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Exploitation of host chemokine signalling by pathogenic mycobacteria

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Chapter 3

The CXCR3-CXCL11 signalling axis mediates macrophage recruitment and dissemination of mycobacterial infection

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Chapter 3

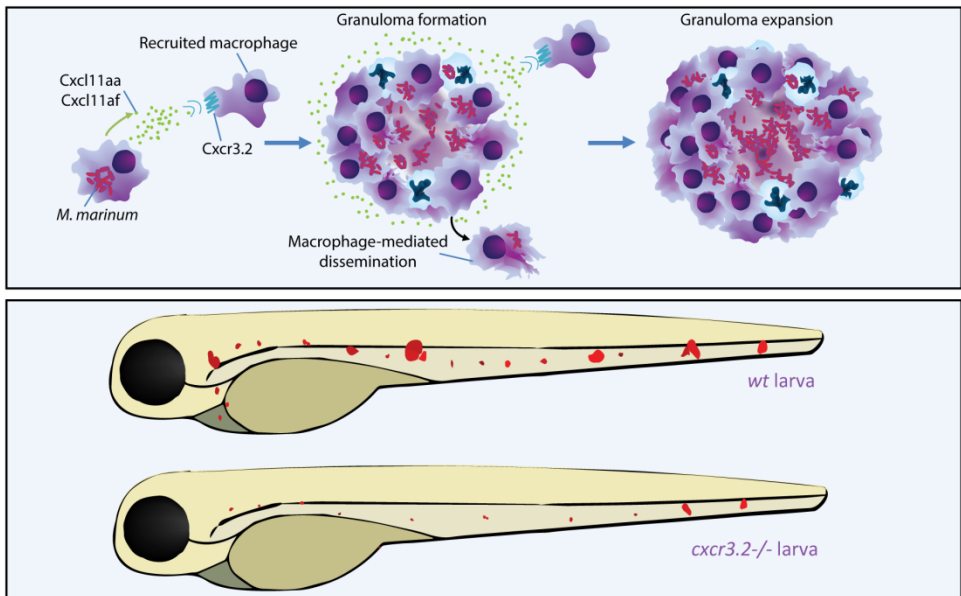
The CXCR3-CXCL11 signalling axis mediates macrophage recruitment and dissemination of mycobacterial infection

Vincenzo Torraca¹, Chao Cui¹, Ralf Boland¹, Jan-Paul Bebelman², Astrid M. van der Sar³, Martine J. Smit², Marco Siderius², Herman P. Spaink¹ and Annemarie H. Meijer¹

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The recruitment of leukocytes to infectious foci depends strongly on the local release of chemoattractant mediators. The human CXC chemokine receptor 3 (CXCR3) is an important node in the chemokine signalling network and is expressed by multiple leukocyte lineages, including T cells and macrophages. The ligands of this receptor originate from an ancestral CXCL11 gene in early vertebrates. Here, we used the optically accessible zebrafish embryo model to explore the function of the CXCR3-CXCL11 axis in macrophage recruitment and show that disruption of this axis increases the resistance to mycobacterial infection. In a mutant of the zebrafish orthologue of *CXCR3* (*cxc3.2*), macrophage chemotaxis to bacterial infections was attenuated, although migration to infection-independent stimuli was unaffected. Additionally, attenuation of macrophage recruitment to infection could be mimicked by treatment with NBI74330, a high-affinity antagonist of CXCR3. We identified two infection-inducible CXCL11-like chemokines as the functional ligands of Cxcr3.2, showing that the recombinant proteins exerted a Cxcr3.2-dependent chemoattraction when locally administered *in vivo*. During infection of zebrafish embryos with *Mycobacterium marinum*, a well-established model for tuberculosis, we found that Cxcr3.2 deficiency limited the macrophage-mediated dissemination of mycobacteria. Furthermore, the loss of Cxcr3.2 function attenuated the formation of granulomatous lesions, the typical histopathological features of tuberculosis, and led to a reduction in the total bacterial burden. Prevention of mycobacterial dissemination by targeting the CXCR3 pathway, therefore, might represent a host-directed therapeutic strategy for treatment of tuberculosis. The demonstration of a conserved CXCR3-CXCL11 signalling axis in zebrafish extends the translational applicability of this model for studying diseases involving the innate immune system.

GRAPHICAL ABSTRACT



In zebrafish, the chemokine receptor Cxcr3.2 is important to guide macrophage recruitment to mycobacterial infection foci, via infection-dependent induction of its ligands Cxcl11aa and Cxcl11af. Cxcr3.2 mutants display reduced trafficking of macrophages to the nascent granuloma lesions, which both limits granuloma expansion and the dissemination of the pathogen via macrophage carriers. As a consequence, *cxcr3.2* mutation is beneficial for the host and reduces susceptibility to the mycobacterial disease.

TRANSLATIONAL IMPACT

Clinical issue - Mycobacteria are the causative agents of chronic, life-long debilitating infectious diseases such as tuberculosis and leprosy. In order to replicate and spread within their host, mycobacteria hijack one of the primary immune defence cells: the macrophage. Recruitment of macrophages relies heavily on the production of chemokines by the infected host. However, the role of chemokine signalling in mycobacterial disease remains poorly explored. CXC chemokine receptor 3 (CXCR3) is an important node in the chemokine signalling network and has been extensively studied in T cells. Emerging evidence suggests that CXCR3 also has important functions in macrophages that might be linked with immune-related diseases.

Results - In this study, the authors used a zebrafish model of tuberculosis to investigate the role of CXCR3 in macrophages during the early stages of mycobacterial infection. They found that mutation of a zebrafish CXCR3 homologue attenuates the infection-dependent recruitment of macrophages and limits the dissemination of the pathogen via macrophage carriers. This results in a reduced formation of granulomatous lesions, typical of mycobacterial disease. Similar attenuation of macrophage attraction to local infections could be achieved by treatment with NBI74330, a high-affinity antagonist of CXCR3. The authors also purified the zebrafish counterparts of the human chemokine (C-X-C motif) ligand 11 (CXCL11) family and demonstrated that two of these are inducible by infection and specifically recruit macrophages via the CXCR3 receptor in the zebrafish model.

Implications and future directions - This study is the first to implicate the CXCR3-CXCL11 signalling axis in macrophage responses that drive the initiation and expansion of mycobacterial granulomas, the pathological hallmark of tuberculosis disease. The beneficial effect of CXCR3 mutation on the control of mycobacterial infection in the zebrafish host should drive further research into the CXCR3-CXCL11 axis as a potential target for host-directed therapy against tuberculosis. Research into such novel therapeutic approaches is important in view of the increasing prevalence of antibiotic-resistant mycobacterial strains. Defects in CXCR3 signalling have also been associated with other immune-related diseases, including cancer and inflammatory disorders. Therefore, the finding that the CXCR3-CXCL11 axis and its sensitivity to pharmacological inhibition are conserved between human and zebrafish has broad implications for the translational value of this model.

INTRODUCTION

Macrophages are extremely dynamic phagocytic cells, able to integrate and respond to a wide spectrum of signals from infected tissues. A variety of receptors on their cell membrane can sense pathogen-associated molecular patterns (PAMPs), which induce the innate immune response¹. Some of these PAMPs, such as N-formylated bacterial peptides, have direct chemoattractant activity on phagocytes². Moreover, a crucial contribution to efficient phagocyte recruitment is provided by lipidic and peptidic chemoattractant factors, produced or activated directly by the host locally at the infection site^{3,4,5}. In this group of compounds, the inflammatory chemokines play a major role. This subclass of small chemotactic proteins is induced upon infection and is able to exert target-specific activities towards subsets of leukocytes, both myeloid and lymphoid⁶. In humans, CXCL9 [also known as MIG (monokine-induced by IFN- γ)], CXCL10 [IP-10 (IFN- γ -inducible protein 10)] and CXCL11 [I-TAC (IFN-inducible T cell α chemoattractant)] are IFN-inducible chemokines and mediate recruitment of T cells, natural killer (NK) cells and monocytes/macrophages at the infection site, predominantly through their cognate G-protein coupled receptor, CXCR3^{7,8}. This signalling axis has been implicated in several physiological activities, including maturation of T cells and vasculogenesis^{9,10}. Additionally, CXCR3 and its ligands have been linked to inflammatory and immune-related diseases, of autoimmune^{9,11,12,13}, infectious^{14,15,16,17} or malignant^{18,19,20,21} nature.

Most of the literature on mammalian systems focuses on the role of this receptor in maturation, priming, activation and migration of T cells^{9,11,22}. However, recent studies have demonstrated that CXCR3 also plays an important role in directing macrophage activities, both under physiological and under pathological conditions^{10,20,23,24}.

The zebrafish embryo model provides a useful platform to study chemokine-dependent cell migration, combining excellent possibilities for intravital imaging with the availability of a vast array of genetic tools²⁵. Homologous relationships between mammalian and zebrafish CXCR4-CXCL12 and CXCR2-CXCL8 receptor-ligand pairs have been well established and studies in zebrafish have contributed significantly to the understanding of the role of these signalling axes in developmental processes, neutrophil motility, long-range neutrophil mobilisation and infection-induced chemotaxis^{26,27,28,29,30}. Based on phylogeny reconstructions, the CXCR3-CXCL11 axis emerged for the first time in a common ancestor of zebrafish and mammals³¹. In placental mammals, amphibians and reptiles, a single copy per haplotype of *CXCR3* is generally present, whereas *CXCR3* was lost in the divergence of avian and marsupial mammalian clades. Several teleost fish show an expansion of the *CXCR3* family^{31,32,33}, including zebrafish, where three copies, namely *cxcr3.1* (ENSDARG00000007358), *cxcr3.2* (ENSDARG00000041041) and *cxcr3.3* (ENSDARG00000070669), are located in tandem on chromosome 16³⁴. The *CXCL9-CXCL10-CXCL11* triplet of CXCR3 ligands in mammals is likely to have originated from a relatively recent common ancestor³⁵. The situation in fish is variegated and, in some cases, specific expansions have taken place. In zebrafish, a cluster of seven putative *cxcl11* genes, which are grouped together in a single locus on chromosome 5, share both homology and synteny with human *CXCL11*³⁴. However, an association between the different isoforms of Cxcl11 ligands and Cxcr3 receptors has not been described, and the *in vivo* relevance of this signalling axis in the zebrafish model has not been addressed.

In previous work we have shown that one of the three *CXCR3* paralogues, *cxcr3.2*, is expressed in macrophages of 1-day-old zebrafish embryos³⁶. In the present study, we used a *cxcr3.2* mutant to investigate the role of Cxcr3 signalling in macrophage mobilisation and function. In agreement with previous morpholino knockdown results, the receptor loss-of-function resulted in the attenuation of macrophage recruitment to local infection with *Salmonella typhimurium*. Moreover, we identified two infection-inducible CXCL11-like chemokines, which act as functional ligands of Cxcr3.2 with chemoattractant activity on macrophages. Finally, we demonstrate here that *cxcr3.2* is required for efficient recruitment of macrophages to *Mycobacterium marinum* infection and for the dissemination of this pathogen into host tissues, which is driven by macrophages. The zebrafish-*M. marinum* host-pathogen pair is widely used to model human tuberculosis and has provided important insights into the interaction of mycobacteria with host macrophages^{37,38,39,40,41,42}. *M. marinum* is closely related to the human pathogen *Mycobacterium tuberculosis*, and the zebrafish model replicates the formation of granulomas, the typical histopathological hallmark of human tuberculosis^{43,44}. The results presented here demonstrate a novel function for the CXCR3-CXCL11 signalling axis in macrophage responses that drive the initiation and expansion of these granulomatous lesions that are crucial for the dissemination of mycobacterial infection.

RESULTS

***cxcr3.2* is expressed in phagocyte populations during zebrafish embryonic and larval development**

We previously reported that *cxcr3.2* expression could be detected by fluorescent *in situ* hybridisation at 1 day post fertilisation (dpf) in phagocytes expressing the macrophage marker *csfl1r* (*colony stimulating factor 1 receptor*) and not in cells positive for the neutrophil marker *mpx* (*myeloperoxidase*)³⁶. However, we were unable to detect its expression with the same method at later stages. To determine whether *cxcr3.2* continues to be expressed in macrophages during the embryonic and larval development, we analysed RNA expression levels from FACS-sorted *mpeg1:mCherry-F⁺* and *mpx:eGFP⁺* cells from the double-transgenic line *Tg(mpeg1:mCherry-F/mpx:eGFP)*. These data show that macrophages (*mpeg1:mCherry-F⁺* population) maintain *cxcr3.2* expression at 2 and at 6 dpf (**Figure 1A–C** and **Supplementary Figure 1**). Expression of *cxcr3.2* could also be detected in neutrophils (*mpx:eGFP⁺* population). In addition, *cxcr3.3* could be detected in both phagocyte types, whereas *cxcr3.1* was not specifically enriched in the sorted cell populations (**Figure 1C** and **Supplementary Figure 1**).

The cxcr3.2^{hu6044} line carries a nonsense mutation in cxcr3.2

Sequencing of an ENU (N-ethyl-N-nitrosourea)-mutagenised zebrafish library resulted in the identification of a *cxcr3.2* mutant allele, *cxcr3.2^{hu6044}*, which carries a T-to-G (deoxythymidine to deoxyguanosine) substitution, creating a premature stop codon. This mutation leads to the interruption of the protein translation after 15 amino acids, before the region that encodes all the transmembrane domains that are essential for the function of the receptor (**Figure 1D** and **Supplementary Figure 2**). The nonsense *cxcr3.2^{hu6044}* mutation is not likely to lead to a functional truncated protein by using a downstream AUG codon as a signal for translation initiation. The second AUG in frame is located 354 nucleotides (118 amino acid residues) downstream from the canonical start codon and use of this codon as a translation start would lead to a truncated product lacking both the most N-terminal extracellular domain and the first two transmembrane domains. Furthermore, because the mutation occurs downstream of all the splicing sites, the possibility of alternative splicing and/or altered pre-RNA maturations seems unlikely and this was excluded by sequencing of the cDNA of *cxcr3.2* in mutants and wildtypes (wt). The *cxcr3.2* locus is closely linked to the loci of *cxcr3.1* and *cxcr3.3* owing to their chromosome proximity. To evaluate the presence of additional alterations in these genes as a consequence of the ENU mutagenesis, we sequenced their genetic loci in the AB/TL wt strain in our facility (used to outcross the mutant) and in two families of *cxcr3.2^{+/-}* and *cxcr3.2^{-/-}* fish. We did not identify additional nonsense mutations, although we could detect several non-synonymous single-nucleotide polymorphisms (nsSNPs), which are described in **Supplementary Table 1**. However, all the nsSNPs that were found in the *cxcr3.2^{-/-}* line were also present in the AB/TL fish line, indicating that these changes are likely to be natural wt polymorphisms and not an effect of the ENU mutagenesis. To address the possible relevance of these nsSNPs with respect to the protein function, we used the PROVEAN software tool (Protein Variation Effect Analyser; <http://provean.jcvi.org>)^{45,46}. None of the nsSNPs was predicted to impact on the protein functionality (**Supplementary Table 1**). Therefore, it is unlikely that single amino acid replacements in the protein sequences of Cxcr3.1 or Cxcr3.3 would affect the phenotype of fish carrying the *cxcr3.2^{hu6044}* nonsense allele.

