expression of *il8* in a spatially restricted pattern around the infection sites, consistent with a function of its protein product in forming a gradient for leukocyte chemotaxis. This function was confirmed by injecting purified Il8 protein into the otic vesicle of 2 dpf zebrafish embryos, which resulted in the specific attraction of neutrophils. The chemoattractant function of zebrafish Il8 on neutrophils supports the orthology relationship with human IL8. Chemoattractant activities of the other three IL8-like zebrafish chemokines remain to be tested.

Like *il8*, *cxcl-c1c* is also highly induced by bacterial challenge in zebrafish embryos. The Cxcl-c1c protein sequence falls outside the main clusters of other CXC chemokines in the phylogenetic tree (Fig. 1), reflecting the rapid evolution of chemokines as compared with other components of the immune system, such as chemokine and pattern recognition receptors, signal transduction proteins, and transcription factors (Stein *et al*., 2007). Previously, we showed local induction of *cxcl-c1c* expression around a tail muscle infection site with *S. typhimurium* wild type bacteria (Stockhammer *et al*., 2009). Here, we extend these observations showing that the *S. typhimurium* Ra strain and *M. marinum* challenge also induce local expression of *cxcl-c1c*. These results strongly suggest that the infection-responsive chemokine Cxcl-c1c plays a role in the host defense response. The *Pichia pastoris* expression system was applied successfully in this study to demonstrate biological activities properties of two other zebrafish chemokines. Therefore, using this system will be a good approach for purifying Cxcl-c1c and testing its activity on macrophage and neutrophil migration in zebrafish embryos.

In chapter 2 we showed that morpholino knockdown of CXC chemokine receptor, Cxcr3.2, reduces macrophage migration in one-day-old zebrafish embryos upon bacterial challenge. However, the chemokine ligand(s) that is involved in this process is currently unknown. We have looked into the CXC chemokine family members in zebrafish that show similarity with human chemokine ligands for CXCR3, which is the human homolog of Cxcr3.2. Phylogeny revealed a cluster of six chemokines that was relatively close to the cluster of human CXCR3 ligands, CXCL9, CXCL10 and CXCL11. Among these six candidates, *cxcl11l* showed highly induced gene expression at all the time points that we examined during *S. typhimurium* and *M. marinum* systemic infections. In addition, *cxcl11* was highly induced at the later stage of *M. marinum* infection. To tests its biological activity, we purified Cxcl11 protein using the *Pichia pastoris* expression system. Injection of

Cxcl11 protein into the hindbrain of one-day-old zebrafish embryos demonstrated its function in attracting macrophages, while no attraction of neutrophils was observed in otic vesicle migration assays. Based on these results we propose Cxcl11 as a putative ligand for receptor Cxcr3.2, which is predominantly macrophage-specific. Cxcr3.2 may have multiple ligands, like its counterpart CXCR3 in human. Therefore, the five other chemokines in the zebrafish Cxcl11-like group remain to be tested using the same strategy. Especially Cxcl11l is a good candidate because of its strong induction at the early stages of infection. In conclusion, we have demonstrated here that the infection-inducible chemokine Cxcl11 has biological activity in chemoattraction of zebrafish embryonic macrophages, which makes it an interesting candidate as a putative ligand for Cxcr3.2.

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Supplementary data

Table S1. Primer sequences for q RT-PCR

Gene	Forward primer	Reverse primer
cxcl-c24e	AACACTGCTGTGCCTGGCTGT	GCCTTGCGGAGGACACCATCA
cxcl14	GTCGGCGGGCTCTGCATATCAA	GTGCTCCGCGACACGTCACA
cxcl-c13b	GGCTGGAGCAGTGCAGTGGA	TTTCCCCTGCTTGGACTCTGGA
cxcl-c1c	TCTTCTGCTGCTGCTTGCGGT	GGTGTCCCTGCGAGCACGAT
cxcl11	GCTGGAGAGGTCAACGGTCAGGA	TGCAAGATGGACTCGGAGGGAAGA
cxc66	TCTGACCTGCCTGATCGCTGGA	TGCCTTTGTCAGCACAGAAGCACC
cxc56	AGGCCAGGCGAGAGCTCCAA	TCCACAAGAAGGGGTCGGTGGT
cxc64	GAGGTGAAAGGCAAAATA	TGCTCCTTTATCAGCACACAAACA
cxcl11l	ACTCAACATGGTGAAGCCAGTGCT	CTTCAGCGTGGCTATGACTTCCAT
cxcl-c5g	AGGGTTGCACTGAGGAACATTGAGA	AGCCCTCCTGATTAATTCCTGGGT
cxcl12a	GCTGGTGCCGTTCCACAGTCA	GGGGCAGTTGGGTGTGTGGAG
cxcl12b	TGCTGGTGTCGCTCCACCCT	TGGAAAGGGCAGCTGGGAGTGT
cxcl-c13d	TGCATCAACCGCCTCCGCAAT	GCTGGATGGGCAGTCGAGCAG
il8l2	ACTGCACTGCTGTGCGCCAA	AGCAGGAGTCACCTTCAGTCCGA
cxcl-c5a	CACTGCCGCAAGGCCGAGAT	TGCCACAGTTGGCATGGCAGT
il8	TGTGTTATTGTTTTCCTGGCATTTC	GCGACAGCGTGGATCTACAG
cxcl-c5c	TCTCAGCGCTGTCGGTGCAT	TTCCCCTCCCTTCCACGACCG

