Exploitation of host chemokine signalling by pathogenic mycobacteria

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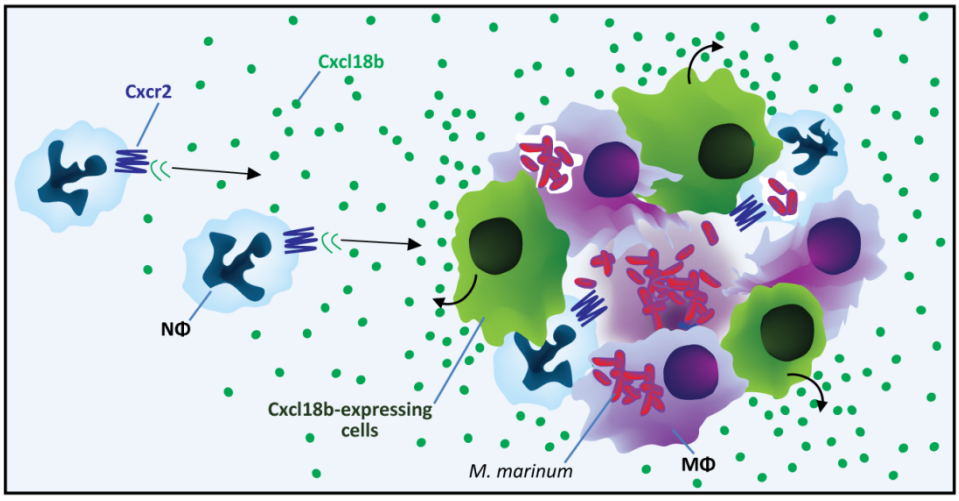
The zebrafish (*Danio rerio*) model has proven successful in many infection/inflammation-related studies. To ensure the translational impact of the zebrafish model in this field, it is important to clarify the homologies between the zebrafish and the human immune systems. Cxcl18b is a chemokine found in zebrafish and conserved in several piscine and amphibian species, although this lineage has not been maintained in the divergence of amniotes. The function of Cxcl18b is yet to be elucidated. However, this ligand represents a valid inflammatory marker in fish, found recurrently upregulated during infection/inflammation. In this study, we produced recombinant zebrafish Cxcl18b and, by local injections *in vivo,* we found that this ligand exhibits specific chemotactic properties towards neutrophils, comparable to those exerted by Cxcl8a, also known as Il8, the best characterised neutrophil chemotactic factor in both humans and teleosts. By pharmacological manipulations, we found that, like Cxcl8a, Cxcl18b requires the chemokine receptor Cxcr2 for optimal chemotaxis, although Cxcl18b, differently from Cxcl8a, could still mediate residual recruitment of neutrophils under conditions of Cxcr2 inhibition. However, the remaining recruitment of Cxcl18b did not rely on expression of *cxcr1* or *cxcr4b,* two other known neutrophil receptors. To visualise the *cxcl18b* expression pattern we generated a *Tg(cxcl18b:eGFP)* reporter line and found that this transgene is induced upon bacterial infection with *Mycobacterium marinum*, in agreement with previous mRNA expression data. The *cxcl18b* reporter expression coincides with the areas of bacterial growth but seems not directly induced in infected cells. Instead, it is predominantly expressed by non-infected cells participating in the initial formation of inflammatory granulomatous lesions that are critical to the pathogenesis of mycobacterial infection. Together, these results suggest that Cxcl18b could be an important contributor to neutrophil chemotaxis in the granuloma microenvironment in the zebrafish model and emphasise the need for a better understanding of how this inflammatory chemokine relates to the better-characterised class of Cxcl8 neutrophil chemotactic factors.

GRAPHICAL ABSTRACT



The chemokine Cxcl18b is able to specifically promote neutrophil chemotaxis. This is at least partly evoked by signalling via the neutrophil chemokine receptor CXCR2. During infection with *M. marinum*, several cells that participate in the formation of the granulomas produce the chemokine ligand Cxcl18b. Generally, these cells are not intracellularly infected and do not express the neutrophil and macorphage markers *lyz* and *mpeg1,* suggesting that they do not represent phagocytic cells, but stromal cells that contribute to the granuloma structure.

INTRODUCTION

The constant interplay between microbes and their hosts is the driving force for the evolution of pathogens and of the host immune system. Complex systems of pathogen virulence and corresponding control mechanisms in their hosts have originated from their co-evolution. Studying infectious diseases in the context of a whole living organism provides the possibility to better understand these mechanisms and to translate them into novel therapeutic strategies. Both the innate and the adaptive immune systems are well conserved among vertebrates and the zebrafish (*Danio rerio*) is an excellent model organism to study the biology of infectious and inflammatory diseases, especially because of the optical accessibility of its early embryonic and larval stages[1,2]. The transparency of the zebrafish allows direct visualisation of cellular migration processes in response to chemotactic cues and infections. To facilitate *in vivo* analysis, many fluorescent reporter lines have been developed that label different immune cell types, including macrophages and neutrophils[3,4]. Additionally, the use of the zebrafish has been extended by combining the optical properties of this model with pharmacological and genetic tools that well apply to this species, due to high permeability to compounds by submersion and to the ease of application of gene editing techniques[5,6].

Chemokines are a family of small cell-signalling proteins that direct the migration of cells expressing the corresponding receptors towards a ligand concentration gradient. The CXC subgroup of chemokines (containing the Cysteine-X-Cysteine motif) is well conserved between mammalian and teleost species. In mammals, this family can be further divided into ELR+ and ELR− chemokines, based on the presence or absence of the Glutamic acid-Leucine-Arginine motif preceding the CXC sequence. ELR+ chemokines include CXCL1-2-3 (GROα-β-γ), CXCL5 (ENA78), CXCL6 (GCP2), CXCL7 (NAP2) and CXCL8 (IL8). These are potent chemoattractants of neutrophils[7,8,9,10,11,12], while ELR− chemokines, such as CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC), are best known for the attraction of lymphocytes and monocyte/macrophages and possess poor chemotactic ability towards neutrophils. In fish, the ELR sequence is not conserved in chemokines that exert neutrophil chemotactic properties[13]. However, it has been demonstrated that the zebrafish Cxcl8/Il8 (Interleukin 8) paralogues, despite being ELR-deficient, are still potent attractors of neutrophils[5,14,15].