Cxcr3.2 signalling in macrophage recruitment and infection dissemination

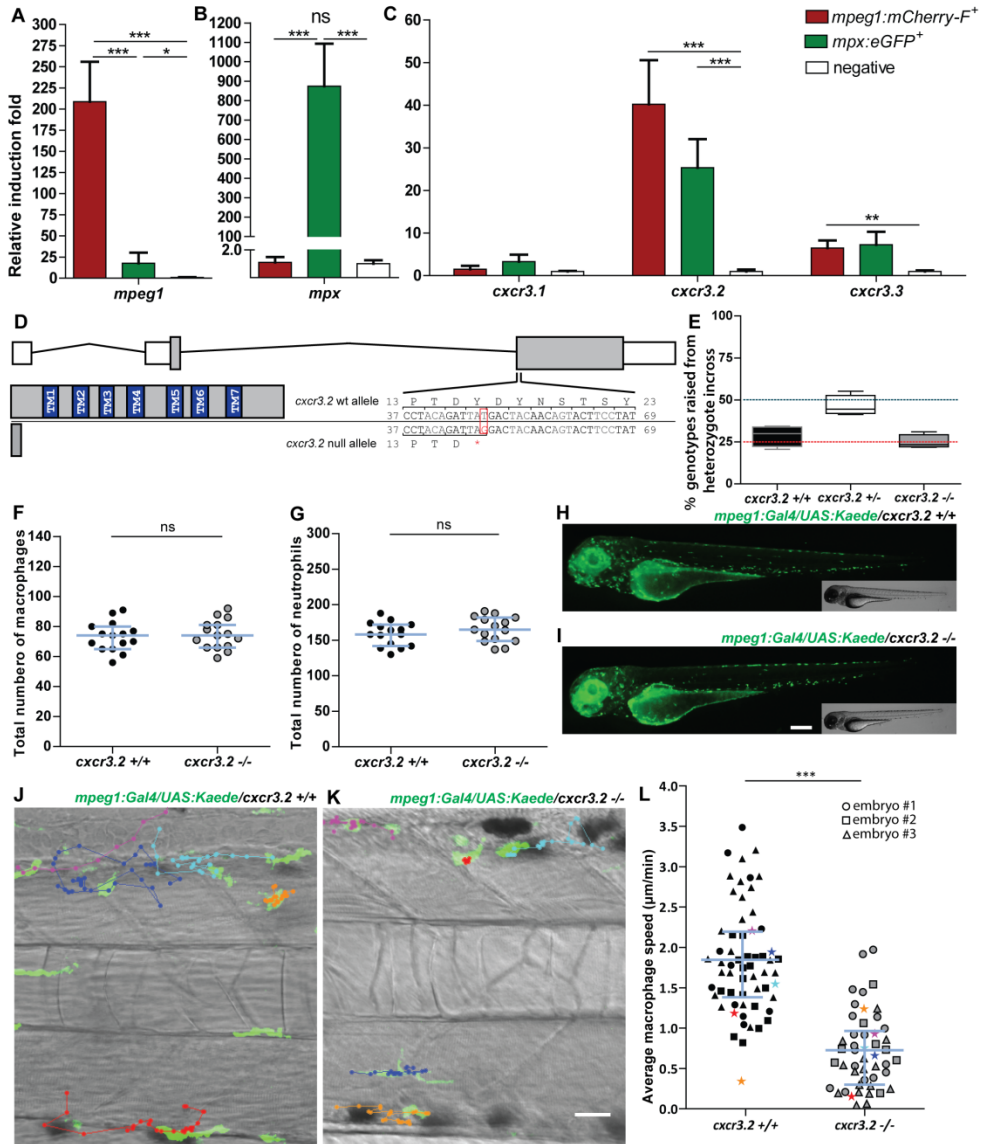


Figure 1. Characterisation of *cxcr3.2*^{-/-} embryos in unchallenged conditions (Legend on the next page).

Figure 1. Characterisation of *cxcr3.2*^{-/-} embryos in unchallenged conditions (Figure on the previous page). **A–C.** Expression of *cxcr3.2* and its paralogues *cxcr3.1* and *cxcr3.3* in FACS-sorted phagocytes. Graphs represent the relative induction fold of the macrophage marker *mpeg1* (A), the neutrophil marker *mpx* (B), and of the *cxcr3* paralogues (C) in FACS-sorted macrophages and neutrophils from the combined transgenic line *Tg(mpeg1:mCherry-F/mpx:eGFP)* at 2 dpf. Expression of *cxcr3.2* and *cxcr3.3* could be detected in both macrophages and neutrophils, whereas *cxcr3.1* was not significantly enriched in the FACS-sorted populations when compared with the non-labelled cell fraction. Sample size (n): five replicates. Errors bars: mean±s.e.m. Reference gene: *elf4a1b*. **D.** Effect of the *cxcr3.2* point mutation. Top: gene structure of *cxcr3.2*. Boxes represent exons, of which the grey parts correspond to the coding sequence. Bottom right: *cxcr3.2* wildtype (wt) and mutant allele. A single T-to-G mutation at nucleotide 48 generates an early stop codon. Bottom left: consequence of *cxcr3.2* mutation at the protein level. In *cxcr3.2* mutant zebrafish, only a peptide of 15 amino acids can be translated, which lacks all the conserved transmembrane domains (TM1-7). Nucleotide and amino acid positions are enumerated from the translation start codon. **E.** Normal viability of *cxcr3.2*^{-/-} mutants. Percentage of genotypes deriving from *cxcr3.2*^{-/-} incross. No significant deviation from the Mendelian 1:2:1 ratio was observed. The genotypes were evaluated on 122 adult fish from four independent breedings. The boxplots represent the area of the distributions between the first and the third quartiles. Whiskers represent the minimum and maximum end points of the distributions. **F–G.** Quantification of macrophages and neutrophils. Combined Leukocyte-plastin (Lp) immunostaining and Myeloperoxidase (Mpx) staining were performed on *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} embryos at 3 dpf and the numbers of stained cells residing in the caudal haematopoietic tissue were counted. Exclusively Lp-stained cells were considered as macrophages (F) and Lp/Mpx double-positive cells as neutrophils (G). No significant differences were detected. Total number of larvae (n) per group in both F and G: 15. Error bars: median and interquartile range. **H–I.** Spatial distribution of macrophages. A macrophage-specific transgenic reporter driven by the *mpeg1* promoter [*Tg(mpeg1:Gal4/UAS:Kaede)*⁶⁶] was crossed into the *cxcr3.2* mutant background. Representative images of the resulting *Tg(mpeg1:Gal4/UAS:Kaede) cxcr3.2*^{+/+} (H) and *cxcr3.2*^{-/-} (I) larvae at 3 dpf show no major differences in the macrophage distribution pattern. Scale bar: 200 µm. **J–K.** Macrophage basal migratory capability. Paths of five representative macrophages in the trunk of *Tg(mpeg1:Gal4/UAS:Kaede) cxcr3.2*^{+/+} (J) and *cxcr3.2*^{-/-} (K) larvae at 3 dpf. Mutant and wt larvae were mounted in agarose on the same dish and behaviour of mutant and wt macrophages were simultaneously followed for 3 hours. Time-lapse images were taken every 6 minutes. The paths were followed and analysed using ImageJ ManualTrack plugin. See also **Supplementary Movies 1–2**. Scale bar: 20 µm. (L) Quantification of basal migration difference. The average speed of individual macrophages was calculated by tracking 15–21 macrophages from three different *Tg(mpeg1:Gal4/UAS:Kaede) cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} larvae (each larva is indicated with a different symbol) and was significantly reduced in *cxcr3.2*^{-/-} macrophages. Total number of tracks (n): 61, 48. Error bars: mean and interquartile range. ns, non-significant; **P*<0.05; ****P*<0.001.

***cxcr3.2* mutation does not affect macrophage development but alters their basal motility**

Crossing of heterozygous *cxcr3.2*^{hu6044} carriers generated homozygous embryos in Mendelian proportions with their heterozygous and wt siblings. Screening of the adult offspring from a heterozygous incross showed that, in laboratory conditions, *cxcr3.2*^{-/-} fish do not exhibit differences in survival and development compared with their wt siblings (**Figure 1E**) and could only be distinguished by genotyping.

Based on combined immunostaining for the pan-leukocyte marker Leukocyte-plastin (Lp) and Mpx activity staining⁴⁷, *cxcr3.2*^{-/-} embryos showed similar numbers of macrophages and neutrophils as their wt siblings (**Figure 1F–G**). With the aim of investigating the relevance of *cxcr3.2* expression in macrophage behaviour, we crossed the *cxcr3.2* mutation into the *Tg(mpeg1:Gal4/UAS:Kaede)* background. Labelled macrophages showed similar numbers and spatial distribution in mutant and wt (**Figure 1H–I**). However, a basal macrophage migratory deficiency was observed in the mutants (**Figure 1J–L** and

Supplementary Movies 1-2). The aberrant motility might be explained by the presence of constitutive quantities of Cxcr3.2 ligands in the macrophage microenvironment, which could contribute to a higher basal activity of *cxcr3.2*^{+/+} macrophages.

Mutation of *cxcr3.2* does not affect chemoattraction of macrophages by Cxcr3.2-independent factors

To test whether the basal motility defect of *cxcr3.2*^{-/-} macrophages affected the stimulus-directed chemoattraction to *cxcr3.2*-independent factors, we locally injected the chemoattractant factors leukotriene B4 (LTB₄) and N-formyl-methionyl-leucyl-phenylalanine peptide (fMLP) into the hindbrain ventricle of embryos at 30 hpf (hours post fertilisation). At this developmental stage, the neutrophil population is not fully differentiated^{48,49} and the population of phagocytes infiltrating the hindbrain upon chemotactic stimulation consists predominantly of macrophages (**Supplementary Figure 3**); therefore, we could use Lp immunostaining to identify recruited macrophages. No significant difference was observed in the numbers of *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} macrophages accumulated to either stimuli in 3 hours (**Figure 2A–H**). In addition, we also employed a previously described chemically induced inflammation (ChIn) assay⁵⁰, using copper sulphate treatment of embryos at 3 dpf to induce acute inflammation of lateral line neuromast hair cells. At this stage, macrophages were counted as Lp-positive and Mpx-negative cells using the combined Lp/Mpx staining. Also in this assay, no significant difference in the numbers of macrophages recruited to the inflamed neuromasts was observed between *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} larvae (**Figure 2I**). Therefore, we concluded that *cxcr3.2* mutation does not affect the capability of macrophages to respond to stimulatory sources independent of Cxcr3.2 signalling and that the basal motility defect does not influence the experimentally induced macrophage recruitment.

Early migration of macrophages to localised infection is affected by mutation of *cxcr3.2* or treatment with a CXCR3 antagonist

To determine whether Cxcr3.2 signalling contributes significantly to the recruitment of macrophages to different types of bacterial infections, we injected either *M. marinum* or *S. typhimurium* into the hindbrain ventricle of embryos at 30 hpf. In both infection models, a significant reduction of the number of macrophages accumulating at the infected site was detected in *cxcr3.2*^{-/-} embryos at 3 hpi (hours post injection) (**Figure 3A**). A similar reduction of macrophage recruitment was also observed when *M. marinum* was locally injected into the otic vesicle at 3 dpf (**Figure 3B**). To visualise the dynamics of the macrophage migration *in vivo*, we used the combined mutant-transgenic line *Tg(mpeg1:Gal4/UAS:Kaede)/cxcr3.2*^{-/-} and followed the early response of *mpeg1*-positive cells to *M. marinum* infection in the otic vesicle of 4 dpf larvae by confocal time-lapse imaging. In agreement with the previous results, a difference in the trend of macrophage recruitment was observed between the mutant and the wt over a time course of 5 hours (**Figure 3C–H, Supplementary Movies 3–4**). Furthermore, at locations distal from the infection site, macrophages in wt larvae showed more frequently an activated morphology with formation of branched protrusions (**Figure 3E**) when compared with the mutant line (**Figure 3H**). To quantify this phenomenon, we classified the distal macrophages of locally infected larvae according to their circularity index (CI), which estimates by an index between 0 and 1 the level of divergence of the cell shape projection from a perfect circle (CI=1).

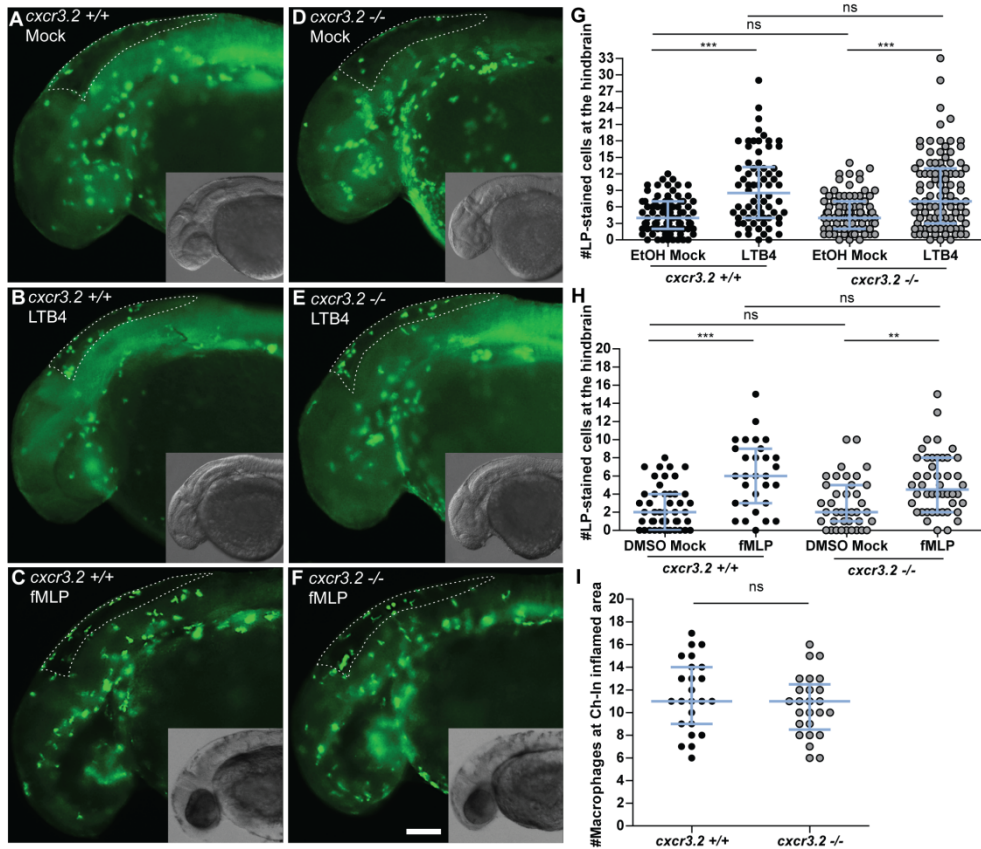


Figure 2. Macrophage chemoattraction by *Cxcr3.2*-independent factors. A–H. Chemoattraction of macrophages by LTB₄ and fMLP. *cxcr3.2*^{+/+} (A–C) and *cxcr3.2*^{-/-} (D–F) embryos at 30 hpf were locally injected into the hindbrain cavity with 10.1 ng/ml (30 nM) LTB₄ (representative images B and E) or with 0.2 mg/ml (0.5 mM) of fMLP (representative images C and F). Mock control injections with the solvents were 0.02% EtOH in PBS for the LTB₄ treatment and 5% DMSO in PBS for the fMLP treatment (representative images A and D). Lp-stained cells accumulated in 3 hours within the hindbrain limits (dotted line) were counted as macrophages, as neutrophils do not significantly contribute to the total number of leukocytes recruited to the hindbrain at this developmental stage (**Supplementary Figure 3**). LTB₄ and fMLP stimulation resulted in significantly increased macrophage recruitment, compared with the mock injection, and this was independent of *cxcr3.2* mutation (G–H). Scale bar in A–F: 100 μ m. Data were accumulated from three independent experiments. Sample size (*n*) in G: 82, 70, 86, 100; Sample size (*n*) in H: 47, 30, 39, 46. Error bars: median and interquartile range. I. Local macrophage recruitment to CuSO₄ chemically-induced inflammation. Copper sulphate treatment was performed on 3 dpf embryos and macrophages accumulated in 3 hours at the damaged neuromasts of the lateral line were counted as Lp-stained cells minus Lp/Mpx double-positive cells, representing neutrophils. Similar numbers of macrophages were recruited to inflamed areas in both *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} embryos. Sample size (*n*): 24, 25. Error bars: median and interquartile range. ns, non-significant; ***P*<0.01; ****P*<0.001.

