RESEARCH ARTICLE

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

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ABSTRACT

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 signaling 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 ortholog of CXCR3 (cxcr3.2), 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 signaling axis in zebrafish extends the translational applicability of this model for studying diseases involving the innate immune system.

KEY WORDS: Macrophage biology, Tuberculosis, Chemokine, CXCR3, CXCL11, Mycobacterium, Zebrafish, Immunology

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 response (Medzhitov and Janeway, 2000). Some of these PAMPs, such as N-formylated bacterial peptides, have direct chemoattractant activity on phagocytes (Schiffmann et al., 1975). Moreover, a crucial contribution to efficient phagocyte recruitment is provided by lipidic and pepticid chemoattractant factors, produced or activated directly by the host locally at the infection site (Ford-Hutchinson et al., 1980; Lira and Furtado, 2012; Sun and Ye, 2012). 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 lymphoid (Groom and Luster, 2011). In humans, CXCL9 [also known as MIG (monokine-induced by IFN-γ)], CXCL10 [IP-10 (IFN-γ-inducible protein 10)] and CXCL11 [I-TAC (T cell chemotactant)] 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 (Janatpour et al., 2001; Loetscher et al., 1996). This signaling axis has been implicated in several physiological activities, including maturation of T cells and vasculogenesis (Liu et al., 2005; Zhou et al., 2010). Additionally, CXCR3 and its ligands have been linked to inflammatory and immune-related diseases, of autoimmune (Bondar et al., 2014; Lacotte et al., 2009; Liu et al., 2005; Müller et al., 2010), infectious (Chakravarty et al., 2007; Cohen et al., 2013; Rosas et al., 2005; Seiler et al., 2003) or malignant (Fulton, 2009; Kawada et al., 2007; Oghumu et al., 2014; Pan et al., 2006) nature. Most of the literature on mammalian systems focuses on the role of this receptor in maturation, priming, activation and migration of T cells (Bondar et al., 2014; Liu et al., 2005; Slüter et al., 2013). However, recent studies have demonstrated that CXCR3 also plays an important role in directing macrophage activities, both under physiological and under pathological conditions (Cuenca et al., 2011; Kakuta et al., 2012; Oghumu et al., 2014; Zhou et al., 2010).

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 tools (Raz and Mahabaleshwar, 2009). 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 signaling axes in developmental processes, neutrophil motility, long-range neutrophil mobilization and infection-induced chemotaxis (David et al., 2002; Deng et al., 2013; Doitsidou et al., 2002; Sarris et al., 2012; Walters et al., 2010). Based on phylogeny reconstructions, the CXCR3-CXCL11 axis emerged for the first time in a common ancestor of zebrafish and mammals (Xu et al., 2014). 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 (Aghaallaei et al., 2010; Chang et al., 2007; Xu et al., 2014), including zebrafish, where three paralogs, cxcr3.1 (ENSDARG00000007358), cxcr3.2 (ENSDARG000000041041) and cxcr3.3 (ENSDARG000000070669), are located in tandem on chromosome 16 (Nomiyama et al., 2013). The CXCL9-CXCL10-CXCL11 triplet of CXCR3 ligands in mammals is likely to have originated from a relatively recent common ancestor (O’Donovan et al., 1999). 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 (Nomiyama et al., 2013). However, an association between the different isoforms of Cxcl11 ligands and Cxcr3 receptors has not been described, and the in vivo relevance of this signaling axis in the zebrafish model has not been addressed.

In previous work we have shown that one of the three CXCR3 paralogs, cxcr3.2, is expressed in macrophages of 1-day-old zebrafish embryos (Zakrzeswka et al., 2010). In the present study we used a cxcr3.2 mutant to investigate the role of Cxcr3 signaling in macrophage mobilization 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 cxcr3.3 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 (Cambier et al., 2014; Clay et al., 2007; Davis et al., 2002; Roca and Ramakrishnan, 2013; Torraca et al., 2014; van der Vaart et al., 2014). 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 (Cronan and Tobin, 2014; Ramakrishnan, 2013). The results presented here demonstrate a novel function for the CXCR3-CXCL11 signaling 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 hybridization at 1 day post fertilization (dpf) in phagocytes expressing the macrophage marker csf1r (colony stimulating factor 1 receptor) and not in cells positive for the neutrophil marker mpx (myeloperoxidase) (Zakrzeswka et al., 2010). 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 analyzed RNA expression levels from FACS-sorted mpeg1:mcherryF<sup>+</sup> and mpx:eGFP<sup>+</sup> cells from the double-transgenic line Tg(mpeg1:mcherryF/mpx:eGFP) (Zakrzeswka et al., 2010). These data show that macrophages (mpeg1:mcherryF<sup>+</sup> population) maintain cxcr3.2 expression at 2 and at 6 dpf (Fig. 1A-C and supplementary material Fig. S1). Expression of cxcr3.2 could also be detected in neutrophils (mpx:eGFP<sup>+</sup> population). In addition, cxcr3.3 could be detected in both phagocyte types, whereas cxcr3.1 was not specifically enriched in the sorted cell populations (Fig. 1C and supplementary material Fig. S1).