Table S2. Primer sequences for whole-mount *in situ* hybridization

Gene	Forward primer	Reverse primer[1]
il8	ATGACCAGCAAAATCATTTCAGTGTG	*TAATACGACTCACTATAGGG*CTTCT TAACCCATGGAGCAG
cxcl-c1c	GTTAAACATAAATAACACCGACTC	*TAATACGACTCACTATAGGG*ACACC CTATAAAACTGAGTA

[1] Reverse primer sequences include the T7 RNA polymerase binding site

Table S3. Amino acid sequences of zebrafish CXC chemokines[1]

Gene	Sequence of amino acids
cxcl-c24e	MRSALLTLLCLAVMLLVQESYQVSSSSYCPCLKLSDGVLRKANIKSYIRQRAG VCHIDAIVFTTVRGITFCADPKLTWVIDAMKFLDKKKAASEPKTTTQPISSTFNA TSMPNTTVNLNTTNTTSDLNTTNTNTTSHLNTANTNTKAQTKRLFTTIQPC
cxcl14	MNRCSTAALFLLVIAIYSLNTEAYKCRCTRKGPKIRYIDVQKLEIKPKHPYCQEK MIFVTMENVSRFKGQEYCLHPRLQSTRNLVKWFKIWKDKHRTFEA
cxcl-c13b	MAFKTLQASVKVLLLLSVCSHFISVKMTAATFIREKCECVKEAGAVQWRKITDY TITPKNPLCNKVQIKLQLSNKEVCLNPESKQGKKLQKCWQKINFNPQRKKVCL TIKKNAPKRLKKL
cxcl-c1c	MAFTPKALLLLLLAVVYVQQGEVLAKIPDRCQCEESSLVNRARRDTIKEFYITP KRPNCDKVEIILTQKPENKTTASGQLCLNPQKQQGQLLQNCWTRLNINNTDS LKMSVCWQ
cxcl11	MKTLAAFLLLSCLIAGEVNGQDRSSRARCFCVDKGLNMVLLKNLEKVEIFPPSP SCNKHEIVVTLKNGAGQKCLNPDSKFTKNVVLKAIGKRMQQSVPHSTTTGTVK SSMTSSTSAPTAFK
cxc66	MKTLAAFLLLTCLIAGKVNGQDNTSRARCFCADKGINMVLLKNIEKVEIFPPSPS CNKNEIVVTLKNGAGQKCLNPDSKFTQNVVLKAIGKRMQQSVPHSTTTGTVKS SMTSSTSAPTAFK
cxc56	MKTLAAVVLLGYLLVIKVEGQARAPRSRCLCADKGVNMVSPKLIEKVDIIPPTPS CGNLEIVVTLKNGAEPKCLSPDSKFTQKYLMKALEKRTLQK
cxc64	MKTAAAFVALGCFLMVEVKGKIPDLKNRCLCADKGANNVNLKTIEKIQIIHPSPS CKRLEIVVTLMKGAGKKCLNPESNLGKNILKALRKKKLTAVRRMNPA
cxcl11l	MKTVTALLLVSLAVVAIEGQHMKSQRCVCLGAGLNMVKPVLIEKIEILPSSPSC GHMEVIATLKNGAGKRCLNPKSKFTKKIIDKIEKNNRNAR
cxcl-c5g	MKTAAFLVFLAFFIFIPGQKKFNRCSCVGKGLDRVALRNIEKFEIIHPSPSCGKQ EIIVTMKSSEQKCLNPESKFTQELIRRALEK
cxcl12a	MDLKVIVVVALMAVAIHAPISNAKPISLVERCWCRSTVNTVPQRSIRELKFLHTP NCPFQVIAKLKNNKEVCINPETKWLQQYLKNAINKMKKAQQQQV
cxcl12b	MDSKVVALVALLMLAFWSPETDAKPISLVERCWCRSTLNTVPQRSIREIKFLHT PSCPFQVIAKLKNNREVCINPKTKWLQQYLKNALNKIKKKRSE
cxcl-c13d	MRCSVFVFLACMTLLSTTEVFAARLPIQQLRCQCVKTYTGKPINPKLIQSLQTIP AGARCKNMEVIATVKKGKTCLNPKDEWVTKIIEGRSVKVPTRGPFTTLPPNST SAPQLTSKM
il8l2	MMKLSVSAFMLLICTTALLCANEGEALPPPQRCQCIKTHSKPPIPKRQVLGLKV TPAGSHCRNEEIIATLKKGQICLNPTETWVISLKEKFAASATKLAATAAPAQTTT TFSTIMTTN
cxcl-c5a	MALRPSLLLAVTAVCCFTIIIECLYDCFSALPMDGFATENKCKCQTTTSSRIPPR LFQKIEILPAGAHCRKAEIITKKDNQAVCLHPEARWVKEMVSKIISKRAERETAM PTVA
il8	MTSKIISVCVIVFLAFLTIIEGMSLRGLAVDPRCRCIETESRRIGKHIKSVELFPPS PHCKDLEIIATLMTTGQEICLDPSAPWVKKIIDRIIVNRKP
cxcl-c5c	MKVSACLINQRLLKKGNIFPELPVRGSMNQIVLILLCALLFGMSLAQSVGHGGG GSQRCRCIGKPYKTVNPRSIQAVDVFQPSPSCSNKEIILTVVEGRGKTKGKGS RKRSKVCLDPNGKQGQRLLKGRWGKKQNQRNRGKKEKNKV

[1]Signal peptide sequences are marked grey.