Cxcr3.2 signalling in macrophage recruitment and infection dissemination

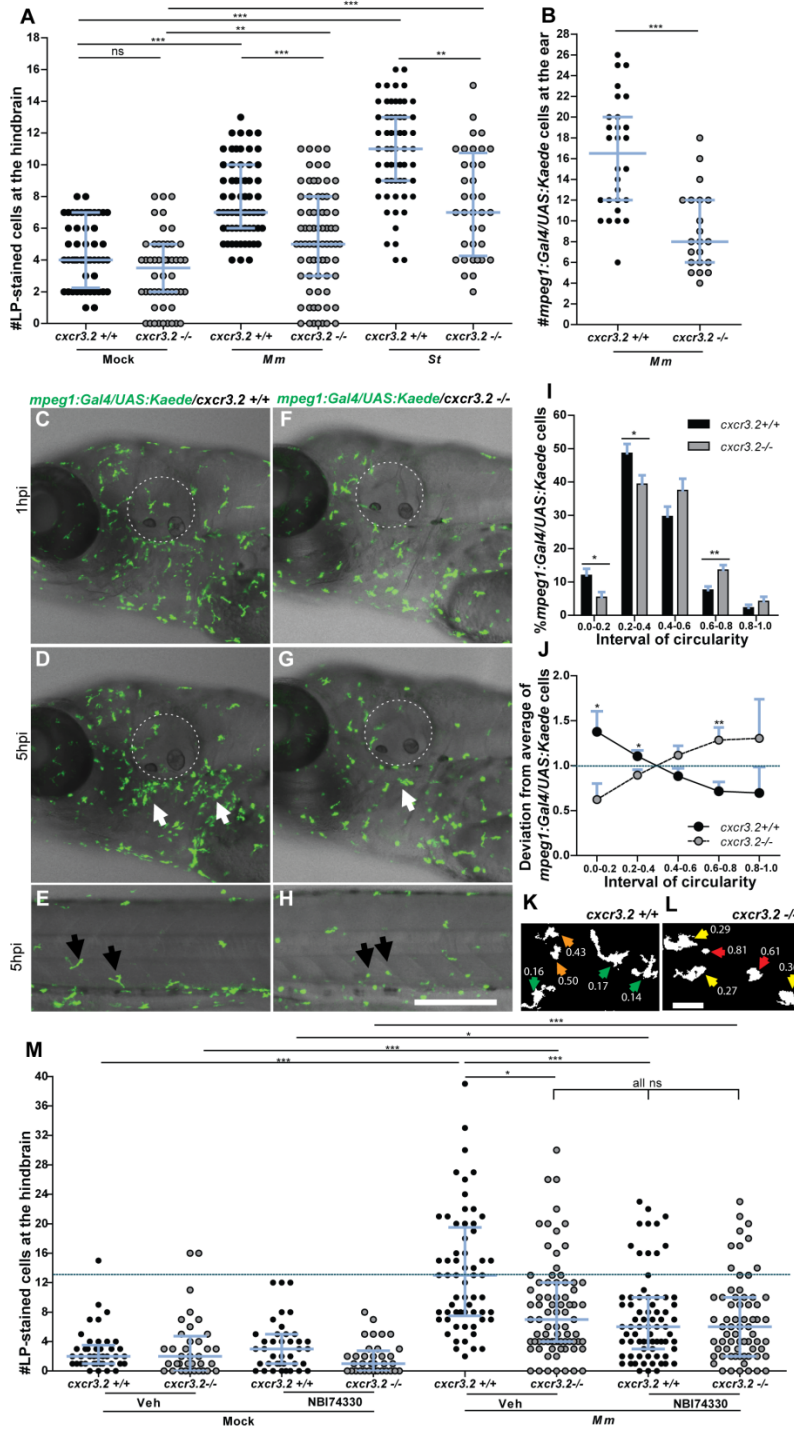


Figure 3. Cxcr3.2-dependent macrophage recruitment to localised bacterial infections (Legend on the next page).

Figure 3. Cxcr3.2-dependent macrophage recruitment to localised bacterial infections (Figure on the previous page). **A.** *M. marinum* and *S. typhimurium* infection in the hindbrain ventricle. *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} embryos were injected at 30 hpf with 200 CFU of *M. marinum* (Mm) or *S. typhimurium* (St), or mock injected with 2% polyvinylpyrrolidone-40 in PBS. Lp-stained cells accumulated in 3 hours within the hindbrain limits were counted as macrophages, because neutrophils do not significantly contribute to the total number of leukocytes recruited to the hindbrain at this developmental stage (**Supplementary Figure 3**). Significant reduction of macrophage chemotaxis to infection is determined by *cxcr3.2* mutation. Data were accumulated from two (St) or three (Mm) independent experiments. Sample size (n): 52, 52, 63, 75, 60, 36. Error bars: median and interquartile range. **B–H.** Macrophage recruitment and systemic activation following *M. marinum* infection in the otic vesicle. *Tg(mpeg1:Gal4/UAS:Kaede) cxcr3.2*^{+/+} and *Tg(mpeg1:Gal4/UAS:Kaede) cxcr3.2*^{-/-} larvae were injected with 200 CFU of *M. marinum* into the otic vesicle (dotted line) at 3 (B) or 4 (C–H) dpf. At 4 hpi of 3 dpf larvae, the accumulation of *mpeg1:Gal4/UAS:Kaede*-positive cells within the perimeter of the otic vesicle was reduced in *cxcr3.2*^{-/-} larvae (B). Following injection at 4 dpf, macrophages are less able to penetrate the otic vesicle, but accumulate in the surrounding area (white arrows in D and G), as shown in representative time course movies (**Supplementary Movies 3–4**) and stills from these movies at 1 hpi (C,F) and 5 hpi (D,G). A reduced accumulation was observed in the *cxcr3.2* mutant. At a distal location in the trunk (E,H), macrophages seemed to show more frequently a branched morphology in *cxcr3.2*^{+/+} and a round morphology in *cxcr3.2*^{-/-} (black arrows in E and H). Sample size (n) in B: 28, 21. Error bars: median and interquartile range. Scale bar in C–H: 250 μ m. **I–L.** Quantification of distal macrophage activation following local *M. marinum* infection. In order to quantify the distal activation of macrophages upon *M. marinum* infection, 200 CFU of bacteria were injected in the otic vesicle of 3 dpf *mpeg1:Gal4/UAS:Kaede* larvae. Images of the macrophages were acquired from the trunk of the infected larvae at 4 hpi and the circularity index (CI) of the distal macrophages was calculated. The graph in I represents the percentage of macrophages residing in the different intervals of CI, whereas the graph in J represents the divergence in distribution of the *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} macrophages in the different classes of CI, calculated as the ratio between the percentage of *cxcr3.2*^{+/+} or *cxcr3.2*^{-/-} macrophages in a certain CI interval and the overall mean percentage [(mutant + wt)/2] of macrophages in that interval (see Materials and Methods). Macrophages of *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} larvae were distributed in a different fashion along the different classes, with the classes of high circularity more populated in the mutants and the classes of reduced circularity more populated in the wildtype (wt). K and L show representative macrophages analysed in I and J and their corresponding CI. Green arrows: $0.0 \leq CI < 0.2$; yellow arrows: $0.2 \leq CI < 0.4$; orange arrows: $0.4 \leq CI < 0.6$; red arrows: $0.6 \leq CI \leq 1.0$. Sample size (n) in I–L: 390 *cxcr3.2*^{+/+} and 293 *cxcr3.2*^{-/-} cells from 14 and 16 embryos, respectively. Error bars: mean $\pm$ s.e.m. Scale bar in K–L: 40 μ m. **M.** Attenuation of macrophage recruitment to *M. marinum* via treatment with the CXCR3 antagonist NBI74330. *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} embryos were bath-exposed to 50 μ M NBI74330 or to vehicle only (Veh; 0.5% DMSO in medium) for 3 hours and then injected in the hindbrain ventricle at 30 hpf with mock or 200 CFU of Mm. Embryos were kept in NBI74330 or vehicle medium for an additional 3 hours and then collected for Lp immunostaining. Treatment with NBI74330 reduced the macrophage chemotaxis to infection in *cxcr3.2*^{+/+} embryos to similar levels as the vehicle-treated *cxcr3.2*^{-/-} embryos, and no significant additive effect of *cxcr3.2* mutation and NBI74330 treatment was observed. Data were accumulated from two independent experiments. Sample size (n): 41, 36, 39, 36, 61, 79, 73, 67. Error bars: median and interquartile range. ns, non-significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

The different intervals of circularity were differently populated in wt and mutant larvae, with the classes of reduced circularity (0 to 0.4) being more populated in *cxcr3.2*^{+/+} larvae and the classes of higher circularity (0.6–0.8) being more populated in *cxcr3.2*^{-/-} larvae (**Figure 3I–L**). These results provide evidence that the Cxcr3.2-dependent signalling pathway mediates a significant component of the macrophage recruitment to pathogens in the early phase of the infection. To determine whether the infection-dependent macrophage recruitment can also be modulated pharmacologically, we tested a chemical inhibitor of human CXCR3, NBI74330⁵¹, which binds with high affinity to a pocket formed by the transmembrane domains of CXCR3. Key amino acid residues in this pocket are conserved

between the human and the zebrafish receptors (**Supplementary Figure 4**). Treatment with this CXCR3 antagonist attenuated the macrophage recruitment to local *M. marinum* infection in *cxcr3.2*^{+/+} embryos to a similar level as that of the vehicle-treated *cxcr3.2*^{-/-} embryos and did not show a cooperative effect with the *cxcr3.2* mutation (**Figure 3M**). These results support the conservation of CXCR3 signalling between fish and mammals.

A group of CXCL11-like chemokines are inducible upon local and systemic infection in zebrafish

Although our analysis of the *cxcr3.2* mutant supports the role of Cxcr3.2 in macrophage chemotaxis to infections, the chemokine ligands that signal via this receptor are unknown. The assignment of ligand-receptor pairs is complicated by the relatively poor conservation of chemokine sequences among vertebrates and the species-specific expansions of the chemokine gene clusters³⁴. However, systematic study of the orthologous relationships between vertebrate chemokines indicated that seven CXCL11-like chemokine genes, located in tandem on chromosome 5 (*cxcl11aa*, *cxcl11ac*, *cxcl11ad*, *cxcl11ae*, *cxcl11af*, *cxcl11ag*, *cxcl11ah*), have evolved in zebrafish as a counterpart to the mammalian CXCL9, CXCL10 and CXCL11 genes³⁴. The amino acid similarity between the CXCL11-like chemokines in zebrafish and human CXCL11 exceeds the similarity that human CXCL9, CXCL10 and CXCL11 show among each other (**Supplementary Figure 5; Supplementary Table 2**). We, therefore, considered the zebrafish CXCL11-like chemokines as putative ligands for the Cxcr3.2 receptor. Because Cxcr3.2 was clearly involved in the early phase of the infection response, we reasoned that the ligands that induce Cxcr3.2-mediated chemotaxis should be promptly upregulated upon local infection. For this reason, we collected RNA samples from whole embryos infected in the hindbrain with 200 colony-forming units (CFU) of either *S. typhimurium* or *M. marinum* at 1 and 3 hpi and designed gene-specific primers for the members of the *cxcl11* gene cluster. Because of the high level of sequence conservation between *cxcl11af* and *cxcl11ag* (only 2 bp difference on the cDNA leading to a single semiconservative residue change of an aspartic acid with a glutamic acid), a promiscuous primer pair was used that can amplify both gene transcripts (*cxcl11af/ag*). Analysis by qRT-PCR revealed that, at 1 hpi, *cxcl11af/ag* showed twofold upregulation with *M. marinum* infection and fourfold upregulation with *S. typhimurium* infection, although no statistical significance was observed compared with the mock-injected controls (**Figure 4A**). At 3 hpi the expression of *cxcl11aa*, *cxcl11ae* and *cxcl11af/ag* was significantly upregulated to levels of two- to 4.5-fold (**Figure 4B**). In particular, *cxcl11aa* displayed the highest levels of induced transcription (~4.5-fold induction) with both the pathogens tested, suggesting this chemokine as an effective signalling ligand of Cxcr3.2 involved in the response to infection. We verified that the genes induced by local infection were also responsive to systemic infection with *M. marinum*. Upregulation of *cxcl11aa* and *cxcl11ae* was detected both at 4 hpi and at 4 dpi (days post injection) during *M. marinum* systemic infection, whereas *cxcl11af/ag* was significantly induced to levels of ~fourfold only at the later stage of infection (**Figure 4C-D**). Additionally, at this time point, *cxcl11ac* and *cxcl11ad* were also significantly upregulated to levels of 1.5- to two-fold (**Figure 4D**).

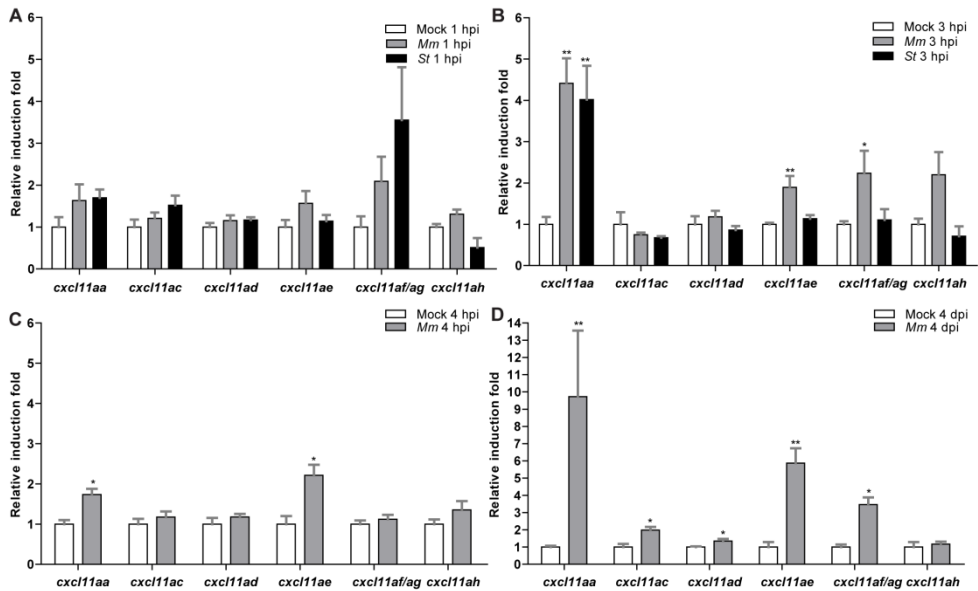


Figure 4. Inducibility of a subset of *cxcl11*-like genes upon bacterial infection. A-B. Quadruplicate pools of 18–20 embryos infected in the hindbrain with 200 CFU of *M. marinum* (Mm) or *S. typhimurium* (St) at 30 hpf or mock-injected with PBS were collected at 1 hpi (A) and 3 hpi (B). A subset of CXCL11-like chemokine genes shows upregulation by qPCR in the infected groups, which becomes significant at 3 hpi. Error bars: mean $\pm$ s.e.m. C-D. Triplicate pools of 18–20 embryos systemically infected with *M. marinum* (Mm) at 1 dpf or mock-injected with PBS were collected at 4 hpi (C) and 4 dpi (D). Expression of *cxcl11aa* and *cxcl11ae* was significantly induced at both time points, whereas *cxcl11ac*, *cxcl11ad* and *cxcl11af/ag* were significantly induced only at 4 dpi. Error bars: mean $\pm$ s.e.m. * P <0.05; ** P <0.01.

Recombinant Cxcl11aa and Cxcl11af exert macrophage chemoattraction *in vivo* in a Cxcr3.2-dependent manner

To assess the chemoattractant properties of the infection-inducible chemokines Cxcl11aa, Cxcl11af and Cxcl11ae, we used *Pichia pastoris* strain X-33 to express recombinant proteins. As a control, we also expressed zebrafish Cxcl8a (Il8), known to be a potent and neutrophil-specific chemoattractant^{27,29}. All three purified CXCL11-like chemokines showed chemoattractant capabilities towards macrophages when locally injected *in vivo* into the hindbrain at 30 hpf (Figure 5), whereas no significant macrophage recruitment was exerted by Cxcl8a (Supplementary Figure 6). Similar levels of these chemokines were injected in the otic vesicle at 54 hpf to evaluate their chemoattractant capabilities towards neutrophils (Supplementary Figure 6B-C). Cxcl11aa and Cxcl11af did not show chemoattraction of neutrophils under these conditions, whereas Cxcl11ae and Cxcl8a exerted significant neutrophil chemoattraction. To determine whether the macrophage chemoattraction is dependent on *cxcr3.2*, hindbrain injections of the recombinant proteins were performed in both wt and *cxcr3.2* mutants. Both Cxcl11aa and Cxcl11af did not stimulate recruitment upon local injection in *cxcr3.2* mutants when compared with their mock controls (Figure 5A,C). In contrast, the chemoattraction of phagocytes mediated by Cxcl11ae was independent of *cxcr3.2* mutation (Figure 5B, Supplementary Figure 6C). Taken together, these results support a direct ligand-receptor interaction between Cxcr3.2 and the chemokines Cxcl11aa and Cxcl11af that mediates the chemoattraction of

macrophages. Differently, *Cxcl11ae*, which exerted a *Cxcr3.2*-independent phagocyte chemoattraction, is likely to signal via a yet-identified receptor.