The *cxcr3.2<sup>mut</sup>* line carries a nonsense mutation in *cxcr3.2*

Sequencing of an ENU (N-ethyl-N-nitrosourea)-mutagenized zebrafish library resulted in the identification of a cxcr3.2 mutant allele, *cxcr3.2<sup>mut</sup>* (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 (Fig. 1D and supplementary material Fig. S2). The nonsense *cxcr3.2<sup>mut</sup>* 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 wild types (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 material Table S1. 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 Analyzer; http://provean.jcvi.org) (Kumar et al., 2009; Choi et al., 2012). None of the nsSNPs was predicted to impact on the protein functionality.
cxcr3.2 embryos showed similar numbers of macrophages and neutrophils as their wt siblings (Fig. 1F,G). With the aim of investigating the relevance of cxcr3.2 expression in macrophage behavior, we crossed the cxcr3.2 mutation into the Tg(mpeg1::gal4/UAS:kaede) background. Labeled macrophages showed similar numbers and spatial distribution in mutant and wt (Fig. 1H,I). However, a basal macrophage migratory deficiency was observed in the mutants (Fig. 1J-L and supplementary material 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 fertilization). At this developmental stage, the neutrophil population is not fully differentiated (Henry et al., 2013; Lieschke et al., 2001) and the population of phagocytes infiltrating the hindbrain upon chemotactic stimulation consists predominantly of macrophages (supplementary material Fig. S3); 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 (Fig. 2A-H). In addition, we also employed a previously described chemically induced inflammation (ChIn) assay (d’Alençon et al., 2010), 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 (Fig. 2I). Therefore, we concluded that cxcr3.2 mutation does not affect the capability of macrophages to respond to stimulatory sources independent of Cxcr3.2 signaling and that the basal motility defect does not influence the experimentally induced macrophage recruitment.

Early migration of macrophages to localized infection is affected by mutation of cxcr3.2 or treatment with a CXCR3 antagonist
To determine whether Cxcr3.2 signaling 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) (Fig. 3A). A similar reduction of macrophage recruitment was also observed when M. marinum was locally injected into the otic vesicle at 3 dpf (Fig. 3B). To visualize 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 (Fig. 3C-H; supplementary material 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 (Fig. 3E) when compared with the mutant line (Fig. 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). 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 (Fig. 3I-L). These results provide evidence that the Cxcr3.2-dependent signaling 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, NBI74330 (Scholten et al., 2014), 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 material Fig. S4). 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 (Fig. 3M). These results support the conservation of CXCR3 signaling between fish and mammals.
Fig. 3. See next page for legend.
orthologous relationships between vertebrate chemokines indicated that seven CXCL11-like chemokine genes, located on tandem in chromosome 5 (cxl11aa, cxl11ae, cxl11ad, cxl11ae, cxl11af, cxl11ag, cxl11ah), have evolved in zebrafish as a counterpart to the mammalian CXCL9, CXCL10 and CXCL11 genes (Nomiyama et al., 2013). 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 material Fig. S5; Table S2). 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 cxll1 gene cluster. Because of the high level of sequence conservation between cxll1af and cxll1ag (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 (cxll1af/ag). Analysis by qRT-PCR revealed that, at 1 hpi, cxll1af/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 (Fig. 4A). At 3 hpi the expression of cxll1aa, cxll1ae and cxll1af/ag was significantly upregulated to levels of two- to 4.5-fold (Fig. 4B). In particular, cxll1aa displayed the highest levels of induced transcription (~4.5-fold induction) with both the pathogens tested, suggesting this chemokine as an effective signaling 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 cxll1aa and cxll1ae was detected both at 4 hpi and at 4 dpi (days post injection) during M. marinum systemic infection, whereas cxll1af/ag was significantly induced to levels of ~fourfold only at the later stage of infection (Fig. 4C,D). Additionally, at this time point, cxll1ae and cxll1ad were also significantly upregulated to levels of 1.5- to two-fold (Fig. 4D).