CXCL8 is the best-studied neutrophil chemoattractant in humans[11,12] and is conserved in zebrafish[5,14,15], while absent in rodents[16,17]. In both zebrafish and mammals, Cxcl8/CXCL8 signals via the receptor Cxcr2/CXCR2[5,12,14]. In mice this receptor still exerts a chemotactic activity towards neutrophils, but this is evoked by binding to other ELR+ chemokines CXCL1-2-3-5-7[12], to the non-chemokine tripeptide PGP (Pro-Gly-Pro, a molecule that derives from extracellular matrix breakdown)[18] and to MIF (macrophage migration inhibitory factor, a non-chemokine component which inhibits macrophage migration but sustains neutrophil recruitment)[19]. The CXCR2 receptor is promiscuous in humans too and can respond to CXCL1-2-3-5-6-7-8[7,8,12], PGP[18,20] and MIF[19]. Finally, in humans, the activity of CXCL8 is non-specific for CXCR2, since this ligand, together with CXCL6-7, can also induce neutrophil chemotaxis via CXCR1, another chemokine receptor, closely related to CXCR2[21]. We currently know that two zebrafish Cxcl8 chemokines, Cxcl8a and Cxcl8bb, require the chemokine receptor Cxcr2 to promote neutrophil chemotaxis[5,14,15]. It is also known that Cxcl8a, differently from its human counterpart, does not require the zebrafish Cxcr1 receptor and chemoattracts neutrophils essentially via Cxcr2[14]. Whether

other lineages of chemokine and non-chemokine ligands are chemotactic towards Cxcr1-2 in zebrafish is not currently known.

A cluster of genes that conserves high sequence homology and synteny with the human CXCL9-10-11 gene cluster also exists in zebrafish[22,23]. Two of these (Cxcl11aa and Cxcl11af) are infection-inducible and require the chemokine receptor Cxcr3.2, an orthologue of mammalian CXCR3. Similarly to the mammalian axis, the Cxcr3.2 receptor induces macrophage recruitment[23]. In addition, macrophages and neutrophils in zebrafish express and are mobilised via Cxcr4b[24] and this receptor has been shown to cross communicate with the human CXCL12, the ligand of human CXCR4[25].

Together with the conservation of the CXCR2, CXCR3, and CXCR4 signalling axes, zebrafish also has a series of other CXC motif chemokines that cannot be unambiguously classified with respect to the mammalian counterparts, due to the short sequence and the fast evolution and divergence of chemokine genes[22]. The absence of the ELR motif in neutrophil-competent teleost chemokines additionally complicates phylogenetic reconstructions and functional classification. Cxcl18b (formerly named Cxcl-c1c) is highly induced in zebrafish upon infection with different pathogens and shares sequence similarities and expression patterns with the zebrafish orthologues of both Cxcl8 and Cxcl11 chemokines (48-53% amino acid similarities to Cxcl8a, Cxcl8bb, Cxcl11aa and Cxcl11af)[22,26,27,28]. Blasting Cxcl18b to the murine and human protein databases reveals instead that murine CXCL3 and human CXCL11 are its two most closely related mammalian chemokines (48% and 47% amino acid similarity, respectively).

Like other inflammatory chemokines, c*xcl18b* transcription was found to rely on the activation of the Myd88-dependent innate immunity signalling pathway during the response to *Edwardsiella tarda*, *Mycobacterium marinum (Mm)* and *Salmonella enterica* serovar Typhimurium[26,27]. Recently, it was also demonstrated that Cxcl18b is upregulated in response to treatment with toxic and pro-apoptotic compounds, which indicates this gene as a marker of inflammation in general[28,29]. However, the function of Cxcl18b has yet to be elucidated and clarification of its role has an important translational significance, since the zebrafish model is being increasingly used to study chemokine axes, leukocyte biology, and inflammatory processes[30]. This study demonstrates that Cxcl18b is an additional neutrophil chemotactic factor. We show that the function of Cxcl18b relies, at least partly on chemokine receptor Cxcr2, and not on Cxcr1 and Cxcr4b. Finally, using a novel reporter line, we show the expression of this chemokine during the pathological inflammation occurring upon mycobacterial infection.

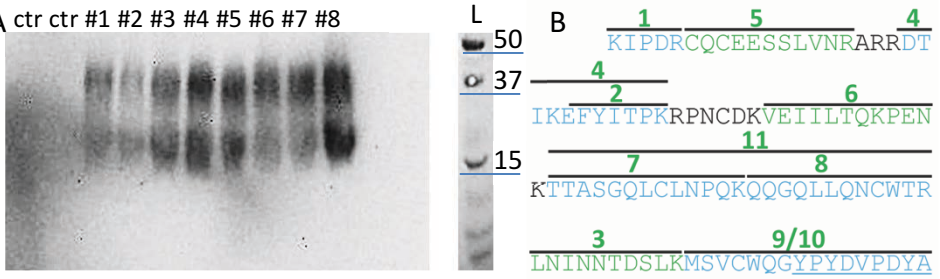
RESULTS

Production and quality confirmation of recombinant Cxcl18b

In order to study the chemotactic properties of Cxcl18b, we set out to produce recombinant protein using a yeast (*Pichia pastoris*) expression system. The coding sequence for Cxcl18b was synthetically generated according to the Ensembl database accession (ENSDARG00000075045/ENSDART00000111598). The sequence was codon optimised for expression in yeast and supplemented with an HA (human influenza haemagglutinin)-tag at the C-terminus, to permit identification with an anti-HA-antibody. Additionally, to facilitate secretion by *Pichia pastoris*, the predicted zebrafish signal peptide of Cxcl18b was replaced with the yeast alpha-factor secretion signal, as a result of cloning of the

Cxcl18b sequence into the pPICZα expression vector. Expression of the construct in successful (Zeocine-resistant) transformants was induced by culturing several isolates in buffered minimal medium supplemented with 0.5% methanol that is used as an inducer of gene expression in this system.