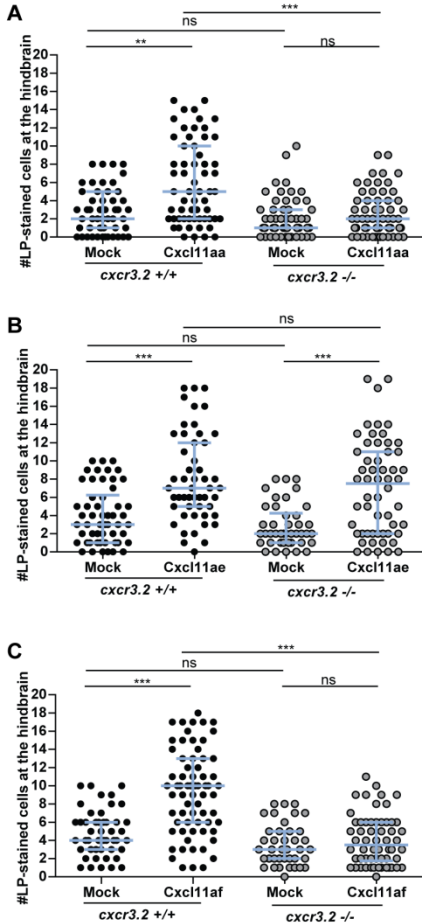


Figure 5. Macrophage chemoattraction by locally injected recombinant chemokines in *cxcr3.2* mutant and wildtype siblings. Recombinant proteins or buffer (mock) were injected into the hindbrain ventricle at 30 hpf and macrophages accumulating in 3 hours within the hindbrain limits were counted as Lp-stained cells. Data were accumulated from three independent experiments. **A.** *Cxcl11aa* (1.2 mg/ml). Sample size (n): 51, 60, 55, 60. **B.** *Cxcl11ae* (0.5 mg/ml). Sample size (n): 54, 51, 42, 56. **C.** *Cxcl11af* (0.5 mg/ml). Sample size (n): 47, 68, 39, 58. Error bars: median and interquartile range. Note that macrophage chemoattraction mediated by *Cxcl11aa* and *Cxcl11af* is abolished by *cxcr3.2* mutation, whereas the chemoattraction mediated by *Cxcl11ae* is independent of *cxcr3.2*. ns, non-significant; ** $P < 0.01$; *** $P < 0.001$.

Mutation of *cxcr3.2* affects mycobacterial infection dissemination and granuloma formation

Pathogenic mycobacteria have the ability to resist intracellular macrophage digestion and they can use the macrophages as a vector for distal dissemination of the infection³⁸. We hypothesised that *cxcr3.2* depletion, preventing a high level of macrophage accumulation to the local infection site, might also prevent extensive dissemination and help to locally restrict the infection. To test this hypothesis we followed *M. marinum* hindbrain infection for 24 hours and evaluated the frequency of infection dissemination in *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} zebrafish embryos. At 24 hpi, almost 50% of the wt embryos displayed dissemination of the infection from the head to the trunk and tail, whereas, in more than 80% of the mutants, the infection remained locally confined (**Figure 6A–F**).

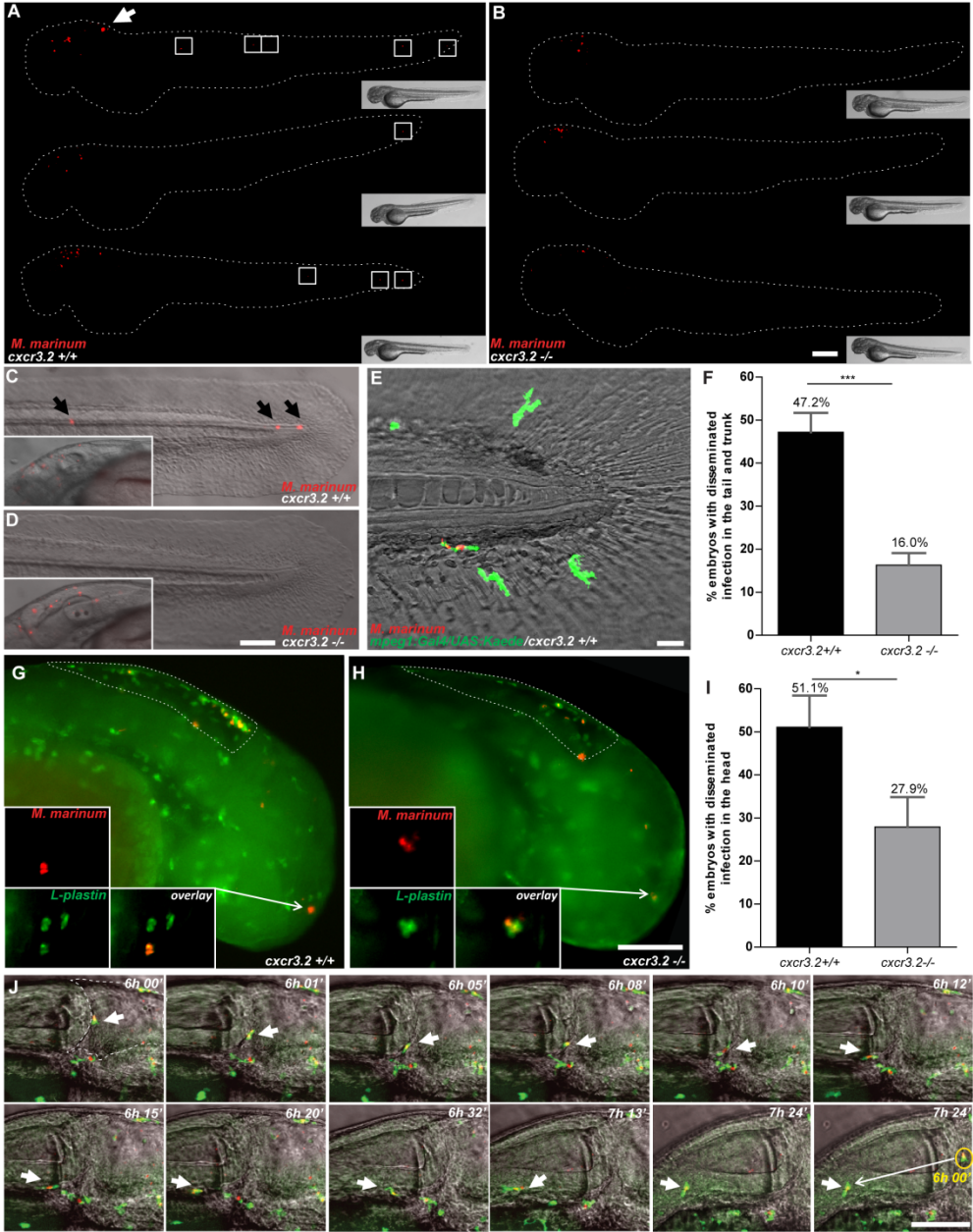


Figure 6. Effect of *cxcr3.2* mutation on dissemination of local mycobacterial infection within 24 hpi (Legend on the next page).

Figure 6. Effect of *cxcr3.2* mutation on dissemination of local mycobacterial infection within 24 hpi (Figure on the previous page). **A-B.** Representative images of *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} embryos with local and disseminated mycobacterial infection. Embryos were infected at 30 hpf by injecting 200 CFU of *M. marinum* into the hindbrain and images were taken at 24 hpi (54 hpf). In *cxcr3.2*^{+/+} embryos, single infected cells are visible distally from the infection (white boxes). Scale bar: 200 μ m. **C-E.** Details of distal infection emerging from hindbrain infection. The black arrows (C) point at single *M. marinum*-infected cells, present in the tail of a *cxcr3.2*^{+/+} fish at 24 hpi but notably absent in the example of a *cxcr3.2*^{-/-} fish (D). Particulars of the infected hindbrains of the same embryos are shown in the boxed inserts on the left, indicating similar levels of local infection. Use of the *Tg(mpeg1:Gal4/UAS:Kaede)* line (E) shows that the infection disseminated from the hindbrain resides in macrophages in *cxcr3.2*^{+/+}. Scale bars in C and D: 100 μ m; scale bar in E: 20 μ m. **F.** Quantification of *M. marinum* infection dissemination in the trunk and tail in *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} embryos. Embryos were scored positive for dissemination if one or more infected macrophages were observed in the trunk or tail region. The graph demonstrates a significant difference in the total percentage of embryos showing infection dissemination at 24 hpi. Data were accumulated from three independent experiments. Sample size (n): 125, 172. Error bars: mean $\pm$ s.e.m. **G-I.** Quantification of *M. marinum* infection dissemination in the head in *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} embryos. Representative figures of *cxcr3.2* wildtype (wt; G) and mutant (H) embryos and quantification (I) of dissemination in the head at 6 hours post *M. marinum* infection in the hindbrain. Embryos were scored positive for dissemination if one or more infected macrophages were observed outside the hindbrain limits (dotted line). Insets in G and H show details of infected macrophages outside the hindbrain. The graph in I demonstrates a significant difference in the total percentage of embryos showing infection dissemination at this time point. Arrows in the figures point at the particular of a disseminated infection. Sample size (n): 47, 43. Error bars: mean $\pm$ s.e.m. Scale bar: 200 μ m. **J.** Time course of a *M. marinum*-infected macrophage egressing from the hindbrain. The image sequence (taken from a *cxcr3.2*^{+/+} embryo) represents over a time course of ~1.5 hours that macrophages (green) can facilitate the dissemination of *M. marinum* (red) that is locally delivered in the hindbrain ventricle. The dashed line in the first image represents the hindbrain limits and arrow points at an infected macrophages adhering to the hindbrain boundary. In the subsequent images the arrow points at the infected macrophage egressing from the hindbrain. The yellow circle in the last image of the sequence contains an inset of the infected macrophage from the first image to represent its initial position (6 hpi) compared with its position at the end of the image sequence (~7.5 hpi). Scale bar: 100 μ m. **P*<0.05; ****P*<0.001.

Dissemination to other areas of the head could be seen already as early as 6 hpi and also this phenotype was attenuated in *cxcr3.2*^{-/-} embryos (**Figure 6G-I**). Disseminated bacteria outside the hindbrain and/or midbrain were residing in phagocytes and, in time course experiments, we could visualise that egression of mycobacteria from the ventricles is facilitated by macrophages (**Figure 6J**), in agreement with previously published results³⁸. When dissemination to the tail and trunk occurred, one to five dissemination foci could be detected in the *cxcr3.2*^{+/+} embryos, whereas *cxcr3.2*^{-/-} embryos never showed more than one or two bacterial clusters distally from the original injection point. At 5 dpi, the bacterial burden in the hindbrain was similar between wt and mutant larvae, but mutants still showed lower levels of dissemination of the infection towards distal areas (**Figure 7A-B**). The infection foci generated distally developed into typical granuloma-like aggregates, as previously described for the zebrafish-*M. marinum* model²⁶. The size of these granulomatous lesions was significantly reduced in the *cxcr3.2*^{-/-} mutant larvae (**Figure 7C-F**). Therefore, we concluded that *cxcr3.2*-mediated signalling strongly influences the dynamics of the infection progression and of granuloma formation. To further investigate the relevance of *cxcr3.2* signalling in the formation of granulomas, we injected 200 CFU of *M. marinum* systemically in 1 dpf embryos via the caudal vein, which, in the wt leads to many granulomatous lesions³⁸. Images of single granulomas at 5 dpi, stained for both macrophages and neutrophils, revealed that granuloma-like aggregates could still be formed in *cxcr3.2* mutants. Similar structures and phagocyte compositions were observed

when lesions of similar sizes in wt and mutant were compared (**Figure 8A-B**). However, it must be noted that a large variation in granuloma structure and composition already exists when comparing different granulomas within the same larva or between different wildtypes, and this makes it very difficult to assess the effect of a mutation on the general architecture of the granulomas.

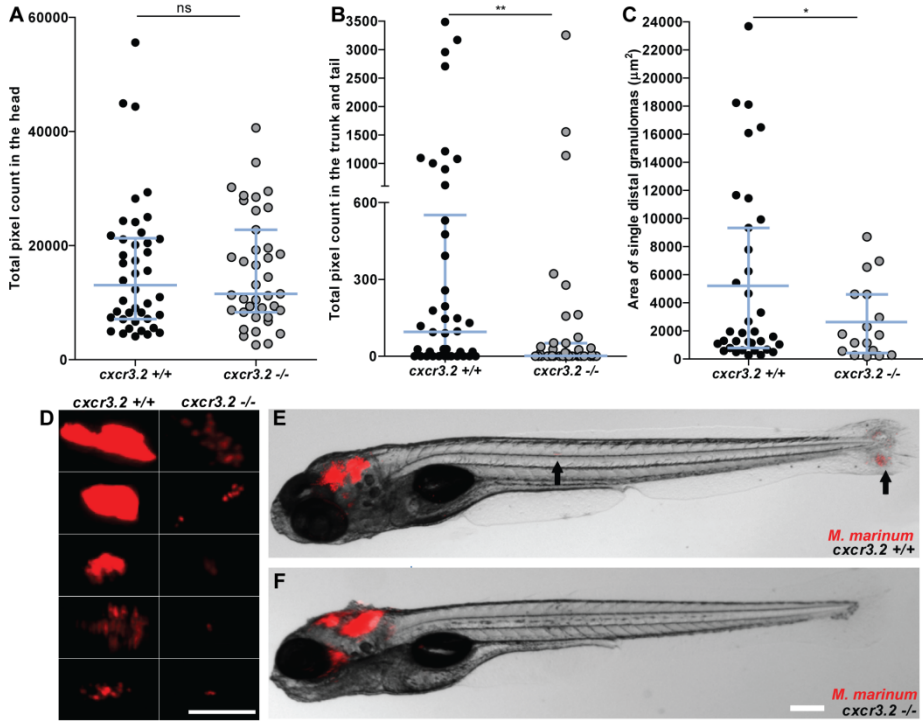


Figure 7. Effect of *cxcr3.2* mutation on dissemination of local mycobacterial infection at 5 dpi. A-B. Quantification of total levels of infection burden in the head and in the trunk and tail of 6 dpf zebrafish larvae. Embryos were injected at 30 hpf in the hindbrain ventricle with 200 CFU of *M. marinum*. Comparable levels of infection are reached locally in the head (A), but disseminated infection burden in the trunk and tail was significantly reduced in *cxcr3.2*^{-/-} larvae (B). Data were accumulated from two independent experiments. Sample size (n): 42, 39. Error bars: median and interquartile range. **C-D.** Size and morphology of distal granulomas in *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} larvae. Distal bacterial clusters that originated occasionally in *cxcr3.2*^{-/-} embryos appeared generally smaller than the ones more frequently formed in the *cxcr3.2*^{+/+} siblings. Size was determined by fluorescent bacterial quantification of single distant granulomas in *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} (C) and five representative images of each are shown (D). Data were accumulated from two independent experiments. Sample size (n): 35 and 17 distal granulomas from 42 and 39 observed *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} embryos, respectively. Scale bar: 200 μm . Error bars: median (A,B) or mean (C) and interquartile range. **E-F.** Late effects of distal infection emerging from hindbrain infection. Representative images of *cxcr3.2*^{+/+} (E) and *cxcr3.2*^{-/-} (F) embryos at 5 dpi. Black arrows point at distal dissemination foci in the *cxcr3.2*^{+/+} larva. Scale bar: 200 μm . ns, non-significant; * $P < 0.05$; ** $P < 0.01$.

Despite this, we observed that *Cxcr3.2* deficiency provided partial protection against mycobacterial infection. Not only did mutants exhibit reduced levels of infection burden (**Figure 8C-E**), but also a reduced number of bacterial clusters (**Figure 8F**) and smaller average bacterial cluster size (**Figure 8G**). Taken together with the results of hindbrain infection, these data demonstrate the important role of *Cxcr3.2*-dependent signalling in guiding macrophage-mycobacteria interactions, and show how this signalling leads to direct effects on the infection progression.