Recombinant Cxll1aa and Cxll1af exert macrophage chemoattraction in vivo in a Cxcr3.2-dependent manner
To assess the chemoattractant properties of the infection-inducible chemokines Cxll1aa, Cxll1af and Cxll1ae, we used Pichia pastoris strain X-33 to express recombinant proteins. As a control, we also expressed zebrafish Cxll8a (I18), known to be a potent and neutrophil-specific chemoattractant (Deng et al., 2013; Sarris et al., 2012). All three purified CXCL11-like chemokines showed chemoattractant capabilities towards macrophages when locally injected in vivo into the hindbrain at 30 hpi (Fig. 5), whereas no significant macrophage recruitment was exerted by Cxll8a (supplementary material Fig. S6). Similar levels of these chemokines were injected in the otic vesicle at 54 hpf to evaluate their chemoattractant capabilities towards neutrophils (supplementary material Fig. S6B,C). Cxll1aa and Cxll1af did not show chemoattraction of neutrophils under these conditions, whereas Cxll1ae and Cxll8a exerted significant neutrophil chemoattraction. To determine whether the macrophage chemoattraction is dependent on ccr3.2, hindbrain injections of the recombinant proteins were performed in both wt and ccr3.2 mutants. Both Cxll1aa and Cxll1af did not stimulate
recruitment upon local injection in cxcr3.2 mutants when compared with their mock controls (Fig. 5A,C). In contrast, the chemoattraction of phagocytes mediated by Cxcl11ae was independent of cxcr3.2 mutation (Fig. 5B; supplementary material Fig. S6C). 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-unidentified receptor.

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 distant dissemination of the infection (Clay et al., 2007). We hypothesized 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 (Fig. 6A-F). 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 (Fig. 6G-I). Disseminated bacteria outside the hindbrain and/midbrain were residing in phagocytes and, in time course experiments, we could visualize that egression of mycobacteria from the ventricles is facilitated by macrophages (Fig. 6J), in agreement with previously published results (Clay et al., 2007). 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 showed lower levels of dissemination of the infection towards distal areas (Fig. 7A,B). The infection foci generated distally developed into typical granuloma-like aggregates, as previously described for the zebrafish-M. marinum model (Davis et al., 2002). The size of these granulomatous lesions was significantly reduced in the cxcr3.2−/− mutant larvae (Fig. 7C-F). Therefore, we concluded that cxcr3.2-mediated signaling strongly influences the dynamics of the infection progression and of granuloma formation. To further investigate the relevance of cxcr3.2 signaling 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 lesions (Clay et al., 2007). 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 (Fig. 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 wild types, and this makes it very difficult to assess the effect of a mutation on the general architecture of the granulomas. Despite this, we observed that Cxcr3.2 deficiency provided partial protection against mycobacterial infection dissemination and granuloma formation.
in the pathogenesis of infectious diseases, autoimmune disorders and the chemokine receptor CXCR3 and its ligands play important roles in infection progression. We considered a cluster of CXCL11-like chemokines as the putative ligands of CXCR3. It is currently unknown whether the Cxcr3.2 receptor, one of the three zebrafish homologs of human CXCR3, interacts with infection-inducible zebrafish homologs of the CXCL11 ligand family and is required for the mobilization of macrophages to different pathogens, such as locally delivered M. marinum or S. typhimurium. Furthermore, mutation of cxc3r.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 signaling in processes as diverse as the recruitment of macrophages to allografts (Kakuta et al., 2012), the macrophage-mediated remodeling of blood vessels (Zhou et al., 2010) and the polarization of macrophages towards an M2 phenotype that promotes tumor progression (Oghumu et al., 2014). Furthermore, CXCR3 signaling has been shown to play a crucial role in the murine neonatal response to sepsis (Cuenca et al., 2011). Like murine neonates, zebrafish embryos and early larvae rely heavily on their innate immune system for defense against infection. During zebrafish embryogenesis, macrophages are the first leukocyte cell type to develop and they express cxc3r.2 from day 1 (Zakrzewska et al., 2010). In mutants of cxc3r.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 signaling 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 and confirmed that two of these, Cxcl11aa and Cxcl11af, are expressed in macrophages. These studies have implicated CXCR3 ligands 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 signaling 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 cxc3r.2 genes in zebrafish are IFN-γ-inducible like their mammalian counterparts, but IFN-γ responsive elements are present in the promoters of these genes (van der Aa et al., 2012). In addition, because we were unable to detect expression of the zebrafish cxc3l1 genes in situ, the cell types producing these chemokines remain to be established. However, qRT-PCR showed rapid upregulation of cxc3l1aa and cxc3l1af/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 cxc3r.3 is expressed in macrophages, but macrophage motility and recruitment defects in the cxc3r.2 mutant line indicate that expression of cxc3r.3 cannot compensate for the loss of function of cxc3r.2. In addition, the expression analysis revealed that also neutrophils express cxc3r.3 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, against mycobacterial infection. Not only did mutants exhibit reduced levels of infection burden (Fig. 8C-E), but also a reduced number of bacterial clusters (Fig. 8F) and smaller average bacterial cluster size (Fig. 8G). Taken together with the results of hindbrain infection, these data demonstrate the important role of Cxcr3.2-dependent signaling in guiding macrophage-mycobacteria interactions, and show how this signaling leads to direct effects on the infection progression.