A



C

CXCLc1c_HA Mass: 11828 Score: 1704 Queries matched: 90 emPAI: 11.70

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Peptide	
17	314.6928	627.3710	627.3704	0.90	1	30	0.019	1	-.KIPDR.C	1
244	449.2398	896.4650	896.4644	0.75	0	43	0.0012	1	K.EFYITPK.R	2
565	566.3031	1130.5916	1130.5931	-1.36	0	35	0.015	1	R.LNINNTDSLK.M	3
1118	677.8694	1353.7242	1353.7180	4.55	1	64	1.3e-05	1	R.DTIKEFYITPK.R	4
1222	691.2958	1380.5771	1380.5762	0.66	0	69	1e-06	1	R.CQCEESSLVNR.A	5
1333	706.4123	1410.8101	1410.8082	1.36	0	74	8.5e-07	1	K.VEIILTQKPENK.T	6
1353	709.3580	1416.7014	1416.7031	-1.23	0	87	8.3e-08	1	K.TTASGQLCLNPQK.Q	7
1784	766.3750	1530.7355	1530.7361	-0.43	0	66	9.5e-06	1	K.QQGQLLQNCWTR.L	8
2938	975.9084	1949.8022	1949.7964	2.95	0	38	0.00064	1	K.MSVCWQGYPYDVPDYA.-	9
2988	983.9038	1965.7930	1965.7914	0.84	0	(30)	0.0039	1	K.MSVCWQGYPYDVPDYA.-	10
4249	977.4811	2929.4215	2929.4287	-2.47	1	72	1.9e-06	1	K.TTASGQLCLNPQKQQGQLLQNCWTR.L	11

Figure 1. Cxcl18b recombinant protein production. A. Western blot analysis of the supernatant derived from eight (#1-#8) isolates of *Pichia pastoris* transformed with Cxcl18b-HA expression vector. The protein was visualised by a HRP-conjugated anti-HA antibody. Lanes marked as ctr refer to the supernatant of two non-transformed isolates. The expected molecular weight of Cxcl18b-HA is 11.5 kDa. However, in the western blot analysis Cxcl18b displayed two bands, one at about 20 kDa and one at about 40 kDa. The 40 kDa band represents most likely a dimer of the 20 kDa product. Increased molecular weight of Cxcl18b as compared to expectation can be explained by glycosylation, difference in salinity/ionic force between the *Pichia pastoris* culture supernatants and the reference protein ladder (L), and high protein concentration in the samples that may delay electrophoretic run. Isolate #8, which appeared to produce the highest levels of recombinant protein, was selected for purification and confirmation by mass spectrometry. **B-C**. Electrospray mass spectrometry analysis of trypsinised Cxcl18b-HA. Virtually all the peptides derived from trypsinisation of Cxcl18b (peptides 1-11) could be detected. Short stretches of the protein could not be identified with statistical significance, because of the high frequency of trypsin digestion sites (.K or .R) on those protein parts (predicted peptides ARR, A, R, PNCD consist of ≤4 residues and are too short for univocal identification). No other zebrafish or *Pichia pastoris* proteins were significantly indicated by the mass spectrometry analysis of purified Cxcl18b. Bars in black in B show the sequence covered by mass spectrometry analysis. The sequence underlined in blue indicates the C-terminal HA tag, used for western blot readout in A. Table of peptide statistics in C was elaborated via Mascott analysis of the Cxcl18b-HA spectrum.

Eight colonies were tested for optimal expression and secretion, by western blot analysis against the HA-sequence on the supernatant of induced cultures (**Figure 1A**). In western blot analysis Cxcl18b displayed two bands (~20 kDa and ~40 kDa), of which the one with the higher molecular weight most likely represents a dimer, as chemokine ligands are known to form very stable dimers[31,32,33,34]. Deviation of molecular weight of the monomer from expectation (11.5 kDa), is most likely due to extensive glycosylation, as multiple glycosylation sites were predicted and chemokines are known to be highly glycosylated[15]. The Cxcl18b-positive clone that appeared to produce the highest levels of chemokine was used for macroscale preparation of Cxcl18b. The supernatant of an induced culture, containing Cxcl18b was concentrated and purified in PBS (phosphate buffer saline) by column filtration. Identity and purity of Cxcl18b in the sample was determined by trypsinisation and electrospray mass spectrometry analysis, which revealed high purity of the protein and undetectable protein contaminations of the recombinant Cxcl18b with *Pichia pastoris* proteins (**Figure 1B-C**).

Cxcl18b induces chemotaxis of neutrophils, but not macrophages

To study the function of Cxcl18b, the isolated chemokine was injected into the hindbrain ventricle of embryos at 2 dpf (days post fertilisation), in neutrophil-specific (*mpx:eGFP*-positive cells[4]) or macrophage-specific (*mpeg1:Gal4/UAS:Kaede*-positive cells[3]) transgenic reporter backgrounds. Embryos were fixed 3 hours post injection (hpi). As a negative control for injections (mock), the supernatant of a non-transformed *Pichia pastoris* culture was processed with the same purification procedure. Injection of either 0.2 or 2 ng of purified recombinant Cxcl18b elicited significantly higher mobilisation of neutrophils when compared to mock, while it did not impact on macrophage recruitment (**Figure 2**), indicating that Cxcl18b has chemotactic specificity toward neutrophils. Injection of either 0.2 or 2 ng of Cxcl18b did not affect the overall level of recruitment in a dose-dependent manner, suggesting that receptor saturation may have occurred.

Cxcl18b requires Cxcr2 receptor, but not Cxcr1 and Cxcr4b, for efficient recruitment of neutrophils

The chemokine receptor Cxcr2/CXCR2 is a key controller of neutrophil chemotaxis in both teleosts and mammalian species[5,12,14]. In mammals, activation of neutrophil migration via CXCR2 can be obtained by stimulation with a wide spectrum of inflammatory CXC chemokines, including CXCL1-2-3-5-6-7-8[7,8,9]. Hence, CXCR2 represents a ligand-promiscuous, but neutrophil-specific, receptor. In zebrafish, 2 functional Cxcl8 ligands (Cxcl8a and Cxcl8bb) of Cxcr2 have been identified[5,14,22]. However, considering the capability of the mammalian CXCR2 to accommodate a multiplicity of chemokines of different lineages, it is very likely that also in zebrafish other CXC chemokines are redundant with Cxcl8 ligands. Therefore, we hypothesised that the neutrophil chemotactic properties of Cxcl18b might also be exerted by activation of Cxcr2. To test this hypothesis we pharmacologically inhibited Cxcr2 with SB225002, a non-peptide inhibitor that can suppress both mammalian and zebrafish CXCR2 activity against its ligands, such as CXCL1 and CXCL8[5,35]. As a positive control, zebrafish Cxcl8a (ENSDARG00000104795/ENSDART00000161996), isolated with a comparable method[23], was used, since it was previously demonstrated that blockade of Cxcr2 with SB225002 can affect Cxcl8-dependent chemotaxis in zebrafish[5]. Cxcl8a and Cxcl18b elicited increased recruitment of neutrophils in control (DMSO vehicle-treated) groups and a significantly diminished chemotactic potency in SB225002-treated groups, which indicated that both

Cxcl8a and Cxcl18b require Cxcr2 to mediate optimal neutrophil chemotaxis (**Figure 3A**). However, the chemotactic capability of Cxcl8a was more severely suppressed by the drug treatment, as compared to the effect exerted by the same treatment on Cxcl18b chemotaxis, since this could still recruit more neutrophils than mock in Cxcr2-depleted condition. We also tested the activity of Cxcl18b under condition of *cxcr1* morpholino knockdown and in a *cxcr4b* mutant line, since these other two receptors are also expressed by zebrafish neutrophils[14,24]. However, Cxcl18b did not display significantly diminished chemoattractant power in *cxcr1* or *cxcr4b* depleted conditions, indicating that these receptors are dispensable for Cxcl18b sensing (**Figure 3B-C**).