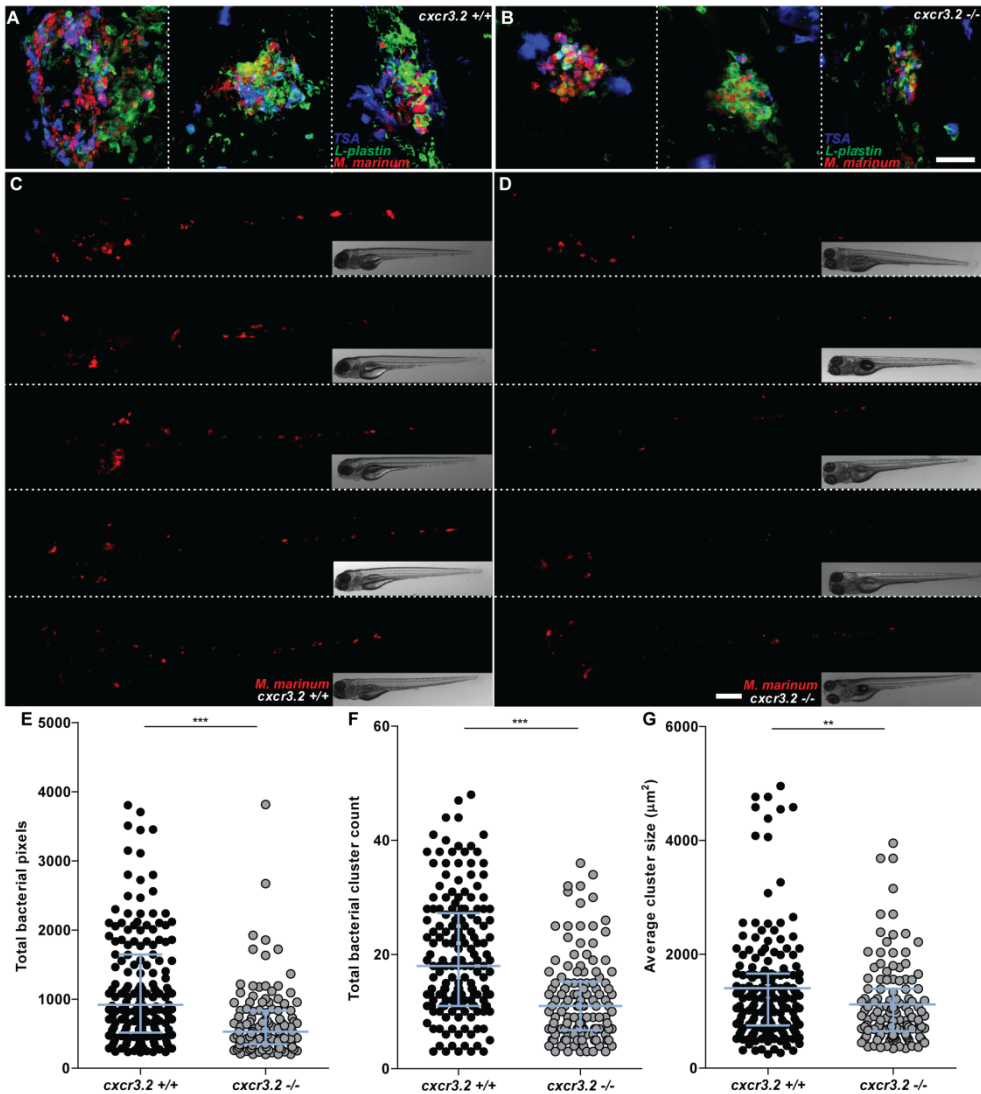


Figure 8. Effect of *cxcr3.2* mutation on granuloma formation following systemic mycobacterial infection (Legend on the next page).

Figure 8. Effect of *cxcr3.2* mutation on granuloma formation following systemic mycobacterial infection (Figure on the previous page). **A–D.** Representative images of granulomas in systemically infected *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} larvae. Embryos were systemically infected at 1 dpf, injecting 200 CFU of *M. marinum* into the caudal vein. Images of three representative granuloma-like structures of wildtype (wt; A) or mutant (B) larvae were taken on samples fixed at 6 dpf and simultaneously stained for Lp and Mpx (TSA stain) to distinguish macrophages (Lp-positive, TSA-negative) from neutrophils (TSA-positive). Images of representative *cxcr3.2*^{+/+} (C) or *cxcr3.2*^{-/-} (D) larvae were acquired live at 6 dpf. Scale bar in A-B: 40 μ m. Scale bar in C-D: 200 μ m. **E–G.** Quantification of the impact of *cxcr3.2* mutation on mycobacterial granuloma formation. Total infection burden (total infection fluorescent pixels; E), total number of bacterial clusters (F) and average area of bacterial clusters (G) are significantly reduced under *cxcr3.2*-deficient conditions. Data were accumulated from three independent experiments. Sample size (n): 170, 122. Error bars: median (E-F) or mean (G) and interquartile range. ** $P < 0.01$; *** $P < 0.001$.

DISCUSSION

The chemokine receptor CXCR3 and its ligands play important roles in the pathogenesis of infectious diseases, autoimmune disorders and cancer^{11,14,15,16,17,19,20,21,22,23,24}. In this study, we report on the function of CXCR3 signalling in macrophage recruitment to infection foci and in the early establishment of mycobacterial granulomas. We found that the *Cxcr3.2* receptor, one of the three zebrafish homologues of human CXCR3, interacts with infection-inducible zebrafish homologues of the CXCL11 ligand family and is required for the mobilisation of macrophages to different pathogens, such as locally delivered *M. marinum* or *S. typhimurium*. Furthermore, mutation of *cxcr3.2* reduced the macrophage-mediated dissemination of *M. marinum*, leading to attenuation of the formation and expansion of granulomatous lesions in both local and systemic models of mycobacterial infection.

CXCR3 is best known as a canonical marker for Th1 cells, but several recent studies have raised interest in the expression of this receptor by macrophages. These studies have implicated CXCR3 signalling in processes as diverse as the recruitment of macrophages to allografts²⁴, the macrophage-mediated remodelling of blood vessels¹⁰ and the polarisation of macrophages towards an M2 phenotype that promotes tumour progression²⁰. Furthermore, CXCR3 signalling has been shown to play a crucial role in the murine neonatal response to sepsis²³. Like murine neonates, zebrafish embryos and early larvae rely heavily on their innate immune system for defence against infection. During zebrafish embryogenesis, macrophages are the first leukocyte cell type to develop and they express *cxcr3.2* from day 1³⁶. In mutants of *cxcr3.2*, or in wt embryos treated with a human CXCR3 antagonist (NBI74330), we observed a significant reduction in the recruitment of macrophages to local bacterial infection in the hindbrain. In contrast, *Cxcr3.2*-deficient macrophages were able to normally migrate in response to chemically induced wounding or towards *Cxcr3.2*-independent chemoattractants, such as LTB₄ and fMLP. These data suggest that *Cxcr3.2* signalling is specifically activated by pathogen-induced chemokine signals. We considered a cluster of CXCL11-like chemokines as the putative ligands of *Cxcr3.2* and confirmed that two of these, *Cxcl11aa* and *Cxcl11af*, exerted chemoattractant activity on macrophages following hindbrain injection of the recombinant proteins. Most likely, *Cxcl11ag*, which is near-identical to *Cxcl11af*, also signals through *Cxcr3.2*. It is currently unknown whether the *cxcl11* genes in zebrafish are IFN- γ -inducible like their mammalian counterparts, but IFN- γ responsive elements are present in the promoters of these genes⁵². In addition, because we were unable to detect expression of the zebrafish *cxcl11* genes *in situ*, the cell types producing these chemokines remain to be established. However, qRT-PCR showed rapid upregulation of *cxcl11aa* and *cxcl11af/ag* gene

expression following infection, supporting their function as the ligands mediating the infection-dependent recruitment of Cxcr3.2-positive macrophages.

Expression analysis on FACS-sorted phagocyte populations showed that also *cxcr3.3* is expressed in macrophages, but macrophage motility and recruitment defects in the *cxcr3.2* mutant line indicates that expression of *cxcr3.3* cannot compensate for the loss of function of *cxcr3.2*. In addition, the expression analysis revealed that also neutrophils express *cxcr3.2* at 2 and 6 dpf. Injection of Cxcl11aa or Cxcl11af into the otic vesicle at 2 dpf did not chemoattract a higher number of neutrophils within 3 hours than mock injections, whereas comparable concentrations of these chemokines were able to recruit macrophages into the hindbrain, and comparable concentrations of Cxcl8a and Cxcl11ae mobilised neutrophils when delivered in the otic vesicle. Different explanations can be given for this effect. Firstly, it is possible that different concentrations of chemokines are required to efficiently chemoattract different cell types. Secondly, the requirement of co-stimulatory signals or cell-specific co-receptors might be different between the phagocyte populations. Thirdly, although macrophages and neutrophils at 2 dpf seem to express comparable levels of *cxcr3.2* mRNA, it remains unknown whether similar protein levels of Cxcr3.2 are exposed on their membranes. It should be noted that macrophages and their progenitors are marked by *cxcr3.2* expression already at 1 dpf, whereas its expression could not be detected at this time point on neutrophil progenitors³⁶. In line with this consideration, it is possible that this different timing in messenger expression impacts the protein levels at 2 dpf.

Macrophages are essential for the dissemination of pathogenic mycobacteria and mediate the formation of both primary and secondary granulomas in the zebrafish host following infection with *M. marinum*^{38,39}. When *M. marinum* was locally injected into the hindbrain ventricle of 1-day-old embryos, almost half of the embryos exhibited dissemination within 24 hours, where single infected macrophages migrated out of the ventricle and localised distally. In *cxcr3.2* mutants, this dissemination of the infection was significantly reduced, which might be a consequence of the diminished macrophage attraction to the primary infection source or a direct effect on the retromigration ability of *cxcr3.2* mutant macrophages. When the bacteria were injected intravenously, *cxcr3.2* mutation reduced the formation and the expansion of granulomas, thereby attenuating the dissemination of bacteria and the overall burden of systemic infection. This phenotype might be explained by the reduced motility of macrophages in *cxcr3.2* mutants because it has been shown that early granulomas in zebrafish larvae expand by spreading of the infection to newly recruited macrophages⁵³. In agreement, the phenotype of *cxcr3.2* mutant larvae resembles those caused by deficiency in other host (*mmp9*) or bacterial (ESAT-6) factors that also impair macrophage recruitment^{53,54}.

The zebrafish larval tuberculosis model is limited to the study of the initial stages of granuloma formation by macrophages in a context where the adaptive immune system is not yet functional. A beneficial effect of CXCR3 mutation has also been observed during chronic infection of BALB/c mice with *Mycobacterium tuberculosis*¹⁴. In this model, the resistance of CXCR3-deficient mice was attributed to the function of CXCR3 in T-cell priming. Another study using C57BL/6 mice showed that CXCR3 mutation affected early granuloma formation after aerosol *M. tuberculosis* infection and correlated this with the invasion of polymorphonuclear neutrophils that produce chemokine signalling via CXCR3¹⁷. Together, the studies in mice and zebrafish models support further investigation

of the CXCR3 signalling axis as a host therapeutic target for tuberculosis. Our study is the first to implicate this signalling axis in macrophage responses that drive the initiation and expansion of mycobacterial granulomas. In future work it will, therefore, be of great interest to investigate how macrophage and T-cell responses determined by CXCR3 signalling cooperate in the control of mycobacterial infections, using adult zebrafish or mammalian models of tuberculosis.

Recently, another chemokine receptor, Ccr2, has also been shown to mediate macrophage recruitment following hindbrain infection of *M. marinum* in zebrafish embryos³⁷. This Ccr2-mediated pathway is dependent on the presence of phenolic glycolipids on the mycobacterial cell surface and it recruits a population of macrophages that are permissive for mycobacterial growth, because activation of the host immune response is largely avoided owing to the presence of other cell surface lipids in virulent mycobacteria (phthiocerol dimycocerosate lipids), which physically mask the underlying PAMPs. *M. marinum* bacteria lacking phenolic glycolipids were still able to recruit macrophages, and morpholino knockdown of either Ccr2 or its ligand Ccl2 attenuated recruitment but did not fully abolish it. These observations indicate that redundant and/or synergistic mechanisms are cooperating in macrophage mobilisation. Combined experiments will be necessary to reveal whether the Ccr2-Ccl2 axis is (partially) redundant or synergistic with the Cxcr3-Cxcl11-mediated macrophage recruitment shown here.

Interestingly, we found that Cxcr3.2 is also involved in the basal motility of macrophages under physiological conditions. We hypothesise that the lower basal motility of macrophages in *cxcr3.2* mutants could be due to the inability to sense small amounts of Cxcr3.2 ligands secreted in the macrophage microenvironment. Possibly, the macrophages themselves could be involved in an autocrine or paracrine secretion of these ligands. Similar mechanisms acting via CXCR3 signalling have already been described in the literature. Keratinocytes have been shown to express CXCL10 and CXCR3 to guide their own migration for re-epithelialisation in a wound-healing response⁵⁵. Similarly, synovial fibroblasts use this ligand-receptor pair to regulate their invasion of joints in rheumatoid arthritis⁵⁶. Furthermore, myeloid cells and haematopoietic progenitors secrete many different chemokines, including CXCR3 ligands, to regulate haematopoiesis in an autocrine or paracrine manner⁵⁷. The autocrine or paracrine production of Cxcr3.2 ligands could potentially work as a local macrophage stimulator, which might significantly contribute to the surveillance activities that macrophages exert in tissues. During mycobacterial disease, similar mechanisms might be stimulated within the core of the granulomatous lesions, and could be involved in the in-and-out trafficking properties of macrophages, characteristic of these dynamic structures^{58,59}. In various animal models of tuberculosis, including the most clinically relevant macaque model, abundant expression of CXCR3 ligands is detected in the core and in the direct neighbourhood of the granulomatous lesions^{59,60,61}. Our study suggests that this is relevant not only for the recruitment of T cells, but also for regulating macrophage activities in the immunopathology of the granulomatous lesion. A number of studies with selective agonists or antagonists of CXCR3 have already shown beneficial effects on inflammation-associated diseases^{62,63} and the zebrafish model might be a suitable model to test their effectiveness on mycobacterial infections.

Concluding, here we propose a dual biological role of Cxcr3.2-Cxcl11aa/af ligand-receptor signalling. First, our results implicate Cxcr3.2 and its ligands in surveillance against

pathogens by promoting the random patrolling of inactive macrophages. Second, we show that this pathway is involved in the mobilisation of macrophages during infection. Depending on the specific interactions of different pathogens with their hosts, a *Cxcr3.2*-dependent response could be beneficial for the resolution of infection or have an unfavourable effect because, on the one hand, it can sustain the recruitment of macrophages to the infection site, but, on the other hand, it can promote the dissemination of bacteria, as in the case of mycobacterial infection.

MATERIALS AND METHODS

Zebrafish lines and maintenance – Zebrafish lines were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). The breeding of adult fish was approved by the local animal welfare committee (DEC) of the University of Leiden (license number: 10612) and adhered to the international guidelines specified by the EU Animal Protection Directive 2010/63/EU. Adult zebrafish were not sacrificed for this study. All experiments in this study were performed on embryos/larvae before the free-feeding stage and did not fall under animal experimentation law according to the EU Animal Protection Directive 2010/63/EU.

Fish lines used in this work were the following: wildtype (wt) strain AB/TL, double-transgenic line *Tg(mpeg1:mCherry-F/mpx:eGFP)*^{64,65} homozygous mutant (*cxcr3.2*^{-/-}) and wt siblings (*cxcr3.2*^{+/+}) of *cxcr3.2*^{hu6044}, *Tg(mpeg1:Gal4-VP16/UAS-E1b:Kaede)*, in short referred to as *Tg(mpeg1:Gal4/UAS:Kaede)*⁶⁶, and the combination of *Tg(mpeg1:Gal4/UAS:Kaede)* with the *cxcr3.2* mutant strain. The *cxcr3.2*^{hu6044} allele was identified by sequencing of an ENU (N-ethyl-N-nitrosourea)-mutagenised zebrafish library and was obtained from the Hubrecht Laboratory and the Sanger Institute Zebrafish Mutation Resource. Heterozygous F2 carriers were outcrossed twice against wt and were subsequently incrossed. Resulting *cxcr3.2*^{-/-} and *cxcr3.2*^{+/+} siblings were raised and used to obtain embryos for all the experiments. The combined mutant-transgenic line *Tg(mpeg1:Gal4/UAS:Kaede/cxcr3.2*^{-/-} or *cxcr3.2*^{+/+}) were obtained by crossing heterozygous carriers with the original transgenic line and subsequently incrossing the heterozygous offspring. For genotyping, genomic DNA was amplified using forward primer 5'-GGCATCTTTTTTGTACAGCCTACAGCTTA-3' and reverse primer 5'-TGGCGATATCGGCGGATAACA-3', amplifying a 201 base pair (bp) product containing the mutation. The forward primer introduces an additional base change, which only in combination with the mutant allele generates the consensus for *DdeI* restriction enzyme. Therefore, the mutant allele was distinguished from the wt by specific digestion into a 174 fragment that can be separated from the undigested wt amplicon on a 2.5% agarose gel. Alternatively, genotyping was performed by KASP assay using the primers 5'-CATCATAGGAAGTACTGTTGTAGTCA-3', 5'-CATCATAGGAAGTACTGTTGTAGTCC-3' and 5'-GGCATCTTTTTTGTACAGCCTACAGATT-3'. Robustness of both methods was verified several times by sequencing of the amplicons.