**DISCUSSION**

The chemokine receptor CXCR3 and its ligands play important roles in the pathogenesis of infectious diseases, autoimmune disorders and cancer (Bondar et al., 2014; Chakravarty et al., 2007; Cohen et al., 2013; Cuenca et al., 2011; Kakuta et al., 2012; Kawada et al., 2007; Oghumu et al., 2014; Pan et al., 2006; Rosas et al., 2005; Seiler et al., 2003; Slütter et al., 2013). In this study, we report on the function of CXCR3 signaling 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 homologs of human CXCR3, interacts with infection-inducible zebrafish homologs of the CXCL11 ligand family and is required for the mobilization of macrophages to different pathogens, such as locally delivered M. marinum or S. typhimurium. Furthermore, mutation of cxc3r.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.
and comparable concentrations of Cxcl8a and Cxcl11ae mobilized 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

Fig. 6. See next page for legend.
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 progenitors (Zakrzewska et al., 2010). 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* (Clay et al., 2007; Davis et al., 2002). 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 localized 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 macrophages (Davis and Ramakrishnan, 2009). 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 (Volkman et al., 2010; Davis and Ramakrishnan, 2009).

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* (Chakravarty et al., 2007). 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 signaling via CXCR3 (Seiler et al., 2003). Together, the studies in mice and zebrafish models support further investigation of the CXCR3 signaling axis as a host therapeutic target for tuberculosis. Our study is the first to implicate this signaling 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 signaling 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 embryos (Cambier et al., 2014). 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 dimycoceroserate 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 abrogate it. These observations indicate that redundant and/or synergistic mechanisms are cooperating in macrophage mobilization. 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 hypothesize 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 signaling have already been described in the literature. Keratinocytes have been shown to express CXCL10 and CXCR3 to guide their own migration for re-epithelialization in a wound-healing response (Kroeze et al., 2012). Possibly, the macrophages themselves could be involved in an autocrine or paracrine secretion of these ligands. Similar mechanisms acting via CXCR3 signaling have already been described in the literature. Keratinocytes have been shown to express CXCL10 and CXCR3 to guide their own migration for re-epithelialization in a wound-healing response (Kroeze et al., 2012). Similarly, synovial fibroblasts use this ligand-receptor pair to regulate their invasion of joints in rheumatoid arthritis (Laragione et al., 2011). Furthermore, myeloid cells and hematopoietic progenitors secrete many different chemokines, including CXCR3 ligands, to regulate hematopoiesis in an autocrine or paracrine manner (Majka et al., 2001). 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 (Chensue, 2013; Fuller et al., 2003). 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 neighborhood of the granulomatous lesions (Aly et al., 2007; Fuller et al., 2003; Khader et al., 2009). 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 (O’Boyle et al., 2012; van Wanrooij et al., 2008), and the zebrafish model might be a suitable model to test their effectiveness on mycobacterial infections.

**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: wild-type (wt) strain AB/TL, double-transgenic line Tg(mpeg1:mcherryF/mpx:eGFP) (Bernut et al., 2014; Renshaw et al., 2006), homozygous mutant (cxcr3.2<sup>−/−</sup>) and wt siblings (cxcr3.2<sup>+/+</sup>) of cxcr3.2<sup>hu6044</sup>, Tg(mpeg1:gal4-VP16/UAS-
E1b:kaede, in short referred to as Tg(mpeg1:gal4/UAS:kaede) (Ellett et al., 2011), and the combination of Tg(mpeg1:gal4/UAS:kaede) with the cxcr3.2 mutant strain. The cxcr3.2hu6044 allele was identified by sequencing of an ENU (N-ethyl-N-nitrosourea)-mutagenized zebrafish library and was obtained from the Hubrecht Laboratory and the Sanger Institute Zebrafish Mutation Resource. Heterozygous F2 carriers were outcrossed twice against wild-type (wt) and were subsequently inbred. 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/cxcr3.2hu6044) was obtained by crossing heterozygous carriers with the original transgenic line and subsequently inbreeding the heterozygous offspring. For genotyping, genomic DNA was amplified using forward primer 5′-GGCATCTTTTTGGACACCTACAGCTA-3′ and reverse primer 5′-TGCCGATACGGCGGATAAAGCA-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