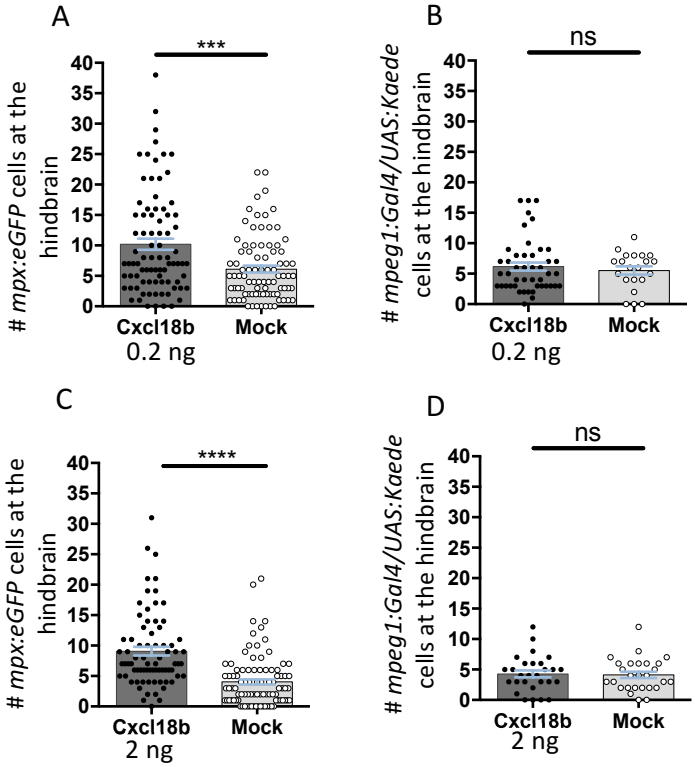


Figure 2. Cxcl18b exerts a neutrophil-specific chemoattraction *(Figure on the previous page)***.** Purified Cxcl18b-HA (0.2 or 2 ng) or mock (isovolumetric) were injected in the hindbrain of 2 dpf zebrafish embryos from *Tg(mpx:eGFP)* or *Tg(mpeg1:Gal4/UAS:Kaede)* lines. Samples were collected at 3 hpi. While Cxcl18b did not significantly chemoattract macrophages more than mock at both concentrations (B,D), Cxcl18b injection induced an increased infiltration of neutrophils to the hindbrain (A,C), indicating the chemotactic capability of this ligand towards neutrophils. Each data point represents an individual embryo. Data are cumulated from 4 (A), 2 (B-C) or 1 (D) replicates.

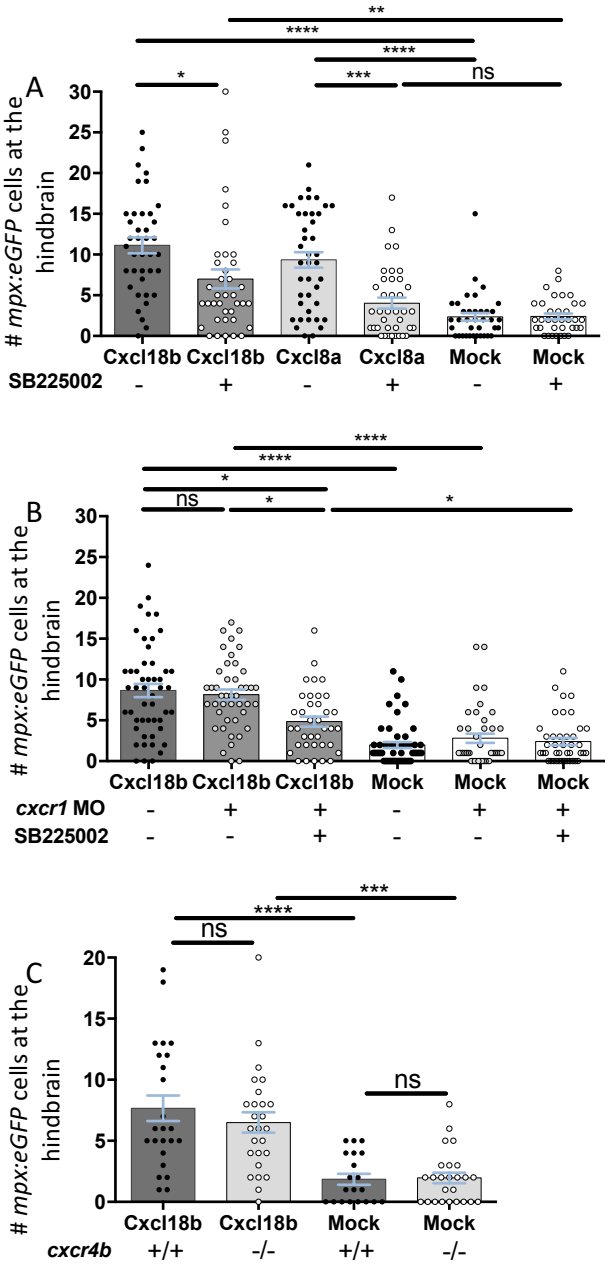


Figure 3. Pharmacological inhibition of CXCR2, but not *cxcr1* knockdown or homozygote mutation of *cxcr4b*, attenuates neutrophil recruitment to Cxcl18b local injections. A. Injections in the hindbrain of Cxcl18b (2 ng) or Cxcl8a (0.5 ng) were performed as in Figure 2. Pharmacological inhibition of Cxcr2 by bath exposure to 5 µM SB225002 affected the chemoattraction of neutrophils to both Cxcl8a and Cxcl18b but did not entirely suppress the chemotactic response to Cxcl18b, suggesting that Cxcl18b could be sensed by an additional receptor. **B.** Morpholino knockdown of *cxcr1* did not affect neutrophil migration to Cxcl18b and did not synergise with Cxcr2 inhibition, as Cxcr1/2 deficient embryos still displayed residual recruitment when compared to mock-injected controls. **C.** *cxcr4b* null mutant embryos did not display impaired neutrophil recruitment to Cxcl18b, indicating that this receptor is not required for the Cxcl18b-dependent chemotaxis of neutrophils. Data are cumulated from 2 (A-B) or 1 (C) replicates.