Embryos were grown at 28.5°C in egg water (60 µg/ml sea salt, Sera Marin, Heinsberg, Germany). For live-imaging or injection assays, larvae were anaesthetised in egg water medium containing 0.02% buffered Tricaine (3-aminobenzoic acid ethyl ester; Sigma-Aldrich, St Louis, MO, USA). To prevent melanisation, larvae were generally maintained in egg water supplemented with 0.003% PTU (1-phenyl-2-thiourea; Sigma-Aldrich).

Sequencing – Sequencing of the full coding sequence of *cxc3.1*, *cxc3.2* and *cxc3.3* was obtained by amplification with primers described in **Supplementary Table 3**. For *cxc3.2* and *cxc3.3*, both genomic and cDNA templates extracted from pools of 15–20 embryos were used. Amplification was performed with Phusion high-fidelity DNA polymerase (Thermo-Scientific, Pittsburgh, PA, USA). DNA amplicons were then gel-extracted on 1.5% agarose and column-purified with PureLink quick gel extraction and PCR purification kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). Sequencing with M13Fw, M13Rv universal primers (incorporated in the amplification primers) or with custom-made primers was outsourced to Baseclear (Leiden, The Netherlands). For *cxc3.1*, sequencing results derive exclusively from genomic DNA amplifications. Amplification of cDNA templates for *cxc3.2* resulted in a band of identical size in mutant, wt and AB/TL, thereby excluding altered exon/intron arrangements attributable to the ENU-mutagenesis per se or to the *cxc3.2*^{hu6044} allele.

Bacterial cultures and infection delivery – Approximately 200 CFU (1 nl) of *M. marinum* strain Mma20 expressing mCherry⁶⁷, or *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) strain SL1027 expressing DsRed⁶⁸, were grown and harvested as described previously^{47,69}. Embryos were staged at 30 hpf and bacteria or mock control [phosphate buffer saline (PBS) supplemented with 0.1% phenol red (Sigma-Aldrich) and 2% polyvinylpyrrolidone-40 (Sigma-Aldrich)] were locally injected in the hindbrain cavity as described previously^{47,69}. Injections of bacteria in the otic vesicle, as shown in **Figure 3**, were performed either at 3 dpf (**Figure 3B,I-L**) or at 4 dpf (**Figure 3C-H**). When infection was delivered systemically, the same dose was instead injected in the caudal vein as in reference⁶⁹. As a control, the same dose was spotted onto plates, incubated and counted. Embryos were kept into fresh PTU egg water, incubated at 28.5°C, and collected for qRT-PCR or used for imaging at 1–6 dpf. In **Figure 8A,B**, embryos were fixed at 6 dpf in 4% paraformaldehyde in PBSTx (1× PBS supplemented with 0.8% Triton X-100; Sigma-Aldrich) and prepared for Myeloperoxidase (Mpx) activity stain with TSA staining kit (PerkinElmer Inc., Waltham, MA, USA), followed by immunostaining against the pan-leukocyte marker Leukocyte-plastin (Lp) as described previously^{47,69}.

FACS-sorting, RNA isolation and qRT-PCR – *Mpeg1:mCherry-F*-positive, *mpx:eGFP*-positive and unlabelled cells were sorted from *Tg(mpeg1:mCherry-F/mpx:eGFP)* at 2 and 6 dpf. FACS-protocol and RNA isolation were performed according to reference⁷⁰. To evaluate the induction of the *cxc11* genes upon infections, pools of 18–20 embryos were collected for RNA isolation, snap-frozen in liquid nitrogen and subsequently stored at –80°C. RNA was extracted using Qiazol reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's guidelines. Residual genomic DNA was removed by DNA-free kit (Ambion, Life Technologies). The cDNA was prepared using the iScript cDNA-synthesis kit (Invitrogen, Life Technologies) and was used as a template for qRT-PCR reaction with iQ SYBR Green Supermix according to the manufacturer's instructions (Bio-Rad Laboratories, Munich, Germany). Specificity of the amplification reaction was analysed using dissociation curves. Each qRT-PCR was performed in technical duplicate and on biological replicates as indicated in the figure legends. Reference genes were *elf4a1b* or *elf5* (eukaryotic translation initiation factor 4a isoform 1b or 5) for FACS-sorted cells and *ppiab* (*peptidylprolyl isomerase ab/cyclophilin a*) for infection experiments. Fold changes were determined using the $\Delta\Delta$ comparative threshold method. Primers are reported in **Supplementary Table 3**.

Production of recombinant chemokines and local injections – Synthetic coding sequences for Cxcl11aa, Cxcl11af, Cxcl11ae and Cxcl8a (included as negative control) were generated (Baseclear) according to database accessions (**Supplementary Table 2**). To enable secretion in yeast, the sequences were codon optimised and the predicted zebrafish signal peptide was replaced with yeast alpha-factor secretion signal, as a result from cloning into pPICZ α expression vector (Invitrogen, Life Technologies). Additionally, a HA (human influenza haemagglutinin)-tag was added at the C-terminus to facilitate the purification process and identification. The recombinant chemokines were produced by *Pichia pastoris* strain X-33 transformed with the chemokine vectors as described previously⁷¹. Proteins were purified via Fast Protein Liquid Chromatography in NaCl salt gradient and finally desalted and concentrated by membrane filtrations on Amicon Ultra Centrifugal filter devices with a nominal molecular weight limit of 3 kilodaltons (Amicon, Merck KGaA, Ireland), using 50 mM sodium phosphate buffer pH 6.5 as a washing and suspension vehicle. Purity and identity of the proteins were confirmed by trypsinisation and electrospray mass-spectrometry. The recombinant chemokines (0.5–1.2 mg/ml), LTB₄ (leukotriene B₄; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 10.1 ng/ml), fMLP (N-formyl-methionyl-leucyl-phenylalanine; Sigma-Aldrich; 0.2 mg/ml) or mocks [sodium phosphate buffer pH 6.5 for the chemokines, 5% DMSO (Sigma-Aldrich) in PBS for fMLP and 0.02% ethanol (Sigma-Aldrich) in PBS for LTB₄] were supplemented with 0.1% phenol red and injected at 30 hpf in the hindbrain ventricle (1 nl) or at 52 hpf in the otic vesicle (0.5 nl) as described previously^{47,69}. In both cases, embryos were fixed at 3 hpi in 4% paraformaldehyde in PBSTx and prepared for Lp immunostaining as in reference⁴⁷. At 30 hpf, the population of fully differentiated leukocytes is represented almost exclusively by macrophages^{48,49}; thereby, we could assume that nearly all the Lp-stained cells able to migrate and infiltrate in the ventricle represented macrophages at this developmental stage. As is shown in **Supplementary Figure 3**, only one to two *mpx*-positive cells [*mpx*-whole mount *in situ* hybridisation as in reference⁴⁷] could be counted within the perimeter of the hindbrain in this experimental setting at 3 hours post local bacterial infection, which is less than 10% of the cells positive for the macrophage marker *mfap4*. At later developmental stages, in order to discern between neutrophils and macrophages, samples were processed also with a neutrophil-specific Mpx activity staining as described previously⁴⁷, by using the leukocyte peroxidase (Myeloperoxidase) staining kit (Sigma-Aldrich) for the histochemical detection of the enzymatic activity of Mpx. Leukocytes accumulated at the injected cavity (macrophages: Lp-positive and Mpx-negative; neutrophils: Mpx-positive) were counted using a Leica MZ16FA fluorescence stereomicroscope (Leica Microsystems, Rijswijk, The Netherlands).

Chemically induced (ChIn) inflammation assay – 3-dpf larvae were exposed to 10 μ M copper sulphate (CuSO₄; Sigma-Aldrich) for 2 hours as described previously⁵⁰. Treated larvae were then fixed and used for combined Mpx activity staining and Lp immunostaining as described above.

Pharmacological treatment with NBI74330 – Bath-treatment with the CXCR3 high-affinity antagonist NBI74330 or vehicle treatment (0.5% DMSO) was started at 27 hpf by exposing dechorionated embryos to 50 μ M of the drug in medium. Embryos were incubated for 3 hours at 28.5°C and then injected in the hindbrain with mock or *M. marinum* as described above. Injected embryos were maintained for an additional 3 hours in 50 μ M NBI74330 or vehicle alone and then fixed in 4% paraformaldehyde/PBSTx and prepared for Lp immunostaining as described previously⁴⁷.

Imaging and image quantification – Fixed or live embryos and larvae were imaged using a Leica MZ16FA fluorescence stereomicroscope. For time-lapse experiments, samples were mounted in 2% low-melting-point agarose (SphaeroQ, Burgos, Spain) and images were acquired with a laser-scanning confocal microscope (Leica TCS SPE, Leica Microsystems or Zeiss Observer 6.5.32, Carl Zeiss, Sliedrecht, The Netherlands). To assess the average speed of macrophages (**Figure 1J-K**), a time-lapse experiment was performed and quantification was obtained on overlaid z-stacks by Fiji/ImageJ software (NIH, Bethesda, MD, USA) using the ManualTrack plug-in as described elsewhere⁷². The average speed was calculated as the average of all the speeds assumed by every single macrophage at each time point. Analysis was performed by cumulating three experiments in which 15–21 macrophages per embryo were followed. To quantify the morphological differences between macrophages in *cxc3.2* mutants and wt, bacteria were injected into the otic vesicle of *Tg(mpeg1:Gal4/UAS:Kaede/cxc3.2^{+/+}* or *cxc3.2^{-/-}*) larvae at 3 dpf and fixed at 4 hpi. Images of macrophages were acquired in the trunk. Perimeter and area of the cells were obtained by Fiji/ImageJ using the Analyse Particles plug-in. The circularity index (CI) corresponding to each cell was obtained by the formula: $CI = 4\pi \text{ (area/perimeter}^2\text{)}$, resulting in an index that ranges from 0 (infinitely branched structure) to 1 (perfect circle). Macrophages were classified in five different intervals of circularity based on their CI (0.0 to 0.19, 0.2 to 0.39, 0.4 to 0.59, 0.6 to 0.79, 0.8 to 1.0) and the average percentages of macrophages in each interval were estimated for *cxc3.2^{+/+}* or *cxc3.2^{-/-}* (**Figure 3I**). To estimate the divergence of distribution of *cxc3.2^{+/+}* and *cxc3.2^{-/-}* macrophages from the overall mean, the percentages in each interval were divided by the average percentage of mutants and wt assumed in that interval, using the formulas: $\text{Deviation}_{(cxc3.2+/+)} = \%cxc3.2^{+/+} / [(\%cxc3.2^{+/+} + \%cxc3.2^{-/-})/2]$ and $\text{Deviation}_{(cxc3.2-/-)} = \%cxc3.2^{-/-} / [(\%cxc3.2^{+/+} + \%cxc3.2^{-/-})/2]$ (**Figure 3J**). To quantify the dissemination of bacterial infection (**Figure 6F,I**), the presence or absence of infection distally from the infected site was evaluated, giving a score of 1 in case of dissemination and a score of 0 in case of absent dissemination. Quantification of total bacterial pixels (**Figure 7A-B** and **Figure 8E**) was obtained using dedicated bacterial pixel count program as in reference⁷³. Total bacterial cluster count (**Figure 8F**) was performed manually from images. Quantification of the area of single distal clusters (**Figure 7C**) and average area of disseminated granulomas (**Figure 8G**) were performed using ImageJ quantification tools as in reference⁷⁴).

Statistical analysis – In the survival test (**Figure 1E**), non-significant deviation from Mendelian rate was evaluated by χ^2 test on four independent replicates. For qRT-PCR, statistical significance was estimated on five (**Figure 1A-C**), four (**Figure 4A-B**) or three (**Figure 4C-D**) biological replicates by two-tailed *t*-tests on ln(n)-transformed relative induction folds. All the other experiments were statistically analysed using GraphPad Prism 4 or 5 (GraphPad Software, La Jolla, CA, USA). Where correction for non-parametric distribution was required (**Figure 1F,G**, **Figure 2**, **Figure 3A,B**, **Figure 3M**, **Figure 5**, **Figure 6**, **Figure 7A,B**, **Figure 8E,F**), comparisons between two groups were performed with two-tailed Mann-Whitney test and comparisons among more than two groups were performed with Kruskal-Wallis test, followed by Dunn's multiple comparison test. When a parametric distribution was assumed (**Figure 1A-C**, **Figure 1L**, **Figure 3I-J**, **Figure 4**, **Figure 8G**), comparisons between two groups were performed with a two-tailed *t*-test. In **Figure 7C**, significance was estimated with an unpaired *t*-test with Welch's

correction, suitable to compare parametric data having different variances. Significance (*P*-value) is indicated with: ns, non-significant; **P*<0.05; ***P*<0.01; ****P*<0.001.

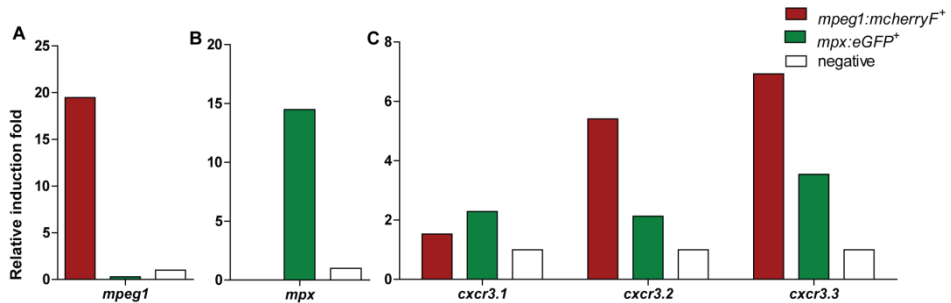
ACKNOWLEDGMENTS

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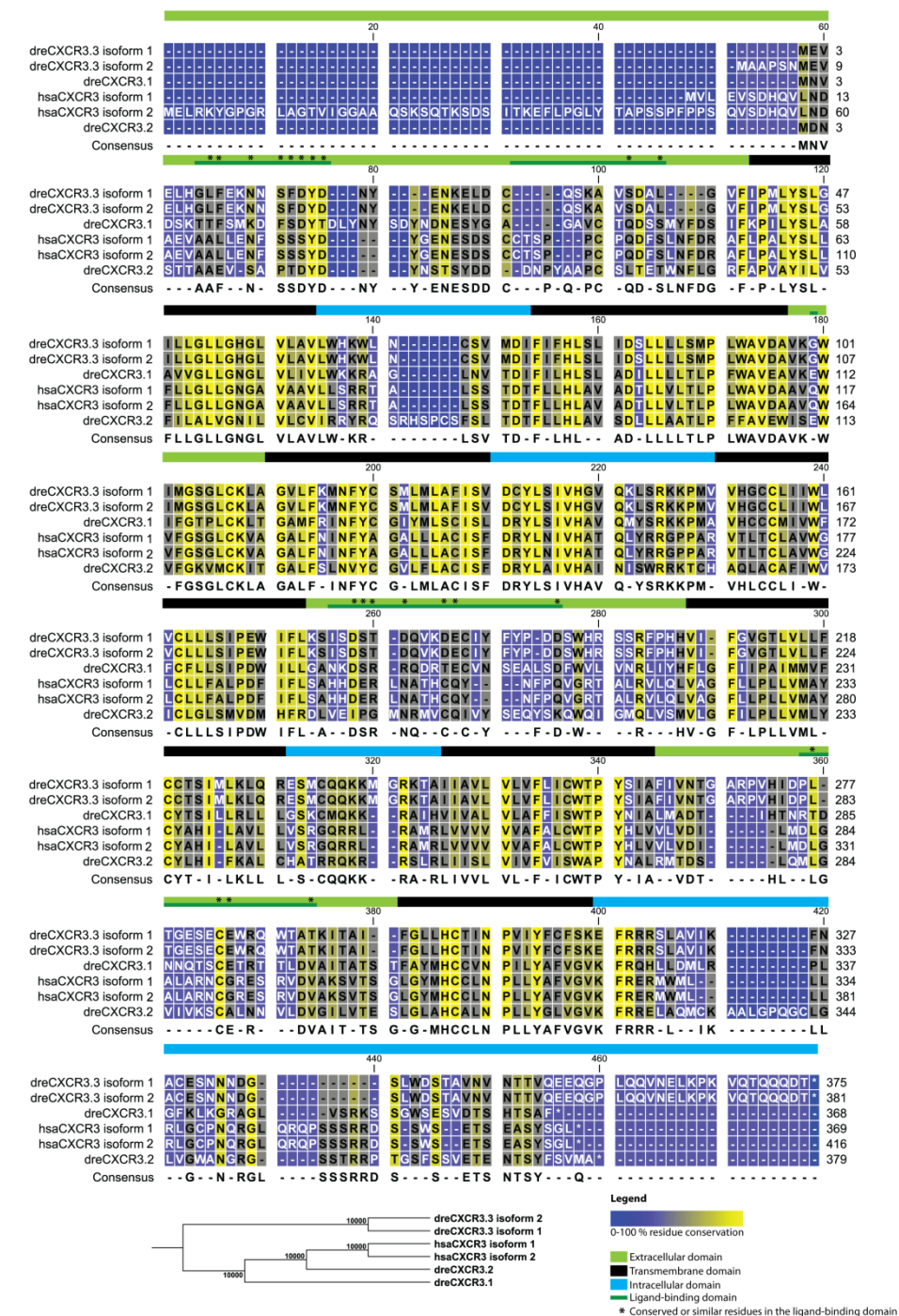
FOOTNOTES

The format and spelling of the contents in this chapter have been adapted from the published article to conform it to the thesis outline.

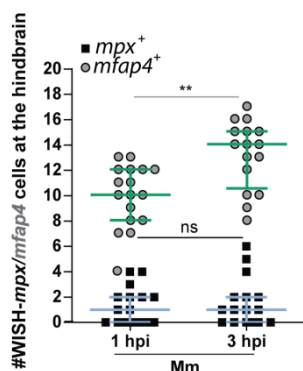


Supplementary Figure 1. Expression of *cxcr3.2* and its paralogues *cxcr3.1* and *cxcr3.3* in FACS-sorted phagocytes at 6 dpf. Graphs represent the relative induction fold of the macrophage marker *mpeg1* (A), the neutrophil marker *mpx* (B), and of the *cxcr3* paralogues (C) in FACS-sorted macrophages and neutrophils from the combined transgenic line *Tg(mpeg1:mCherry-F/mpx:eGFP)* at 6 dpf. Expression of *cxcr3.2* and *cxcr3.3* could be detected in both macrophages and neutrophils, while *cxcr3.1* was not significantly enriched in the FACS-sorted populations when compared to the non-labelled cell fraction. Reference gene: *elf5*.

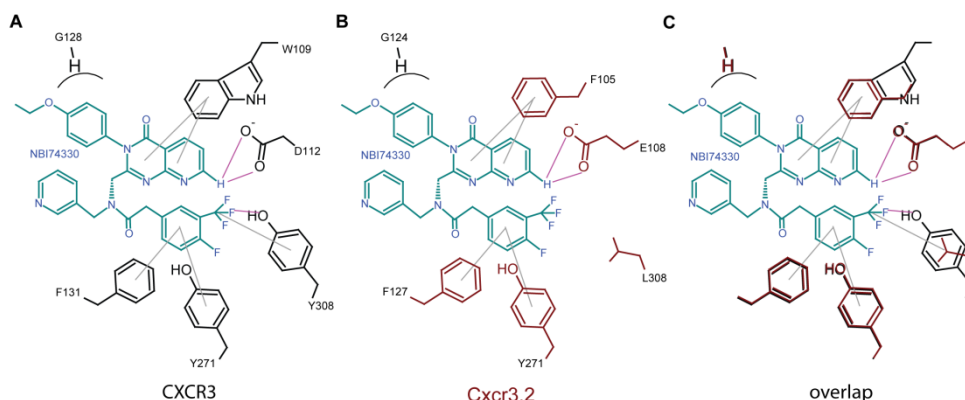
Chapter 3



Supplementary Figure 2. Protein sequence multiple alignment of human and zebrafish CXCR3 chemokine receptors (Figure on the previous page). Residue colour from blue to yellow indicates increasing degree of amino acid conservation. The alignment and the tree were obtained using CLC main workbench 6.8.4 by Neighbour-Joining Algorithm. Gap costs were given with a penalty score of 10 for each gap open, and an additional score of 1 per each extension; no cost was associated to end gaps. Extracellular (light green bars), Transmembrane (black bars), and Intracellular (light blue bars) domains were predicted with CLC main workbench 6.8.4. Ligand binding domains (dark green bars) and conserved residues or similar residues within the binding domains (black asterisks) were predicted according to reference⁷⁵. The numbers at the tree nodes denote the bootstrap for 10000 replicates. Single alignment of the predicted zebrafish chemokine receptor proteins to the canonical isoform of CXCR3 (hsaCXCR3 isoform 1) were performed with clustalO algorithm (<http://www.uniprot.org/align>) and provided the following % of identity: dreCXCR3.1 (ENS DARG00000007358): 39.1%, dreCXCR3.2 (ENS DARG000000041041): 35.7%, dreCXCR3.3 (ENS DARG00000070669) isoform 1: 29.8% and dreCXCR3.3 isoform 2: 29.6%.

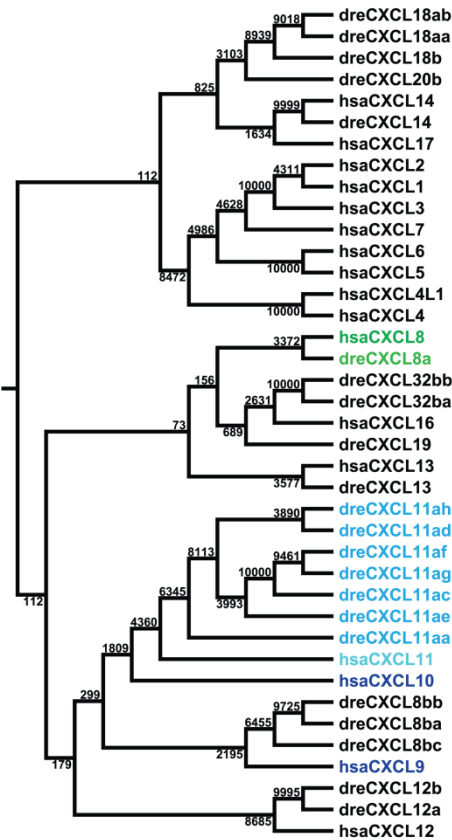


Supplementary Figure 3. Macrophages are the predominant phagocyte cell type recruited to local hindbrain infection in 31-33 hpf embryos. Embryos were locally injected into the hindbrain cavity at 30 hpf with 100 CFU of *M. marinum* and fixed at 1 and 3 hpi. Double fluorescent *in situ* hybridisation was performed with *mfap4* as a macrophage marker and *mpx* as a neutrophil marker³⁶. The average number of *mfap4*-positive macrophages in the hindbrain at 1 or 3 hpi exceeds the average number of *mpx*-positive neutrophils approximately 10-fold and a significant difference between the time points was observed only for macrophages. Sample size (n): 17, 16. Error bars: median and interquartile range. ns, non-significant; **P<0.01.



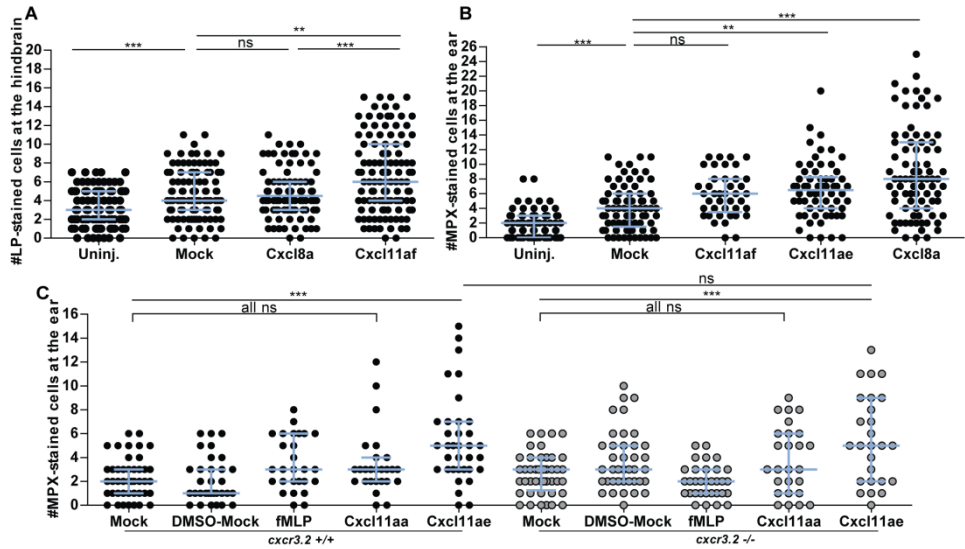
Supplementary Figure 4. Predicted molecular docking of NBI74330 into the transmembrane minor pocket of Cxcr3.2 The figure represents the high conservation of the transmembrane minor pocket of zebrafish Cxcr3.2 with human CXCR3. **A-B.** Side chains of proposed interacting residues are shown in black for CXCR3 (A) and in red for Cxcr3.2 (B). The ligand NBI74330 is shown in cyan. Suggested receptor/ligand interactions are depicted as coloured lines. Polar and hydrogen bonding interactions are shown as pink lines, whereas aromatic interactions are shown as grey lines. **C.** Overlay of the CXCR3 and Cxcr3.2 binding pocket. Prediction of ligand binding for CXCR3 is according to reference³¹. Predictions of ligand binding for Cxcr3.2 is according to sequence alignment and amino acid similarity in the corresponding positions between CXCR3 and Cxcr3.2.

Supplementary Figure 4. Predicted molecular docking of NBI74330 into the transmembrane minor pocket of Cxcr3.2



Supplementary Figure 5. Phylogenetic tree of human and zebrafish chemokine protein sequences. The alignment and the tree were obtained using CLC main workbench 6.8.4 by Neighbour-Joining Algorithm. Gap costs were given with a penalty score of 10 for each gap open, and an additional score of 1 per each extension; no cost was associated to end gaps. Additionally, the CXC motif of the chemokine was set as fixed alignment point. The numbers at the tree nodes denote the bootstrap for 10000 replicates. Light blue: Cxcl11-like cluster, containing human CXCL11 and seven zebrafish Cxcl11-like chemokines. Dark blue: human CXCL9 and CXCL10. Green: Cxcl8-like cluster, containing human CXCL8 (IL8) and zebrafish Cxcl8a (IL8). Nomenclature according to reference³⁴.

Cxcr3.2 signalling in macrophage recruitment and infection dissemination



Supplementary Figure 6. Chemoattractive effects of recombinant chemokines on different phagocytes.

A. Chemoattraction of macrophages to the hindbrain ventricle. Recombinant proteins or buffer (mock) were injected into the hindbrain ventricle of wildtype (AB/TL) embryos at 30 hpf and Lp-stained cells accumulating in 3 hours within the hindbrain limits were counted as macrophages. Cxcl11af but not Cxcl8a significantly attracted macrophages. Sample size (n): 106, 100, 94, 112. Error bars: median and interquartile range. **B-C.** Chemoattraction of neutrophils to the otic vesicle. Recombinant proteins or mock were injected into the otic vesicle of wildtype (AB/TL) embryos (B) or *cxcr3.2*^{+/+} and *cxcr3.2*^{-/-} siblings (C) at 54 hpf and neutrophils accumulating in 3 hours within the otic vesicle were counted after Mpx activity staining. Neutrophil attraction by recombinant Cxcl8a (Il8) (B) was in agreement with previous reports²⁷. Note that Cxcl11ae significantly recruited neutrophils in all zebrafish lines, while Cxcl11af (B), Cxcl11aa (C), and fMLP (C) did not exert significant neutrophil attraction above mock injections. Sample size (n) in B: 75, 77, 41, 62, 83. Sample size (n) in C: 44, 32, 27, 26, 35, 44, 39, 32, 26, 26. Error bars: median and interquartile range. ns, non-significant; **P<0.001.

Chapter 3

Protein	Amino acid position	Strain	Variant	PROVEAN score	Predicted effect
Cxcr3.1	203	Reference*	E	0.000	Neutral
		AB/TL	E/K	-0.945	Neutral
		<i>cxcr3.2</i> ^{+/+}	E/K	-0.945	Neutral
		<i>cxcr3.2</i> ^{-/-}	E	0.000	Neutral
Cxcr3.1	292	Reference	E	0.000	Neutral
		AB/TL	E/K	-1.803	Neutral
		<i>cxcr3.2</i> ^{+/+}	E	0.000	Neutral
		<i>cxcr3.2</i> ^{-/-}	K	-1.803	Neutral
Cxcr3.1	351	Reference	K	0.000	Neutral
		AB/TL	R	0.645	Neutral
		<i>cxcr3.2</i> ^{+/+}	R	0.645	Neutral
		<i>cxcr3.2</i> ^{-/-}	R	0.645	Neutral
Cxcr3.2	16	Reference	Y	0.000	Neutral
		AB/TL	Y	0.000	Neutral
		<i>cxcr3.2</i> ^{+/+}	Y	0.000	Neutral
		<i>cxcr3.2</i> ^{-/-}	STOP	<i>cxcr3.2</i> ^{hu6044}	Non-sense
Cxcr3.2	93	Reference	V	0.000	Neutral
		AB/TL	I	0.048	Neutral
		<i>cxcr3.2</i> ^{+/+}	I	0.048	Neutral
		<i>cxcr3.2</i> ^{-/-}	I	0.048	Neutral
Cxcr3.3	48	Reference	M	0.000	Neutral
		AB/TL	L	0.332	Neutral
		<i>cxcr3.2</i> ^{+/+}	L	0.332	Neutral
		<i>cxcr3.2</i> ^{-/-}	L	0.332	Neutral
Cxcr3.3	75	Reference	C	0.000	Neutral
		AB/TL	C/P	-0.547	Neutral
		<i>cxcr3.2</i> ^{+/+}	C/P	-0.547	Neutral
		<i>cxcr3.2</i> ^{-/-}	C	0.000	Neutral
Cxcr3.3	95	Reference	S	0.000	Neutral
		AB/TL	T	2.931	Neutral
		<i>cxcr3.2</i> ^{+/+}	T	2.931	Neutral
		<i>cxcr3.2</i> ^{-/-}	T	2.931	Neutral
Cxcr3.3	236	Reference	E	0.000	Neutral
		AB/TL	E/D	-0.328	Neutral
		<i>cxcr3.2</i> ^{+/+}	E/D	-0.328	Neutral
		<i>cxcr3.2</i> ^{-/-}	E	0.000	Neutral
Cxcr3.3	238	Reference	M	0.000	Neutral
		AB/TL	M/L	-0.611	Neutral
		<i>cxcr3.2</i> ^{+/+}	M/L	-0.611	Neutral
		<i>cxcr3.2</i> ^{-/-}	M	0.000	Neutral
Cxcr3.3	244	Reference	M	0.000	Neutral
		AB/TL	M/I	-0.473	Neutral
		<i>cxcr3.2</i> ^{+/+}	M/I	-0.473	Neutral
		<i>cxcr3.2</i> ^{-/-}	I	-0.473	Neutral
Cxcr3.3	297	Reference	T	0.000	Neutral
		AB/TL	S	-0.463	Neutral
		<i>cxcr3.2</i> ^{+/+}	S	-0.463	Neutral
		<i>cxcr3.2</i> ^{-/-}	S	-0.463	Neutral
Cxcr3.3	327	Reference	L	0.000	Neutral
		AB/TL	L/Q	-2.562	Neutral
		<i>cxcr3.2</i> ^{+/+}	L/Q	-2.562	Neutral
		<i>cxcr3.2</i> ^{-/-}	Q	-2.562	Neutral
Cxcr3.3	346	Reference	D	0.000	Neutral
		AB/TL	D/E	0.098	Neutral
		<i>cxcr3.2</i> ^{+/+}	D/E	0.098	Neutral
		<i>cxcr3.2</i> ^{-/-}	D	0.000	Neutral
Cxcr3.3	350	Reference	V	0.000	Neutral
		AB/TL	V/E/D	0.895/1.275	Neutral
		<i>cxcr3.2</i> ^{+/+}	V/E/D	0.895/1.275	Neutral
		<i>cxcr3.2</i> ^{-/-}	D	0.895	Neutral
Cxcr3.3	378	Reference	Q	0.000	Neutral
		AB/TL	Q/E	-0.077	Neutral
		<i>cxcr3.2</i> ^{+/+}	Q/E	-0.077	Neutral

Supplementary Table 1. Non-synonymous single nucleotide polymorphisms in *cxcr3.1*, *cxcr3.2*, and *cxcr3.3* coding sequences (Legend on the next page).