Fig. 8. Effect of cxcr3.2 mutation on granuloma formation following systemic mycobacterial infection. (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 wild-type (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 μm. Scale bar in C,D: 200 μm. (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.
generates the consensus for DdeI restriction enzyme. Therefore, the mutant allele was distinguished from the wt by specific 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′-CATCATAGGAAATCTGGATGACA-3′, 5′-CATCATAGGAAATCTGGATGACA-3′ and 5′-GGCATCTTTCGTTTATCAGATC-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 anesthetized in egg water medium containing 0.02% buffered Tricaine (3-aminobenzoic acid ethyl ester; Sigma-Aldrich, St Louis, MO, USA). To prevent melanization, 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 cxcr3.1, cxcr3.2 and cxcr3.3 was obtained by amplification with primers described in supplementary material Table S3. For cxcr3.2 and cxcr3.3, 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, Waltham, MA, 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 cxcr3.1, sequencing results derive exclusively from genomic DNA amplifications. Amplification of cDNA templates for cxcr3.2 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 cxcr3.2 mutant allele.

Bacterial cultures and infection delivery

Approximately 200 CFU (1 nl) of M. marinum strain Mma20 expressing mCherry (van der Sar et al., 2004), or Salmo nella enterica serovar Typhimurium (S. typhimurium) strain SL1027 expressing DsRed (van der Sar et al., 2003), were grown and harvested as described previously (Benard et al., 2012; Cui et al., 2011). 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 (Benard et al., 2012; Cui et al., 2011). Injections of bacteria in the otic vesicle, as shown in Fig. 3, were performed either at 3 dpf (Fig. 3B-L-J) or at 4 dpf (Fig. 3C-H). When infection was delivered systematically, the same dose was instead injected in the caudal vein as in Benard et al. (2012), 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 Fig. 8A,B, embryos were fixed at 6 dpf in 4% paraformaldehyde in PBS (1× PBS supplemented with 0.8% Triton X-100; Sigma-Aldrich) and prepared for Myeloperoxidase (Mpx) activity staining with TSA staining kit (PerkinElmer Inc., Waltham, MA, USA), followed by immunostaining against the pan-leucocyte marker Leucocyte-Plastin (Lp) as described previously (Cui et al., 2011).

FACS-sorting, RNA isolation and qRT-PCR

Mpeg1::mcherryF-positive, mpx::eGFP-positive and unlabeled cells were sorted from Tg(mpeg1::mcherryF/mpx::eGFP) at 2 and 6 dpf. FACS-protocol and RNA isolation were performed according to Rougeot et al. (Rougeot et al., 2014). To evaluate the induction of the cxcl11 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 analyzed using dissociation curves. Each qRT-PCR was performed in technical duplicate and on biological replicates as indicated in the figure legends. Reference genes were eif4a1b or eif5 (eukaryotic translation initiation factor 4a isoform 1b or S) for FACS-sorted cells and ppiab (peptidylprolyl isomerase ab/cyclolin a) for infection experiments. Fold changes were determined using the ΔΔ comparative threshold method. Primers are reported in supplementary material Table S3.

Production of recombinant chemokines and local injections

Synthetic coding sequences for Cxcl11aa, Cxcl11af, Cxcl11ae and Cxclg1a (included as negative control) were generated (Baseclear) according to database accessions (supplementary material Table S2). To enable secretion in yeast, the sequences were codon optimized and the predicted zebrafen signal peptide was replaced with yeast alpha-factor secretion signal, as a result from cloning into pPIC9A expression vector (Invitrogen, Life Technologies). Additionally, a HA (human influenza hemagglutinin)-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 previously (Wu and Letchworth, 2004). 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 suspending vehicle. Purity and identity of the proteins were confirmed by trypsinization and electrospray mass-spectrometry. The recombinant chemokines (0.5-1.5 mg/μl), LTβ (leukotriene B4; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 10.1 ng/ml), IMLP (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 IMLP and 0.02% ethanol (Sigma-Aldrich) in PBS for LTβ] 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 (Benard et al., 2012; Cui et al., 2011). In both cases, embryos were fixed at 3 hpi in 4% paraformaldehyde in PBSx and prepared for Lp immunostaining as in Cui et al. (2011). At 30 hpf, the population of fully differentiated leukocytes is represented almost exclusively by macrophages (Henry et al., 2013; Lieschke et al., 2001); therefore, 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 material Fig. S3, only one to two mpx-positive cells [mpx-whole mount in situ hybridization as in reference (Cui et al., 2011)] 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 mfp4. 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 previously (Cui et al., 2011), 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 (Chln) inflammation assay