To address whether redundant function between Cxcr1 and Cxcr2 might explain that *cxcr1* morpholino injection did not impair neutrophil recruitment, we blocked Cxcr1/2 signalling by *cxcr1* knockdown and simultaneous Cxcr2 pharmacological inhibition. Similar as in the case of Cxcr2 inhibition (**Figure 3A**), simultaneous blockade of Cxcr1 and Cxcr2 significantly reduced neutrophil recruitment, but some residual chemotactic activity was observed (**Figure 3B**).

A *Tg(cxcl18b:eGFP)* reporter lines labels mycobacterial-induced inflammation

Since previous expression studies indicated that Cxcl18b is induced in response to several bacterial infections[26,27], we constructed a *Tg(cxcl18b:eGFP)* reporter line to visualise and longitudinally follow the expression pattern of *cxcl18b in vivo*. To validate inflammation-dependent induction of the reporter line, we injected embryos with *Mycobacterium marinum* (*Mm*), a fish pathogen which leads to the formation of granulomatous lesions in the zebrafish species and is widely used as a surrogate model for tuberculosis[36]. These inflammatory lesions consist of immune cell aggregates that have migrated and collected bacteria at the infection focus.

The initial stages of granuloma formation mostly consist of macrophages, while neutrophils are mostly recruited at a more advanced stage. Granulomas in the zebrafish embryonic/larval model form without requiring the presence of adaptive immune cells, as these lesions can be generated at developmental stages that anticipate ontogenesis of lymphocytes[36]. When *Tg(cxcl18b:eGFP)* embryos were challenged with *Mm*, the *cxcl18b*-driven eGFP accumulated in the areas where initial aggregates were forming (**Figure 4**), consistent with previous evidence reported by transcriptomic and *in situ* gene expression studies[26,27]. At 36 hpi, cells residing in the caudal haematopoietic tissue and endothelial cells appeared to upregulate the transgene. (**Figure 4E-F**). As the infection progressed, the expression of the reporter continued to accumulate at the nascent granulomas in the cells surrounding the lesion. However, confocal imaging of the infected larvae indicates that the infected cells are not the main producers of *cxcl18b*, rather this is produced by non-infected cells in the immediate surrounding tissue (**Figure 5A-F**). However, these *cxcl18b*-expressing cells localised in tight proximity to the cells where most of the infection was residing, participating in the formation of the nascent granulomatous aggregates. At 3-4 dpi (days post infection), highly *cxcl18b*-expressing cells could be found within the granuloma lesion. Considering the capability of some of the Cxcl18b^{+} cells to emit long dendrites (**Figure 5C-F**), we investigated whether these cells could represent uninfected phagocytes (macrophages and neutrophils). Therefore, we crossed the *Tg(cxcl18b:eGFP)* transgenic line with *Tg(lyz:DsRed)* or *Tg(mpeg1:mCherry-F)* lines and infected the offspring with *Mm*-mCrimson to induce Cxcl18b expression. As expected, the infection resided essentially in c*xcl18b*-negative cells, although the nascent granuloma aggregates contained several c*xcl18b*-positive cells. Strikingly, the c*xcl18b* transgene expression did not overlap with *lyz* and *mpeg1* transgenes, indicating that Cxcl18b is expressed by non-phagocytic cells that participate in the granuloma aggregate. Since we identified Cxcl18b as a potent neutrophil chemoattractant and neutrophils are known to be attracted to the granulomatous aggregates[37,38], we propose that Cxcl18b-producing non-phagocytic cells that compose the granuloma stroma might be important to drive neutrophil recruitment to the mycobacterial infection foci.

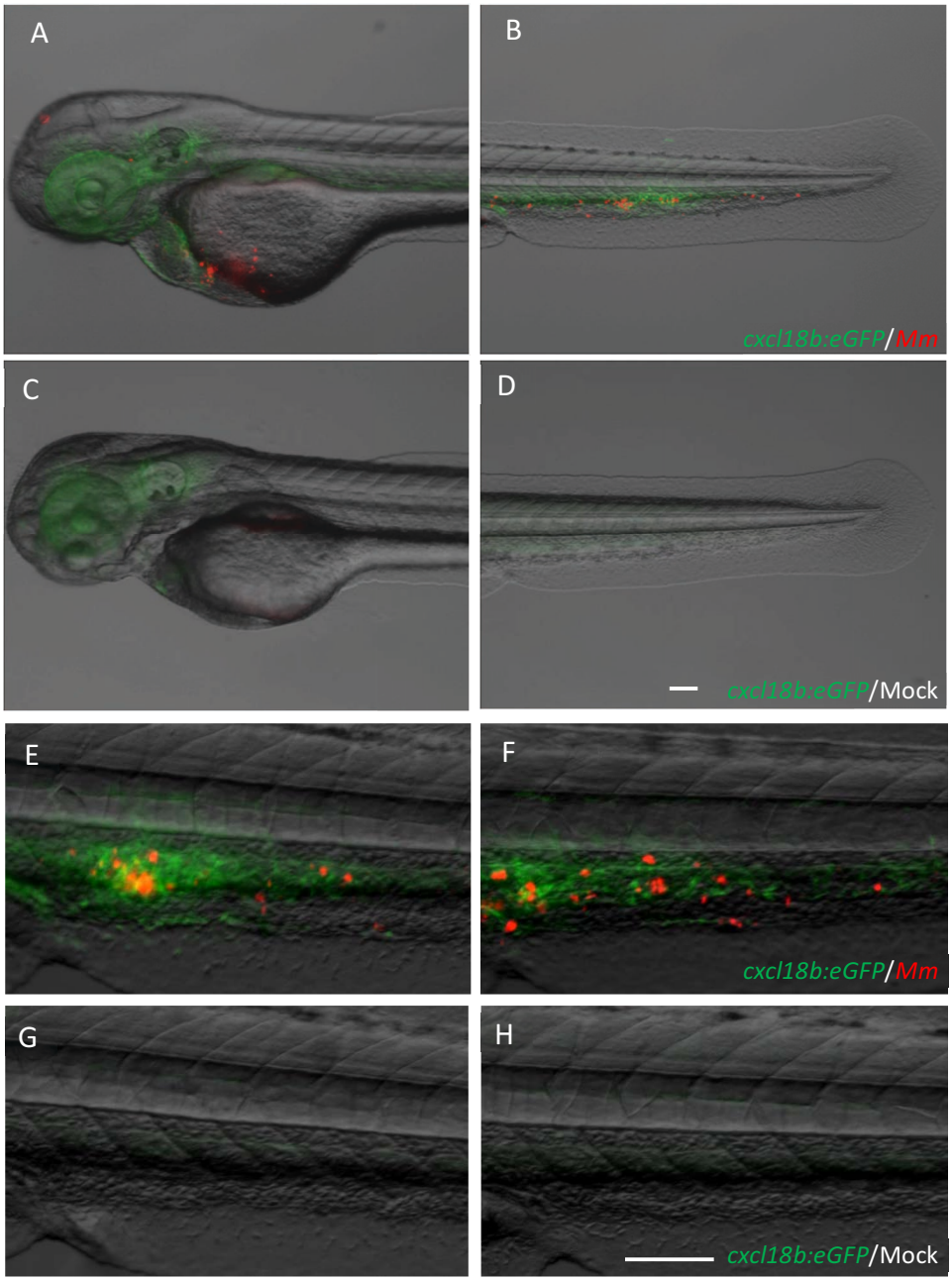


Figure 4. *cxcl18b:eGFP* is expressed by the tissue surrounding *M. marinum* infection sites. Injections of approximately 200 CFU of *M. marinum* M-mCrimson (*Mm*) via the caudal vein (A-B and E-F), but not mock injection (2% PVP in PBS, C-D and G-H) induced expression of the *cxcl18b:eGFP* transgene in the infected areas, especially localised around the sites of bacterial growth. Images were taken at 36 hpi. Scale bars: 100 µm.

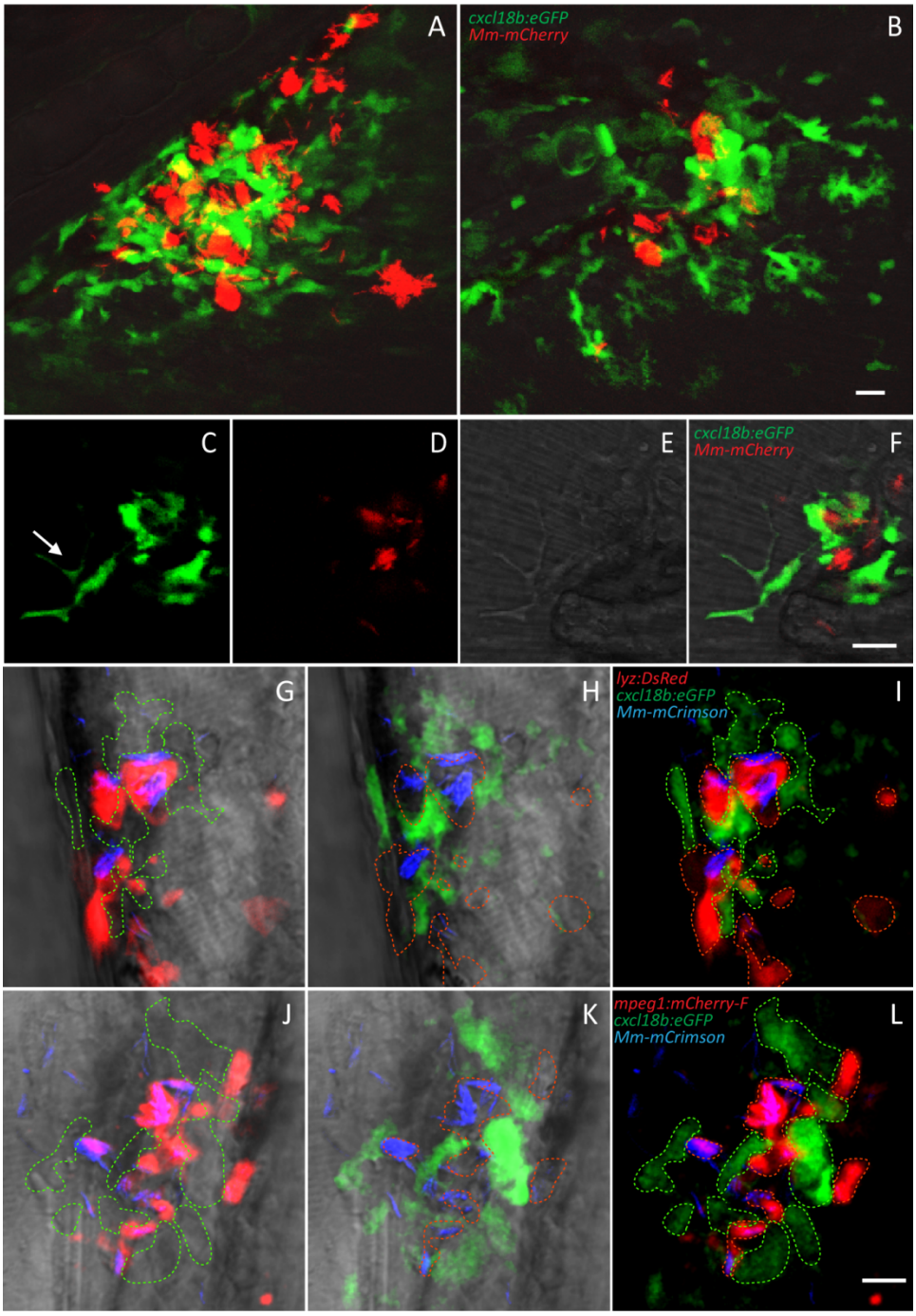


Figure 5. *cxcl18b:eGFP* is mostly expressed by non-infected cells accumulated at the nascent granuloma *(Legend on the next page)*.

Figure 5. *cxcl18b:eGFP* is expressed by non-infected cells accumulated at the nascent granuloma *(Figure on the previous page)*. **A-F.** During the formation of granulomas, the cells that express high levels of *cxcl18b:eGFP* consist predominantly of uninfected cells that participate in the cell aggregates initiating the granuloma. Phenotypic inspection shows that some of these cells can emit long protrusions (arrow in C). **G-L.** Cxcl18b-expressing cells do not represent phagocytic cells since this marker does not overlap with established neutrophil and macrophage markers l*yz* (G-I) and *mpeg1* (J-L). Images are representative of granulomas forming at 3 dpi (A-F) or 4 dpi (G-L). A-B are multiple z-stack maximum projections, C-L are split channels and overlay of an individual optical layer. In G-L outlines of green and red cells are marked with dashed lines of the corresponding colour. Scale bars: 10 µm.

DISCUSSION

Our study reports that the inflammatory chemokine Cxcl18b, a reliable marker of inflammation in zebrafish, displays expression patterns and chemotactic properties towards neutrophils, similar to those of the zebrafish Cxcl8 paralogues. Both Cxcl8 chemokines and Cxcl18b require the chemokine receptor/neutrophil marker Cxcr2 to mediate an optimal neutrophil emigration[5].

Our study therefore indicates that, like in mammalian species, the zebrafish Cxcr2 is a promiscuous receptor, able to respond to a variety of CXC ligands[12]. However, while Cxcl8a heavily relies on Cxcr2 activity to induce chemotaxis, Cxcl18b can still elicit significant (although diminished) neutrophil recruitment in Cxcr2-depleted conditions, indicating that another neutrophil receptor can mediate residual Cxcl18b chemotaxis in Cxcr2-blocked conditions. We excluded the possibility that this receptor may be Cxcr4b or Cxcr1, other two chemokine receptors expressed by the neutrophil lineage which can affect neutrophil chemotaxis in zebrafish and/or mammals[21,24], since Cxcl18b could still elicit normal recruitment of neutrophil in a *cxcr4b* mutant line or in *cxcr1* knockdown conditions. These data are in line with previous studies in zebrafish that demonstrated that Cxcr1 is also not required to sense the zebrafish Cxcl8a and Cxcl8bb chemokines that essentially recruit via Cxcr2[5,14], and with the fact that Cxcr4b mostly elicits cell recruitment via the Cxcl12a ligand[39]. The residual recruitment of neutrophils upon Cxcl18b injection can be explained either with the existence of another neutrophil receptor that contributes to Cxcl18b-mediated recruitment, or with the possibility that the Cxcr2 inhibitor may not have completely suppressed Cxcr2 function, which could lead to some residual neutrophil recruitment when saturating doses of chemokine ligands are provided.

In this study, we have also constructed a *cxcl18b* reporter line that can be used to further investigate what cell types are involved in the production of the Cxcl18b neutrophil-chemotactic cue. Our analysis of the Cxcl18b transgene expression indicates that non-phagocytic cells at the infection site are responsible for *cxcl18b* expression in response to *Mm* bacterial challenge. This observation suggests an important contribution of stromal cells in the granuloma microenvironment to the development of inflammation. A better understanding of responses in the granuloma microenvironment is particularly relevant in view of the emerging role of inflammation in the pathogenesis of tuberculosis, with several recent studies emphasising the need for a well-balanced inflammatory response in order to provide protective functions yet prevent pathological consequences[40,41]. Additional combination of *Tg(cxcl18b:eGFP)* with other reporter lines for inflammatory phagocytes or inflamed tissues (e.g. *tnfa*[42] and *il1b*[43] reporters) and transcriptional profiling of *cxcl18b*-expressing cells may further help to elucidate the nature and the importance of the *cxcl18b*

positive cells in the promotion of inflammation and in driving neutrophilic infiltration in mycobacterial granulomas.

In a recent study, Cxcl18b was the most upregulated chemoattractant detected at 4 hours post tail fin amputations[44], indicating that Cxcl18b is also induced upon sterile acute inflammation. Notably, in this study the expression of Cxcl18b was fully suppressed by treatment with the glucocorticoid beclomethasone. Treatment with this anti-inflammatory drug coincided with the abolishment of neutrophil recruitment to the wound, while it did not affect macrophage recruitment[44]. Given our evidence that Cxcl18b is a powerful and neutrophil-specific chemotactic cue, it is very likely that neutrophil chemotaxis was impaired by beclomethasone treatment owing at least partly to suppression of *cxcl18b* induction. Previous studies performed in a similar model showed that, at 1 hour post wounding (hpw), the two zebrafish Cxcl8a and Cxcl8bb genes were also significantly induced. While induction of Cxcl18b was not addressed at 1 hpw, induction of Cxcl8a and Cxcl8bb was found to be very transient and significantly dropped down at 4 hpw[5]. Taken together, these studies suggest that Cxcl8a-bb and Cxcl18b might represent differently timed chemotactic cues which may be responsible for the recruitment of different waves of neutrophils during acute and chronic inflammatory responses. The recombinant Cxcl8a and Cxcl18b proteins and the c*xcl18b* reporter line presented here will possibly aid in studying the dynamics of *cxcl18b* expression and permit to clarify shared and distinct functions of Cxcl8 isoforms and Cxcl18b in orchestrating neutrophil function. Understanding to what extent Cxcl18b is redundant with Cxcl8 isoforms and whether the two clades of chemokines also display specific functions in the regulation of the inflammatory response could also shed light on the divergences and/or convergences existing between fish and mammalian chemokine axes and on the evolutionary diversification of chemokine ligands.

MATERIALS AND METHODS

Zebrafish lines and embryo/larvae handling – Zebrafish were handled in compliance with the local (Leiden University) animal welfare policies and were maintained according to standard protocols (zfin.org). All experiments in this study were performed on 2 dpf embryos, therefore prior the free feeding stage and did not fall under animal experimentation law according to the EU Animal Protection Directive 2010/63/EU. Embryos were kept in egg water (60 µg/ml sea salt; Sera Marin) at 28.5°C. To prevent pigmentation, embryos were maintained in water supplemented with 0.003% PTU (1-phenyl-2-thiourea, Sigma-Aldrich). For the recruitment assays, neutrophils and macrophages were labelled with the transgenes *Tg(mpx:eGFP^{i114})*[4] or *Tg(mpeg1:Gal4-VP16^{gl24}/UAS-E1b:Kaede^{s1999t})*[3] [in short referred to as *Tg(mpeg1:Gal4/UAS:Kaede)*], respectively. For confocal microscopy (**Figure 5**), neutrophil and macrophage lines *Tg(lyz:DsRed^{nz50})*[45] and *Tg(mpeg1:mCherry-F^{ump2})*[46] were single crossed to the *cxcl18b:eGFP* line (see below). To address chemotactic properties of Cxcl18b via Cxcr4b, mutants (*cxcr4b$^{-/-}$*) and wildtype siblings (*cxcr4b$^{+/+}$*) of *(cxcr4b^{t26035})*[47], crossed into the *Tg(mpx:eGFP^{i114})* background were used.

Production and verification of recombinant Cxcl18b – The coding sequence for Cxcl18b (ENSDARG00000075045/ENSDART00000111598) was optimised for expression in yeast, supplemented with an HA (human influenza haemagglutinin)-tag at the C-terminus and cloned into pPICZα expression vector (Invitrogen, Life Technologies). The expression plasmid was linearised with SacI and transformed into *Pichia pastoris* strain X-

33 as described previously[23,48]. Successful transformants were selected on YPD-agar (1% yeast extract 2% peptone 2% dextrose 2% agar, Sigma-Aldrich) by resistance to 100 µg/ml Zeocine and reselected on 1000 µg/ml Zeocine plates. Highly resistant clones were selected for expression efficiency by liquid culturing the isolates in 10 ml YPD medium (30ºC, 180 rpm, O/N), transfer to 2.5 ml buffered minimal methanol medium (100 mM potassium phosphate buffer pH 6, 1.34% yeast nitrogen base, $4 \cdot 10^{-5}$% biotin, 0.5% methanol, Sigma-Aldrich), and cultured (30ºC, 180 rpm) for five days (with additional 0.5% methanol supplemented daily). The supernatant of the cultures was probed for anti-HA reactivity on western blot, using a custom-made horseradish peroxidase-directly conjugated antibody. One clone was selected for large-scale culture (250 ml). Cxcl18b-HA accumulated in the supernatant was concentrated and purified in PBS by filtration between columns with a cut-off of 50 kDa and 3 kDa (Amicon Ultra Centrifugal filters, Merck KGaA). Identity and purity of Cxcl18b in the sample was determined by trypsinisation and electrospray mass spectrometry analysis, which revealed high quality of the protein and undetectable protein contaminations of the recombinant Cxcl18b with *Pichia pastoris* proteins (Figure 1B-C). To obtain a mock control for injections, isolation was performed from the supernatant of non-transformed isogenic *P. pastoris*, cultured in identical condition (with the exception of Zeocine 100 µg/ml present in the starter plate and pre-culture for the transformed isolate). We confirmed chemokine purity and identity by in-solution trypsinisation and electrospray mass spectrometry. Quantification was obtained by BCA-assay (Micro BCATM Protein Assay Kit, Thermo Fisher Scientific, Life Technologies), the protein concentration assessed by the assay in the mock is subtracted from the total Cxcl18b protein concentration.

Recruitment assays – Cxcl18b, Cxcl8a or mock (produced as described above), were diluted in PBS to the desired concentration (0.2 ng/nl and 2 ng/nl) and injected (1 nl) into the hindbrain ventricle of 2 dpf embryos. Prior and during injections, embryos were anaesthetised in egg water medium containing 0.02% buffered Tricaine (3-aminobenzoic acid ethyl ester; Sigma-Aldrich). The embryos were fixed (O/N) at 3 hours post injection (hpi) in PBSTx (1× PBS supplemented with 0.8% Triton X-100, Sigma-Aldrich) containing 4% paraformaldehyde. Subsequently, the embryos were washed in PBS and the fluorescently labelled cells within the hindbrain perimeter were counted (blinded) using a Leica MZ16FA fluorescence stereomicroscope.

Pharmacological inhibition of Cxcr2 – For the Cxcr2 inhibition assays, we followed and adapted the protocol used in reference[5]. Briefly, 2 dpf larvae were preincubated for 1 hour at 28.5°C in presence or absence of the selective nonpeptide inhibitor SB225002 (Sigma-Aldrich) at a concentration of 5 µM in egg water. Since the compound is initially suspended in DMSO, the control group was exposed to the same concentration of DMSO (Sigma-Aldrich) alone (0.05%) as in the SB225002 group. Upon injection, embryos were returned and maintained in SB225002 or vehicle treatment until they were fixed (3 hpi) in 4% paraformaldehyde in PBSTx O/N.

Knockdown of Cxcr1 – 3 nl of 75 µM *cxcr1* morpholino (5′-TGTCAGGATACTAAACTTACCAGTC-3′, targeting exon1-intron 1 splicing site, Gene tools) or the same volume and concentration of a standard control morpholino (5′-CCTCTTACCTCAGTTACAATTTATA-3′, Gene tools) were injected at 1 cell stage in zebrafish fertilised eggs, according to previous reports[14].

Cloning of *cxcl18b* promoter and construction of *Tg(cxcl18b:eGFP)* reporter line – 3.04 kb of *cxcl18b* promoter immediately proximal to the transcriptional start were amplified from genomic DNA derived from a pool of AB/TL embryos, using the following amplification primers: *XhoI-Cxcl18bFw:* 5′-GGGCCCCTCGAGGTCTCCTCATGCATTGACTAC-3′ and *BamHI-Cxcl18bRv:* 5′-GGGCCCGGATCCAATTGCTGCAAACTATATGTAGG-3′. The PCR resulted in a single band on gel electrophoresis at the expected molecular weight. The primers supplemented the sequence with a unique XhoI site at the 5' end, a unique BamHI site at the 3' end and with exceeding 5′-GGGCCC-3′ extremities preceding both restriction sites to facilitate digestion. XhoI and BamHI extremities were activated by double enzymatic digestion to permit cloning into a custom-adapted pTol2^{+} destination vector, upstream of the eGFP coding sequence. Briefly, the destination vector was derived from *pTol2^{+}/coro-1a:eGFP-SV40pA* vector, previously described and kindly provided by the Wen lab[49]. The *7.03* kb *coro-1a* promoter sequence was fully removed by double restriction and gel extraction and replaced by a multiple cloning site, containing a BamHI proximal to the eGFP transcription start and an XhoI site more distally. Both vector and *XhoI-cxcl18b-BamHI* constructs where BamHI/XhoI digested and ligated together to obtain *pTol2^{+}/cxcl18b:eGFP-SV40pA*. The plasmid was purified and injected into zebrafish fertilised eggs together with the Tol2-transposase mRNA, according to previous reports[3]. Several founders were identified and appeared very similar in basal eGFP expression. One eGFP-positive founder was selected, outcrossed to AB/TL and the positive F1 offspring was raised to adulthood. Further single crosses of the F1 founders to AB/TL produced significantly >25% positive animals, suggesting the presence of multiple integrations.

Mycobacterial infection and image acquisition – *Tg(cxcl18b:eGFP)* embryos were injected with 200 CFU of mCrimson-labeled *M. marinum* strain M by the caudal vein injection route (**Figure 4**) or with 50 CFU via the trunk injection route (**Figure 5**). Bacteria were handled, prepared and injected as previously described[23,50]. Stereo-fluorescence images in **Figure 4** were taken with a Leica MZ16FA fluorescence stereomicroscope connected to a Leica DFC420C camera (Leica Microsystems). Confocal images were acquired with a Leica TCS SPE microscope equipped with a HCX APO L U-V-I 40x/WATER objective (Leica Microsystems).

Statistical analysis – All data were analysed using GraphPad Prism 5 (GraphPad Software). For comparison between two groups (**Figure 2**), a Mann-Whitney test was used. For comparisons between more than two groups (**Figure 3**) a Kruskal-Wallis test was used, followed by Sidak's multiple comparisons post-hoc test for selected groups. Significance (P-value) is indicated as: ns (non-significant); * (P<0.05); ** (P<0.01); *** (P<0.001); **** (P<0.0001). Error bars in all the graphs are mean±s.e.m.

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