Cxcr3.2 signalling in macrophage recruitment and infection dissemination

Supplementary Table 1. Non-synonymous single nucleotide polymorphisms in *cxcr3.1*, *cxcr3.2*, and *cxcr3.3* coding sequences (Legend on the next page). Several non-synonymous single nucleotide polymorphisms (nsSNPs) are present in *cxcr3.1*, *cxcr3.2*, and *cxcr3.3* genes that are inherited in association with the ENU-mutagenised *cxcr3.2*^{hu6044} allele. Except for the Cxcr3.1 E292K variant, all the nsSNPs linked to the *cxcr3.2*^{-/-} fish did not differ from the ones represented also in the *cxcr3.2*^{+/+} fish. Furthermore, all nsSNPs, including the Cxcr3.1 E292K variant, were detected also in the AB/TL wt strain. Analysis using the PROVEAN software tool (Protein Variation Effect Analyser, <http://provean.jcvi.org>)^{45,46}, predicts that the nsSNPs are unlikely to have an impact on the protein functionality. The threshold to score the possibility of protein non functionality was set to -4.1⁴⁶. Note: Amino acid notations for Cxcr3.3 are represented accordingly to their position in Cxcr3.3 splicing isoform 2. No additional amino acid replacements were specifically found in the splicing isoform 1. *Reference: according to ENSEMBL accessions.

Name	Gene Accession	Protein Sequence
hsaCXCL9	ENSG00000138755	mkksgvflilgiillvlgvqTPVVRKGR C SCISTNQGTIHLQSLKDLKQFAPSPSC EKIE I ATLKNGVQT C LNPD SADVKELIKKWEK Q V S QKKKQKNGKKHKQKK KVLKVRKSQRSRQKKT
hsaCXCL10	ENSG00000169245	mnqtailiccliftltsgiqVPLSRTVR C TCISISNPVNPRSLEKLEIIPASQ F CPR VE I ATMKKKGEKR C LNPE SKAIKNLLKAVSKER S KRSP
hsaCXCL11	ENSG00000169248	msvkgmaialavilcatvvqgFPMFKRGR C LCIGPGVKAVKVADIEKASIMYPS NN C DKIEV I TLKENKGQR C LNPKSKQAR L IKKVERKNF
dreCxcl11aa	Gene ID: 798892	mktvtallvslavvaiegQHMKSQR C VLGAGLNMVKPVLIEKIEILPSSP S CG HMEV I ATLKNGAGKR C LNPKSKFTKKIIDKIEKN R NAR
dreCxcl11ac	Gene ID:100334604	mktlaafllltcliagkvngQDNTSRAR C F CADKGINMVLLKNIKVEIFPPSP S C NKNE I VVTLKNGAGQK C LNPD SKFTQNVVLAIGK R MQQSVPHSTTTGT VKSSMTSSTSAPTAFK
dreCxcl11ad	Gene ID: 567656	mktlaavvllgylvikvegQARAPSR C LCADKGVNMVSPKLEKVDIIPPTP S C GNLE I VVTLKNGAEPK C LPDSKFTQKYLMALEK R TLQK
dreCxcl11ae	ENSDARG00000092423	mkttaafvlflafifipgQKKFN R CS C VGKGLDRVALRNIKFEI I HPSP S CGKQEI IVTMKSSEQK C LNPE SKFTQELIRRALEK
dreCxcl11af	ENSDARG00000094706	mktlaaflllslciagevngQDRSSRAR C FCVDKGLNMVLLKNLDKVEIFPPSP S C CNKHE I VVTLKNGAGQK C LNPD SKFTKNVVLKAIGK R MQQSVPHSTTTGT TVKSSMTSSTSAPTAFK
dreCxcl11ag	ENSDARG00000093779	mktlaaflllslciagevngQDRSSRAR C FCVDKGLNMVLLKNLEKVEIFPPSP S C NKHE I VVTLKNGAGQK C LNPD SKFTKNVVLKAIGK R MQQSVPHSTTTGT VKSSMTSSTSAPTAFK
dreCxcl11ah	ENSDARG00000095747	mkttaafvalgcflmvevkgKIPDLKN R CLCADKGANNVNLTIEKIQI I HPSP S C CKRLE I VVTLMKGAGK C LNPE SNLGKNILKALR K KLTA VRRMNPA
dreCxcl8a	Gene ID: 100002946	mtskiisvcvivflaftiiegMSLRGLAVDP R CRCIETESRRIGKHIKSVELFPSP H CKDLE I ATLMTTGQE I CLDPSAP V VKKIIDRIIV R NRP
hsaCXCR3 isoform 1	ENSG00000186810	MVLE V SDHQVLNDAEVAALLENFSSSYDGENESDSCCTSPPCPDQFSLN FDRAFLPALYSLLFLLGLLNGAVAAVLLSRRTALSTDTFLHLAVADTLLV LTLPWLAVDAAVQWVFGSGLCCKVAGALFNINFYAGALLACISFDRLNI VHATQLYRRGPPARVTLTCLAVWGLCLLFPALDFILSAHHDRLNATHC QYNFPQVGR ¹ TALRVLQVLVAGFLLPLLVMAYCYAHILAVLLVSRGQRRRLRA MRLVVVVVAFALCWTPYHLVVLDLMDLGLARNCGRESRVDVAKSV TSLGLYMHCLNPLLYAFVGKFRERMMWMLLLRLGCPNQRGLQRQPSS SRDSSWSETSEASYSL

Supplementary Table 2. List of chemokines and chemokine receptors used in this work with accession codes and protein sequences (continued on the next page). Red letters: amino acids at the splicing sites of the mRNA; blue letters: conserved CXC-chemokine motifs. Part in grey: active chemokine upon removal of the signal peptide.

Chapter 3

Name	Gene Accession	Protein Sequence
hsaCXCR3 isoform 2	ENSG00000186810	MELRKYGPGRLAGTVIGGAAQSKSQTKSDSITKEFLPGLYTAPSSFPFPSQ VSDHQVLNDAEVAALLENFSSSYDYGENESDSCCTSPPCPQDFSLNFDRA FLPALYSLLFLLGLLGNGAVAVALLSRRRTALSSTDTFLLHLAVADTLVLTLPL WAVDAAVQWVFGSGLCKVAGALFNINFYAGALLACISFDRLYNIVHAT QLYRRGPPARVTLTCLAVWGLCLLFPDFIFLSAHHDERLNATHCQYNF PQVGRTALRVLQLVAGFLLPLVMAYCYAHILAVLLVSRGQRRLRAMRLV VVVVVAFALCWTPYHLVVLVDILMDLGALARNCGRESRVDAKSVTSGL GYMHCCLNPLLYAFVGVKFRERMWMLLLRLGCPNQRGLQRQPSRSSRRD SSWSETSEASYSGL
dreCxcr3.1	ENSDARG00000007358	MNVDSKTTFSMKDFSDYTDLYNYSYNDNESYGAGAVCTQDSSMYFDSI FKPILYSLAAVVGLGNGLVVLVWKKRAGLNVTDIFILHLSLADILLTLPLF WAVEAVKEWIFGTPLCKLTGAMFRINFYCGIYMLSCISLDRLYSIVHAVQ MYSRKKPMAVHCCCMIVWFFCFLSIPDWILLGANKDSRRQRDRTECVNS EALSDFWVLVNRLIYHFLGFIIIPAIMMVFCYTSILLRLLGSKCMQKKRAIH VIVALVLAFFISWTPYNIALMADTIHTNRTDNNQTSCESTRITLDVAITATST FAYMHCCVNPILYAFVGVKFRQHLLDMLRPLGFKLKGRAGLVSRSKSSGW SESVDTSHTSAF
dreCxcr3.2	ENSDARG000000041041	MDNSTTAAEVSAPTDYDYNSTSYDDDNPYAAPCSLTETWNFLGRFAPVA YILVFILALVGNILVLCVIRRYRQSRHSPCSFSLTDTFLLHLAVSDLLAATLPF FAVEWISEWVFGKVMCKITGALFSLNVYCGVLFACISFDRLAIVHAINIS WRRKTCHAAQLACAFIWWICGLSMVDMHFRDLVEIPGMNRMVCQIVYS EQYSKQWQIGMQLVSMVLGFILPLVMLCYLHIFKALCHATRQKRRSL RLIISLVIVFVISWAPYNALRMTDSLQMLGVIVKSCALNNVLDVGILVTESL GLAHCALNPLLYGLVGVKFRRELAQMCKAALGPQGCLGLVGWANGRGS STRRPTGSFSSVETENTSIFYFSVMA
dreCxcr3.3 isoform 1	ENSDARG000000070669	MEVELHGLFEKNNSFDYDNYENKELDCQSKAVSDALGVFIPMLYSLGILL GLLGHGLVLAVLWHKWLNCVMDIFIFHLSLIDSLLLSMLPLWAVDAVKG WIMGSGLCKLAGVLFKMNFYCSMLMLAFISVDCYLSIVHGVQKLSRKKP MVVHGCCLIIWLVCLLSIPEWIFLKSISDSTDQVKDECIYFYPDDSWHRS RFPHHVIFGVGTLLVLCCTSIMLKLQRESMCQQKKMGRKTAIIAVLVLF LICWTPYSIAFIVNTGARPVIDPLTGESECEWRQWTATKITAIFGLLHCTI NPVIYFCFSKEFRRRSLAVIKFNACESNNNDGSLWDSTAVNVNTTVQEEQ GPLQQVNELKPKVQTQQQDT
dreCxcr3.3 isoform 2	ENSDARG000000070669	MAAPSNMEVELHGLFEKNNSFDYDNYENKELDCQSKAVSDALGVFIPML YSLGILLGLLGHGLVLAVLWHKWLNCVMDIFIFHLSLIDSLLLSMLPLWA VDAVKGWIMGSGLCKLAGVLFKMNFYCSMLMLAFISVDCYLSIVHGVQK LSRKKPMVVHGCCLIIWLVCLLSIPEWIFLKSISDSTDQVKDECIYFYPDDS WHRSSRFPHHVIFGVGTLLVLCCTSIMLKLQRESMCQQKKMGRKTAIIA VLVLVFLICWTPYSIAFIVNTGARPVIDPLTGESECEWRQWTATKITAIFG LLHCTINPVIYFCFSKEFRRRSLAVIKFNACESNNNDGSLWDSTAVNVNTT VQEEQGPLQQVNELKPKVQTQQQDT

Supplementary Table 2. List of chemokines and chemokine receptors used in this work with accession codes and protein sequences (continued from the previous page).

Cxcr3.2 signalling in macrophage recruitment and infection dissemination

Primers used in qRT-PCR reactions

Gene Name	Gene Accession	Sequence (5'-3')
<i>cxcr3.1</i>	ENSDARG00000007358	Fw: CTTTCCTGCATCAGTCTCGACC Rv: TGACGTCTGGAGTCTTTGTTGG
<i>cxcr3.2</i>	ENSDARG000000041041	Fw: CCTCTGTTGGTAATGCTGTATTGC Rv: ACACGATGACTAAGGAGATGATGAG
<i>cxcr3.3</i>	ENSDARG000000070669	Fw: GCTCTCAATGCCTCTCTGGG Rv: GACAGGTAGCAGTCCACACT
<i>ppiab</i>	ENSDARG000000042247	Fw: ACACTGAAACACGGAGGCAAAG Rv: CATCCACAACCTTCCCGAACAC
<i>cxcl11aa</i>	Gene ID: 798892	Fw: ACTCAACATGGTGAAGCCAGTGCT Rv: CTTGACGTGGCTATGACTTCCAT
<i>cxcl11ac</i>	Gene ID: 100334604	Fw: TCTGACCTGCCTGATCGCTGGA Rv: TGCCTTTGTGACACAGAAGCACC
<i>cxcl11ad</i>	Gene ID: 567656	Fw: AGGCCAGGCGAGAGCTCCAA Rv: TCCACAAGAAGGGGTCGGTGGT
<i>cxcl11ae</i>	ENSDARG000000092423	Fw: AGGGTTGCACTGAGGAACATTGAGA Rv: AGCCCTCTGATTAATTCCTGGGT
<i>cxcl11af/ag</i>	ENSDARG000000094706 ENSDARG000000093779	Fw: GCTGGAGAGGTCAACGGTCAGGA Rv: TGCAAGATGGAAGTCCGAGGGAAGA
<i>cxcl11ah</i>	ENSDARG000000095747	Fw: GAGGTGAAAGGCAAAATA Rv: TGCTCCTTTATCAGCACACAAACA

Primers used for amplification and sequencing of *cxcr3.1*, *cxcr3.2* and *cxcr3.3*

- Amplification of genomic DNA templates

Gene Name		Sequence (5'-3')
<i>cxcr3.1</i>	Set 1	Fw: GTTGTAAACGACGGCCAGTATGAATGTTGACTCAAAAACAAC Rv: CAGGAAACAGCTATGACCTATTATCCAAAACATTCTTTCTCTAC
<i>cxcr3.1</i>	Set 2	Fw: GTAAACGACGGCCAGGGGAAACGGCTTGGTTCTGA Rv: CAGGAAACAGCTATGACTGTATGCATGAAACAGCAAAGAGAA
<i>cxcr3.1</i>	Set 3	Fw: GTAAACGACGGCCAGGATTGGAACAACCTTTTTCAGCA Rv: CAGGAAACAGCTATGACCTTGAAGCCAGAGGTCGTA
<i>cxcr3.1</i>	Set 4	Fw: GTTGTAAACGACGGCCAGTCTTCATTGTCATCTTCTTCATCAG Rv: CAGGAAACAGCTATGACCTCAGAAAGCAGATGTGTGGG
<i>cxcr3.2</i>		Fw: GTAAACGACGGCCAGGGCGCCTTATAATGCTCTGC Rv: CAGGAAACAGCTATGACCCATGGAAGCTTCAGTTTTTAC
<i>cxcr3.3</i>	Set 1	Fw: GTAAACGACGGCCAGTTCCAGCAGATGCGTTACA Rv: CAGGAAACAGCTATGACGTGCGCAAGCAAACGTAAC
<i>cxcr3.3</i>	Set 2	Fw: GTAAACGACGGCCAGGTTCCGCCAAGCTTTACTTT Rv: CAGGAAACAGCTATGACTAAACAGCGTCTTTACCAGATCCC
<i>cxcr3.3</i>	Set 3	Fw: GTAAACGACGGCCAGGACTGCTACCTGTCCATCGTT Rv: CAGGAAACAGCTATGACGCTCATGTCTAAATACAGTTTGCTG

- Amplification of cDNA templates

Gene Name	Sequence (5'-3')
<i>cxcr3.2</i>	Fw: GTTGTAAACGACGGCCAGTATGGACAACCTCAACAGCCGACG Rv: CAGGAAACAGCTATGACCTCAGGCCATGACAGAAAAGTACGAAGTG
<i>cxcr3.3 isoform2</i> Fw	Fw: GTTGTAAACGACGGCCAGTATGGCAGCACCTTCAAACATGG
<i>cxcr3.3 isoform1</i> Fw	Fw: GTTGTAAACGACGGCCAGTATGGAGGTAGAGCTTCACGG
<i>cxcr3.3</i> common Rv	Rv: CAGGAAACAGCTATGACCTCATGTATCCTGCTGCTGGG

- Primers used for sequencing

Gene Name	Sequence (5'-3')
M13 universal Fw	Fw: GTAAACGACGGCCAG
M13 universal Rv	Rv: CAGGAAACAGCTATGAC
<i>Cxcr3.2</i> E1 cDNA Rv	Rv: CGCCAGGATAAACACCAAG
<i>Cxcr3.3</i> cDNA Rv	Rv: GGCAGTCCAGTTCTTTGTTCT

Supplementary Table 3. List of qRT-PCR, amplification, and sequencing primers used in this work.

HYPERLINK TO SUPPLEMENTARY MOVIES

Supplementary Movies are available online at the following URL:

https://drive.google.com/open?id=0B_188Sfgn4xoUkRVNjNKdINhSEE

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