3-dpf larvae were exposed to 10 μM copper sulphate (CuSO4; Sigma-Aldrich) for 2 hours as described previously (d’Alençon et al., 2010). 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 μM 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 μM NB174330 or vehicle alone and then fixed in 4% paraformaldehyde/PBSTx and prepared for Lp immunostaining as described previously (Cui et al., 2011).

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 (SpheroQ, Burgos, Spain) and images were acquired with a laser-scanning confocal microscope (Leica TCS SPE, Leica Microsystems or Zeiss Observer D.5.32, Carl Zeiss, Sliedrecht, The Netherlands). To assess the average speed of macrophages (Fig. 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 elsewhere (Meijering et al., 2012). The average speed was calculated as the average of the 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 cxcr3.2 mutants and wt, bacteria were injected into the otic vesicle of Tg(mpeg1:gal4/UAS:kaede/cxcr3.2−/− or cxcr3.2−/−) 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 Analyze Particles plug-in. The circularity index (CI) corresponding to each cell was obtained by the formula: CI=4π(area/perimeter²), 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 cxcr3.2−/− or cxcr3.2−/− (Fig. 3I). To estimate the divergence of distribution of cxcr3.2−/− and cxcr3.2−/− 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: Deviation(cxcr3.2−/−)=(%cxcr3.2−/−−%cxcr3.2−/−)/2 and Deviation(cxcr3.2−/−)=(%cxcr3.2−/−−%cxcr3.2−/−)/2 (Fig. 3I). To quantify the dissemination of bacterial infection (Fig. 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 (Fig. 7A,B and 8G) was performed manually from images. The area of single distal clusters (Fig. 7C) and average area of disseminated granulomas (Fig. 8G) were performed using ImageJ quantification tools as in Elks et al. (2013).

Statistical analysis
In the survival test (Fig. 1E), non-significant deviation from Mendelian rate was evaluated by χ² test on four independent replicates. For qRT-PCR, statistical significance was estimated on five (Fig. 1A-C), four (Fig. 4A,B) or three (Fig. 4C,D) biological replicates by two-tailed t-tests on ln(n) transformed relative induction folds. All the other experiments were statistically analyzed using GraphPad Prism 4 or 5 (GraphPad Software, La Jolla, CA, USA). Where correction for non-parametric distribution was required (Fig. 1F,G; Fig. 2; Fig. 3A,B; Fig. 3M; Fig. 5; Fig. 6; Fig. 7A,B; Fig. 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 (Fig. 1A-C; Fig. 1L; Fig. 3J; Fig. 4; Fig. 8G), comparisons between two groups were performed with two-tailed t-test. In Fig. 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.

Acknowledgements
We thank Ewa Snaar and Claudia Tulotta for valuable discussions. We are grateful to Fons Verbeek and Alex Nezhinsky (Leiden Institute of Advanced Computer Science) for making their pixel quantification software available prior to publication and to Erica Benard for RNA samples of M. marinum systemically infected embryos. For providing the zebrafish knockout allele cxcr3.2−/−, we thank the Hubrecht Laboratory and the Sanger Institute Zebrafish Mutation Resource.

Competing interests
The authors declare no competing or financial interests.

Author contributions
V.T., C.C. and A.H.M. conceived and designed the experiments. V.T., C.C., R.B. and J.-P.B. performed the experiments. V.T. and C.C. analyzed the data. A.M.v.d.S., M.S., M.J.S. and H.P.S. provided tools and reagents and gave valuable suggestions. V.T. and A.H.M. wrote the manuscript and the final version was approved by all authors.

Funding
This work was supported by the Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Ministry of Education, Culture and Science, the European Commission 7th framework project ZF-HEALTH (contract number HEALTH-F4-2010-242048), and the European Marie-Curie Initial Training Network FisTPharma (contract number PITN-GA-2011-288290). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Supplementary material
Supplementary material available online at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.017756/-/DC1

References


Figure S1: Expression of cxcr3.2 and its paralogs 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 paralogs (C) in FACS-sorted macrophages and neutrophils from the combined transgenic line Tg(mpeg1:mcherryF/mpx:eGFP) at 6 dpf. Expression of cxcr3.2 and cxcr3.3 could be detected in both macrophages and neutrophils, while cxcr3.3 was not significantly enriched in the FACS-sorted populations when compared to the non-labeled cell fraction. Reference gene: eif5.

Figure S2: Protein sequence multiple alignment of human and zebrafish CXCR3 chemokine receptors. Residue color 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 Trotta et al., 2009 (Trotta et al., 2009). 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 (ENSDARG0000007358): 39.1%, dreCXCR3.2 (ENSDARG00000041041): 35.7%, dreCXCR3.3 (ENSDARG00000070669) isoform 1: 29.8% and dreCXCR3.3 isoform 2: 29.6%.
Figure S3: 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* hybridization was performed with *mfap4* as a macrophage marker and *mpx* as a neutrophil marker (Zakrzewska et al., 2010). 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.

Figure S4: 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. 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 colored lines. Polar and hydrogen bonding interactions are shown as pink lines, whereas aromatic interactions are shown as gray lines. C: overlay of the CXCR3 and Cxcr3.2 binding pocket. Prediction of ligand binding for CXCR3 is according to ref. (Scholten et al., 2014). 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.
Figure S5: 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 (II8). Nomenclature according to ref. (Nomiyama et al., 2013).
Figure S6: 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 wild type (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 wild type (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 (II8) (B) was in agreement with previous reports (Deng et al., 2013). 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<0.01; ***P<0.001.
Movie S1. Macrophage basal motility in cxcr3.2+/+ embryos. Paths of 5 representative macrophages of the trunk. Macrophages were followed contemporary for 3 hours and confocal time lapse images were taken every 6 minutes. The paths were followed and analyzed using Image J ManualTrack plugin. cxcr3.2-/- macrophages (compare with supplementary material Movie S2) have a lower basal movement capability than cxcr3.2+/+ macrophages. See also Fig. 1J-L. Scale bar depicted in the first photogram: 20 μm.

Movie S2. Macrophage basal motility in cxcr3.2-/- embryos. Paths of 5 representative macrophages of the trunk. Macrophages were followed contemporary for 3 hours and confocal time lapse images were taken every 6 minutes. The paths were followed and analyzed using Image J ManualTrack plugin. cxcr3.2-/- macrophages have a lower basal movement capability than cxcr3.2+/+ macrophages (compare with supplementary material Movie S1). See also Fig. 1J-L. Scale bar depicted in the first photogram: 20 μm.

Movie S3. Macrophage recruitment following M. marinum infection in the otic vesicle in cxcr3.2 +/- larvae. A Tg(mpeg1:gal4/UAS:kaede) cxcr3.2-/- larva was injected with 200 CFU of M. marinum into the otic vesicle (dotted line) at 4 dpf. The video represents the situation monitored from 1 to 5 hpi, with acquisition every 5 minutes. See also Fig 3. It should be noted that since acquisitions start at 1 hpi, a difference in macrophage number in the surrounding of the otic vesicle is already present between mutants and wt. Scale bar depicted in the first photogram: 250 μm.
Movie S4. Macrophage recruitment following \textit{M. marinum} infection in the otic vesicle in \textit{cxcr3.2}^- larvae. A \textit{Tg(mpeg1:gal4/UAS:kaede) cxcr3.2}^- larva was injected with 200 CFU of \textit{M. marinum} in the otic vesicle (dotted line) at 4 dpf. The video represents the situation monitored from 1 to 5 hpi, with acquisition every 5 minutes. See also Fig 3. It should be noted that since acquisitions start at 1 hpi, a difference in macrophage number in the surrounding of the otic vesicle is already present between mutants and wt. Scale bar depicted in the first photogram: 250 μm
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*Reference: according to ENSEMBL accessions

Table S1: Non-synonymous single nucleotide polymorphisms in Cxcr3.1, Cxcr3.2, and Cxcr3.3 coding sequences. 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-mutagenized Cxcr3.3<sup>4hu6044</sup> allele. Except for the Cxcr3.1 E292K variant, all the nsSNPs linked to the Cxcr3.2<sup>**</sup> fish did not differ from the ones represented also in the Cxcr3.2<sup>**</sup> 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 Analyzer, http://provean.jcvi.org) (Kumar et al., 2009; Choi et al., 2012), 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 -4.1 (Choi et al., 2012). 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.
Table S2. List of chemokines and chemokine receptors used in this work with accession codes and protein sequences. Red letters: amino acids at the splicing sites of the mRNA; blue letters: conserved CXC-chemokine motifs. Part in gray: active chemokine upon removal of the signal peptide.
### Primers used in qRT-PCR reactions

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| cxcr3.1  | ENSDARG00000007358 | Fw: CTTCCTGCATCACTCGTCGACC  
Rv: TGACGTTCGGAGCTCTTGGGG |
| cxcr3.2  | ENSDARG000000041041 | Fw: CTCCTGGTGTAAGCTGTAGGAG  
Rv: ACACGATGACTAGAGATGAGG |
| cxcr3.3  | ENSDARG00000070669 | Fw: GCTCTCAATGGATTCTCTGTCCT  
Rv: GCAGGATGACTAGAGATGAGG |
| ppiab    | ENSDARG00000042247 | Fw: ACACGACACAGGAGGAAGAAG  
Rv: CATCCCAACACCTCTCCGAAAC |
| cxcl11aa | Gene ID: 798892 | Fw: ACTCAACATGGAGCAGCAGTGT  
Rv: TGCCCTGCTGAGCAAGAAGCC |
| cxcl11ac | Chr5: 44501563-44502267 (-) | Fw: GTGACTGACTAGAGATGAGGAAG  
Rv: AGCGGATGACTAGAGATGAGG |
| cxcl11ad | Gene ID: 567656 | Fw: ACACGACACAGGAGGAAGAAG  
Rv: CATCCCAACACCTCTCCGAAAC |
| cxcl11ae | ENSDARG00000092423 | Fw: AGCGGATGACTAGAGATGAGGAAG  
Rv: AGCGGATGACTAGAGATGAGG |
| cxcl11af/ag | ENSDARG00000092423 | Fw: ACACGACACAGGAGGAAGAAG  
Rv: CATCCCAACACCTCTCCGAAAC |
| cxcl11ah | ENSDARG00000097547 | Fw: AGCGGATGACTAGAGATGAGGAAG  
Rv: AGCGGATGACTAGAGATGAGG |

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

#### Amplification of genomic DNA templates

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| cxcr3.1  | Set 1 Fw: GTTGTAAACGAGCAGCAGCAGTATGAAAGCTTCAAAACAAAC  
Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |
| cxcr3.1  | Set 2 Fw: GTTGTAAACGAGCAGCAGCAGTATGAAAGCTTCAAAACAAAC  
Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |
| cxcr3.1  | Set 3 Fw: GTTGTAAACGAGCAGCAGCAGTATGAAAGCTTCAAAACAAAC  
Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |
| cxcr3.1  | Set 4 Fw: GTTGTAAACGAGCAGCAGCAGTATGAAAGCTTCAAAACAAAC  
Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |
| cxcr3.2  | Set 1 Fw: GTGTTAAACGAGCAGCAGCAGTATGAAAGCTTCAAAACAAAC  
Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |
| cxcr3.3  | Set 1 Fw: GTGTTAAACGAGCAGCAGCAGTATGAAAGCTTCAAAACAAAC  
Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |
| cxcr3.3  | Set 2 Fw: GTGTTAAACGAGCAGCAGCAGTATGAAAGCTTCAAAACAAAC  
Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |
| cxcr3.3  | Set 3 Fw: GTGTTAAACGAGCAGCAGCAGTATGAAAGCTTCAAAACAAAC  
Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |

#### Amplification of cDNA templates

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Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |
| cxcr3.3 isoform2 Fw | Fw: GTTGTAAACGAGCAGCAGCAGTATGAAAGCTTCAAAACAAAC  
Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |
| cxcr3.3 isoform1 Fw | Fw: GTTGTAAACGAGCAGCAGCAGTATGAAAGCTTCAAAACAAAC  
Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |
| cxcr3.3 common Rv | Fw: GTTGTAAACGAGCAGCAGCAGTATGAAAGCTTCAAAACAAAC  
Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |

### Primers used for sequencing

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Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |
| M13 universal Rv | Fw: GTTGTAAACGAGCAGCAGCAGTATGAAAGCTTCAAAACAAAC  
Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |
| Cxcr3.2 E1 cDNA Rv | Fw: GTTGTAAACGAGCAGCAGCAGTATGAAAGCTTCAAAACAAAC  
Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |
| Cxcr3.3 cDNA Rv | Fw: GTTGTAAACGAGCAGCAGCAGTATGAAAGCTTCAAAACAAAC  
Rv: CAGGAAAACAGCTATGACCTATTACAAACATTCCTCTAC |

Table S3. List of q-RTPCR, amplification, and sequencing primers used in this work.

### References

Please see the main article for references not listed below: