Cxcl8 (IL-8) Mediates Neutrophil Recruitment and Behavior in the Zebrafish Inflammatory Response

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Neutrophils play a pivotal role in the innate immune response. The small cytokine CXCL8 (also known as IL-8) is known to be one of the most potent chemoattractant molecules that, among several other functions, is responsible for guiding neutrophils through the tissue matrix until they reach sites of injury. Unlike mice and rats that lack a CXCL8 homolog, zebrafish has two distinct CXCL8 homologs: Cxcl8-11 and Cxcl8-12. Cxcl8-11 is known to be upregulated under inflammatory conditions caused by bacterial or chemical insult but until now the role of Cxcl8s in neutrophil recruitment has not been studied. In this study we show that both Cxcl8 genes are upregulated in response to an acute inflammatory stimulus, and that both are crucial for normal neutrophil recruitment to the wound and normal resolution of inflammation. Additionally, we have analyzed neutrophil migratory behavior through tissues to the site of injury in vivo, using open-access phagocyte tracking software PhagoSight. Surprisingly, we observed that in the absence of these chemokines, the speed of the neutrophils migrating to the wound was significantly increased in comparison with control neutrophils, although the directionality was not affected. Our analysis suggests that zebrafish may possess a subpopulation of neutrophils whose recruitment to inflamed areas occurs independently of Cxcl8 chemokines. Moreover, we report that Cxcl8-12 signaled through Cxcr2 for inducing neutrophil recruitment. Our study, therefore, confirms the zebrafish as an excellent in vivo model to shed light on the roles of CXCL8 in neutrophil biology. The Journal of Immunology, 2013, 190: 000–000.
lacking the ELR motif, teleost Cxcl8s possess high homology with human CXCL8 (33, 34). Despite the description of both lineages in zebrafish, so far it has only been reported that the cxcl8-l1 gene is induced in inflammation (35). To date, neither the expression of cxcl8-l2 in this context nor the involvement of these chemokines in neutrophil recruitment has been addressed in the zebrafish.

The objective of this study was to understand in vivo the role of zebrafish Cxcl8s on neutrophil behavior and function in the inflammation elicited by tissue injury. First, our analysis indicated that both cxcl8-l1 and cxcl8-l2 were upregulated in wound inflammation. Importantly, we observed that recruitment of neutrophils toward the wound was significantly reduced in the absence of these Cxcl8s. By analyzing in vivo neutrophil migration and behavior with new open source tracking algorithms, PhagoSight, we have unexpectedly observed that in the absence of these chemokines, the velocity of the neutrophils migrating to the wound was significantly increased in comparison with normal controls. Furthermore, we have found that Cxcr2 mediated neutrophil recruitment to wounds and that Cxcl8-l2 signaled through this receptor to promote neutrophil recruitment in vivo. Overall, these observations led us to propose that zebrafish Cxcl8 chemokines are both required for efficient neutrophil recruitment in inflammation. Our data further support the idea that the zebrafish may possess, at least at the larval stage, a subpopulation of neutrophils whose recruitment to inflamed areas occurs independently of Cxcl8 chemokines.

**Materials and Methods**

**Characterization of zebrafish CXCL8**

A search for cxcl8 was performed at the Ensembl (http://www.ensembl.org/index.html) zebrafish database (zv8 and zv9) to check for the localization of CXCL8s in the zebrafish genome. Further genomic DNA analysis was performed to obtain accurate exon/intron sequences. Protein sequence alignments of zebrafish Cxcl8-11 (XP_001342606) and Cxcl8-12 (HF574400) with human CXCL8 (NP_000575) were generated using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

**Zebrafish husbandry**

All experiments with live animals were performed using protocols approved by the European Union Council Guidelines (86/609/EU) and the Bioethical Committee of the University of Murcia (approval no. 333/2008). Zebrafish fertilized eggs were obtained from natural spawning of wild-type (obtained from the Zebrafish International Research Center) and the transgenic (Tg) (mpx:gfp)i114 (22) line held at our facilities following standard husbandry practices. Animals were maintained in a 12 h light/dark cycle at 28.5˚C.

**Morpholinol knockdown**

The following splice blocking morpholinol (MO)-modified antisense oligonucleotides (Gene Tools) were injected into 1-cell-stage fertilized eggs (2–6 ng/egg): MO cxcl8-l1 E1/I1 (4 ng/egg), MO cxcl8-l1 E2/I2 (6 ng/egg), MO cxcl8-l2 E1/I1 (4 ng/egg), MO cxcl8-l2 I2/E3 (2 ng/egg) (see Table I). For assessment of morphant efficacy, total RNA was prepared from 3 d postfertilization (dpf) whole larvae using TRIzol reagent and purified in parallel to control for the specificity of the identified MO-mediated effects.

**Zebrafish tail tissue sample collection and gene expression analysis**

At 3 dpf, larvae were anesthetized in embryo medium with 0.16 mg/ml ethyl 3-aminobenzoate (tricaine; Sigma-Aldrich) and complete transection of the tail fin tip was performed with a disposable sterile scalpel. Larvae were recovered in embryo medium at 28.5˚C. At the time points indicated, larvae were anesthetized again with 0.16 mg/ml tricaine and, using a sterile scalpel, the body portion between the cloaca and the wound (tail fin tip) was excised from 80 larvae at each time point. Tail tissue samples were then pooled and frozen in liquid nitrogen. Total RNA was extracted from cell pellets with TRIZol reagent (Invitrogen) and purified with an RNAqueous Micro Kit total RNA purification system (Ambion), following the manufacturer’s instructions, and first-strand cDNA synthesized as above. Real-time PCR was performed with an ABI Prism 7500 instrument (Applied Biosystems) using SYBR Green (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95˚C, followed by 40 cycles of 15 s at 95˚C, 1 min at 60˚C, and finally 15 s at 95˚C, 1 min at 60˚C, and 15 s at 95˚C. For each mRNA, gene expression was normalized against the expression of the ribosomal protein S11 gene (rps11) in each sample. The primers used are shown in Table II. In all cases, PCR was performed with triplicate samples and repeated at least twice. Statistical analysis was performed using two-way ANOVA with Bonferroni posttest in Graph Prism 5 software.

**Tail fin wounding**

At 3 dpf, larvae were anesthetized in embryo medium with 0.16 mg/ml tricaine. Complete transection of the tail fin tip was then performed with a disposable sterile scalpel and fish were maintained in 1% (w/v) low melting point agarose (Sigma-Aldrich) dissolved in embryo medium supplemented with 0.16 mg/ml tricaine. The success of transection was immediately confirmed by fluorescence stereomicroscope MZ16FA (Leica) equipped with green fluorescent filters. After solidification, embryo medium with 0.16 mg/ml tricaine solution was added to keep embryos hydrated during experiments. Thereafter, images were captured at the selected times while animals were kept in their agar matrices with added medium at 28.5˚C.

**SB225002 pharmacological treatment**

For CXCR2 inhibition assays, we chose to use a bath immersion method. Briefly, larvae were preincubated 1 h at 28˚C in presence or absence of the selective nonpeptide inhibitor SB225002 (Tocris Bioscience) at a final concentration of 5 μM diluted in embryo medium supplemented with 1% DMSO. In tail fin wounding experiments, during recovery larvae were kept in embryo medium supplemented with 1% DMSO in presence or absence of SB225002 until imaging at 6 h postwounding (hpw).

**Production of recombinant Cxcl8-l2**

Recombinant zebrafish Cxcl8-l2 was produced in Escherichia coli. Briefly, the open reading frame of cxcl8-l2 encoding the mature protein without the signal peptide (residues 27–118) was synthesized, cloned in vector E3, produced as an N-terminal 6xHis fusion protein in E. coli, obtained from inclusion bodies with 6 M guanidine hydrochloride, and purified by a Ni2+Trap column (GenScript).

**Otic injection**

At 3 dpf larvae were preincubated in presence or absence of SB225002 (Tocris Bioscience) as described before, anesthetized in embryo medium with 0.16 mg/ml tricaine, and mounted in 1% of low melting point agarose. Injection in the otic vesicle of 1 nl PBS, Cxcl8-l2 recombinant protein at 30 μM, or leukotriene B4 (LTB4) at 30 nM was performed. Embryo medium with 0.16 mg/ml tricaine solution supplemented with

### Table I. MOs used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>MO Name</th>
<th>MO Sequence (antisense)</th>
<th>Target</th>
<th>[Mo], ng/egg</th>
</tr>
</thead>
<tbody>
<tr>
<td>cxcl8-l1</td>
<td>MO cxcl8-l1 E1/I1</td>
<td>5′-GGTTTGGCATGTTACACTTTACCTGCA-3′</td>
<td>E1/I1</td>
<td>4</td>
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<tr>
<td>cxcl8-l1</td>
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<tr>
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<td>E1/I1</td>
<td>4</td>
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<td>5′-GGCGGGTGGAGAAAAACACATGATC-3′</td>
<td>I2/E3</td>
<td>2</td>
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</tbody>
</table>

In the subsequent experiments employing the above mentioned MOs, standard control MOs (MO StdC) purchased from GeneTools were used in parallel to control for the specificity of the identified MO-mediated effects.
1% DMSO and, when required, with 5 µM SB225002 was added on the top to keep embryos hydrated during the experiments. Images were taken 1 h after injection.

**Image acquisition and processing**

For each experiment 3 dpf morphant and control larvae were imaged in three independent experiments. Images were taken from wounded or control larvae mounted as described above. Different methods of image acquisition were used according to the requirements of each experiment. Briefly, for total neutrophil counts and number of neutrophils at site of injury/injection, images were taken using a Leica MZ16F fluorescence stereo microscope. Time-lapse images from wounded tail fins were acquired using a Zeiss 5 Live confocal line-scanning microscope with a numerical aperture of 1 and ×20 water immersion objective in z-stack mode every 3 min until 6 hpw and assembled into time-lapse movies. For neutrophil PhagoSight analysis, time-lapse images were taken using a Zeiss Axiovert 200 fully motorized, inverted, wide-field fluorescence microscope using a numerical aperture of 0.8 and ×20 objective in z-stack mode every 2 min until 6 hpw. All data were processed using ImageJ (http://rsb.info.nih.gov/ij/).

**Neutrophil response analysis using PhagoSight**

The time-lapse movies from morphant and control larvae were processed using PhagoSight (http://www.phagosight.org.uk), an open-source neutrophil tracking software developed in Matlab. This software allowed us to generate quantitative measurements with which we were able to perform an unbiased comparison of neutrophil migratory behavior between cxcl8 morphants and control larvae and between resting and inflammatory conditions. This was done by analyzing either neutrophil speed or velocity oriented/lateral with respect to the wound as well as distinguishing the movement before the neutrophil reached the wound (referred to as “in translation”) and after it reached the wound (referred to as “in exploration”). Among the many parameters compared, the following were analyzed: the meandering index, the number of neutrophils that leave the wound, the total number of tracks generated by the neutrophils, the number

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**Table II. Primers used to analyze gene expression in this study**

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Use</th>
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<td>F</td>
<td>5’-GTGCCCATCTACGAGGGTTA-3’</td>
<td>PCR</td>
</tr>
<tr>
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<td>XM_001342570</td>
<td>F</td>
<td>5’-CCAGCTGAATGAGCTCTCTC-3’</td>
<td>PCR/qPCR</td>
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<td>F1</td>
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<td>F5</td>
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<td>NM_001025504</td>
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<tr>
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<td>NM_213377</td>
<td>F</td>
<td>5’-ACAGAAATGCCCCTCTCAG-3’</td>
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<td></td>
<td>R</td>
<td>5’-GCCTCTTCTCAAAAAAAAAGGTTG-3’</td>
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**FIGURE 1.** cxcl8-l1 and cxcl8-l2 mRNA levels are upregulated in wounded zebrafish tail fin tissue. Tail fins from 3 dpf zebrafish larvae were wounded and mRNA levels of indicated genes were determined by qPCR in tail fin tissue at 0, 1, and 4 hwp (80 tail fins per time point). Gene expression was normalized against rps11 and expressed as fold change compared with transcript expression levels of 3 dpf tail fin tissue from unwounded larvae (0 hwp). Each bar represents the mean ± SEM of triplicated samples. The p values were calculated using one-way ANOVA and Bonferroni multiple comparison test. **p < 0.01, ***p < 0.001.
of tracks that enter the wound, the number of tracks that enter the wound and leave, and the time point at which the neutrophils enter the wound.

Tail fin injury resolution assay

To address whether inflammation resolved normally in cxcl8-l1 morphants, 3 dpf morphant and control larvae were wounded as previously described and recovered at 28°C in embryo medium until live imaging acquisition. Larvae were then mounted in 0.5% (w/v) low melting point agarose in embryo medium with 0.16 mg/ml tricaine. Tail fin images were taken using a Zeiss Axiovert 200 fully motorized, inverted, wide-field fluorescence microscope using a numerical aperture of 0.8 and ×20 objective in a z-stack mode at 6, 24, and 48 hpw. Larvae were allowed to recover between acquisitions at 28°C in embryo medium. Images were processed with ImageJ, and neutrophil counts were performed at the indicated time points.

Statistical analysis

All error bars indicate SEM. For gene expression experiments data are shown as mean ± SEM of three separate experiments. One-way ANOVA with Bonferroni posttest was used for Figs. 1, 2D, 3–5, and 7E and for Supplemental Figs. 1E, 4, and 5A; a two-tailed Mann–Whitney U test was used for Fig. 7B; and a two-way ANOVA with Bonferroni posttest was used for Figs. 2B, 3 (il1b and ptgs2b), and 6B and for Supplemental Fig. 1D.

FIGURE 2. The cxcl8 morphants display a reduced zebrafish neutrophil recruitment in acute inflammation. Tail fins of Tg(mpx:gfp)i114 previously microinjected with MO StdC, MO cxcl8-l1, and MO cxcl8-l2 were transected at 3 dpf. (A) Representative maximum intensity projections of 6 h confocal time-lapse microscopy of 3 dpf Tg(mpx:gfp)i114 control and morphant larvae, acquired at 3-min intervals. This sequence is shown in Supplemental Video 1. Scale bar, 100 μm. (B) Counts of fluorescent neutrophils at the wound were made at 1, 2, 4, and 6 hwp. Data are presented as means ± SEM (n = 30 performed as three independent experiments). The p values were calculated using two-way ANOVA and a Bonferroni multiple comparison test. At 1 hwp no significant differences in neutrophil number were observed for both morphants in comparison with control larvae (p > 0.05). For MO cxcl8-l1 significant decreases were observed at 2 hwp (p < 0.01) and from 4 to 6 hwp (p < 0.001). As for MO cxcl8-l2, we have observed a significant decrease from 2 to 6 hwp (p < 0.001). (C) Differential interference contrast and GFP wide-field fluorescence microscope micrographs from 3 dpf control and morphant larvae. (D) Total neutrophil counts in whole larvae for each condition. Each bar represents means ± SEM (n = 20 performed as two independent experiments). The p values were calculated using one-way ANOVA and a Bonferroni multiple comparison test (p > 0.05, no significant differences were observed).
Results

Expression of both cxcl8 genes is upregulated in wounded zebrafish tail fin tissue

In the carp, it has been shown that members of Cxcl8 lineages, namely Cxcl8-l1 and the Cxcl8-l2, play a role in acute inflammation. Consistently, the mRNA levels of both carp Cxcl8 genes are upregulated under these conditions (33). In view of this, we first addressed the expression of both zebrafish cxcl8 at different time points in 3 dpf Tg(mpx:gfp)i114 larvae under acute inflammatory conditions using quantitative PCR (qPCR). In this study, we made use of the tail fin transection model, a validated model to investigate acute inflammation (16–23). We found that the mRNA levels of cxcl8-l1 and cxcl8-l2 were significantly increased in injured tail fin tissue and peaked at 1 hpw (Fig. 1).

Levels of il1b and ptgs2b (cox2b) mRNA were used as positive controls of inflammation in qPCR experiments (Fig. 1).

Genetic inhibition of both cxcl8 genes attenuate zebrafish neutrophil recruitment in acute inflammation

Next, we decided to knock down both Cxcl8 by using splice-blocking MOs. To design these MOs, we had to know the complete genomic sequence for both chemokines. Whereas cxcl8-l1 was already properly annotated in the zv8 database, for cxcl8-l2 we first needed to use an EST sequence (EH557944) to design primers to perform RT-PCR analysis and sequencing. Next, after confirming the full coding sequence, we performed a BLAST search of the zebrafish genome from Ensembl using the zv9 database and we were able to identify the full cxcl8-l2 sequence on chromosome 7, position 8658505:8665964, opposite strand. This gene is now annotated at Gene EMBL with the accession number HF674400 (http://www.ebi.ac.uk/ena/). Two different splice-blocking MOs were designed and tested for each gene to generate viable cxcl8-l1 and cxcl8-l2 morphants. Thus, we used MO cxcl8-l1 E1/I1 (referred to as MO cxcl8-l1) and MO cxcl8-l1 E2/I2 to generate cxcl8-l1 morphants and MO cxcl8-l2 E1/I1 (referred to as MO cxcl8-l2) and MO cxcl8-l2 I2/E3 for cxcl8-l2 morphants (Supplemental Fig. 1A, 1B). At 3 dpf mpx:GFP morphant larvae were injured and neutrophil recruitment was quantified at different time points from 1 to 6 hpw. Our results show that cxcl8-l1 and cxcl8-l2 knockdown significantly attenuated neutrophil recruitment to injured zebrafish tail fins (Fig. 2A, 2B, Supplemental Videos 1–3, Supplemental Fig. 1C, 1D). Furthermore, none of the MOs used to knock down cxcl8-l1 or cxcl8-l2 affected the total neutrophil number at 3 dpf in whole larvae (Fig. 2C, 2D, Supplemental Fig. 1E, 1F).

Although all four MOs reduced neutrophil recruitment, we decided to use MO cxcl8-l1 and MO cxcl8-l2 for further studies because they had higher efficiencies in CXCL8 knockdown and thus affected neutrophil recruitment more significantly than did the other two.

cxcl8-l2 knockdown impairs cxcl8-l1 induction after wounding

Although both genes were important for neutrophil recruitment in zebrafish, MO cxcl8-l2 caused a larger inhibition of the neutrophil response. Considering this, we further asked whether knockdown of a given cxcl8 could affect the expression of the other. By qPCR analysis, we observed that both morphants expressed higher basal levels of the other cxcl8 gene (Fig. 3). Upon wounding, the cxcl8-l2 mRNA levels were not affected in the cxcl8-l1 morphant. In contrast, cxcl8-l1 transcript levels were significantly reduced in cxcl8-l2 morphants when compared with control conditions. Moreover, in both cxcl8 morphants, we observed that the mRNA levels of two inflammation control markers, namely il1b and ptgs2b, were also significantly reduced (Fig. 3).
Neutrophil migratory behavior is affected in the absence of either Cxcl8

Our main objective was to study in vivo the function of Cxcl8s on the migratory behavior of neutrophils in acute inflammation. For such purpose, time-lapse images were acquired every 2 min from uninjured or previously injured 3 dpf larvae tail fins. Imaging was performed simultaneously for control conditions and for both cxcl8 morphants. To inspect neutrophil migratory behavior in detail, the acquired data sets were further analyzed using PhagoSight, software developed in Matlab (http://www.phagosight.org.uk/). PhagoSight provides an array of measurements that capture neutrophil behavioral traits, the most important of which are presented in Figs. 4 and 5, Supplemental Fig. 2, and Supplemental Movies 4–6.

In wounded larvae, we first confirmed that in both cxcl8 morphants, a significant lower number of neutrophils were being mobilized and recruited to the wound in the first 6 hpw (Fig. 4A–C). Moreover, neutrophils in cxcl8-l2 morphants were observed to enter the wound at significantly later time points than in control and cxcl8-l1 morphants (Fig. 4E). The ratio between the total number of tracks and the number of tracks in the wound (Fig. 4C), the number of tracks that crossed the wound and back (Fig. 4D), and the number of neutrophil time points in the wound (Fig. 4F) were not significantly affected.

Interestingly, the knockdown of either cxcl8-l1 or cxcl8-l2 did not affect the meandering index of the neutrophil movement in the tail fin tissue under acute inflammatory conditions (Fig. 5A). Unexpectedly, the speed of neutrophils in both cxcl8 morphants was significantly higher when compared with the control condition (Fig. 5B). Consistently, the mean speed from neutrophils moving toward the wound (i.e., in translation) was significantly increased in both cxcl8 morphants (Fig. 5C). Furthermore, the mean neutrophil speed was also significantly reduced for neutrophils moving within the wound (i.e., in exploration) in the absence of Cxcl8-l2 chemokine (Fig. 5D). Both lateral and oriented velocities (Supplemental Fig. 2A–F) were significantly increased in both cxcl8 morphants, indicating that the overall neutrophil speed was increased and not just the speed toward the wound.

In Fig. 5E, we present representative three-dimensional tracking plots of wounded tail fins of 3 dpf larvae from control, cxcl8-l1, and cxcl8-l2 morphants. Tracks were plotted highlighting the faster ones. As mentioned above, a significantly lower number of neutrophils were recruited to the wounds at higher speeds in both cxcl8 morphants in comparison with normal conditions. For the unwounded conditions, no significant differences were observed for neutrophil migratory speed, directionality, or number of neutrophils migrating to the tail (Supplemental Fig. 2G–L). Additionally, neutrophils migrated extravascularly, through the tissue matrix, in both wild-type and cxcl8 morphants (Fig. 5E, Supplemental Movies 1–6).

Inflammation resolution in wounded tail fin tissue is affected by the absence of Cxcl8 chemokines

Having established that neutrophil recruitment to wound heavily depends on both Cxcl8s, we further addressed whether inflammation resolution is affected by the absence of these chemokines. For these experiments, we wounded 3 dpf control and morphant larvae at the tail fins and counted the number of neutrophils present at the wound at 6, 24, and 48 hpw. Control larvae had a significantly reduced number of neutrophils at 24 hpw when compared with

![FIGURE 4](http://www.jimmunol.org/)

PhagoSight analysis confirms that CXCL8s morphants display a reduced zebrafish neutrophil recruitment in acute inflammation. Tailfins of Tg (mpx:gfp)i114 previously microinjected with MO StdC, MO cxcl8-l1, and MO cxcl8-l2 were transected at 3 dpf and time-lapse movies were performed under a wide-field fluorescence microscope (MO StdC = 270 tracks, MO cxcl8-l1 = 147 tracks, and MO cxcl8-l2 = 128 tracks from n = 10 larvae as three independent experiments) and further analyzed by PhagoSight. Distinct parameters were determined to study neutrophil migratory behavior, namely: (A) total number of tracks; (B) number of tracks entering the wound; (C) ratio of number of tracks in wound/total number of tracks; (D) number of tracks crossing the wound and back; (E) tracks enter the wound time; (F) number of hops in wound. Longer horizontal bars represent the means and shorter horizontal bars represent SEM. The p values were calculated using one-way ANOVA and a Bonferroni multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001.
6 hpw (Fig. 6), indicating a successful inflammation resolution. However, in both cxcl8 morphants, a reduction of the number of recruited neutrophils was not observed at either 24 or 48 hpw. Interestingly, cxcl8-l2, and to some extent cxcl8-l1, morphant larvae showed a faster healing and regenerative capacity than did control larvae (data not shown).

**Pharmacological inhibition of Cxcr2 impairs neutrophil recruitment to recombinant Cxcl8-l2**

To address whether also in zebrafish Cxcl8 signals through Cxcr2 for inducing neutrophil recruitment, we designed two different in vivo recruitment assays using a human CXCR2 selective non-peptide inhibitor, SB225002 (36). The presence of SB225002 at 5 μM significantly reduced the number of neutrophils recruited to the wound upon tail fin injury (Fig. 7A, 7B). Owing to the fact that cxcl8-l2 was induced at higher levels than cxcl8-l1 in wounded tissue (Fig. 1), recombinant Cxcl8-l2 protein was produced (Fig. 7C). Consistently with its chemotactic function, injection of the recombinant chemokine into the otic vesicle increased neutrophil recruitment to the ear in comparison with control conditions (Fig. 7D, 7E). When recombinant protein injection was performed into the otic vesicle of larvae pretreated with SB225002 so as to inhibit Cxcr2, the number of neutrophils recruited to the ear was reduced to a basal level (Fig. 7D, 7E). In contrast, LTB4-induced neutrophil recruitment was unaffected by SB225002 (Fig. 7D, 7E), confirming the specificity of this inhibitor.

**Discussion**

CXCL8 is an important chemokine that mediates neutrophil migration, accumulation, and function at sites of inflammation (37,

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**FIGURE 5.** PhagoSight analysis reveals that cxcl8s morphants have an increased zebrafish neutrophil recruitment velocity in acute inflammation. Tail fins of Tg(mpx:gfp)i114 previously microinjected with MO StdC, MO cxcl8-l1, and MO cxcl8-l2 were transected at 3 dpf and time-lapse movies were made in wide-field fluorescence microscope (MO StdC = 270 tracks, MO cxcl8-l1 = 147 tracks, and MO cxcl8-l2 = 128 tracks from n = 10 larvae as three independent experiments) and further analyzed by PhagoSight. Distinct parameters were determined to study neutrophil migratory behavior such as: (A) meandering index; (B) mean speed; (C) mean speed in translation; (D) mean speed in exploration. Longer horizontal bars represent the mean values and shorter horizontal bars represent SEM. (E) Three-dimensional photomicrograph of a typical tracking experiment, with lines indicating the path of neutrophil movement for 6-h time lapse during the recruitment phase of inflammation and colors indicating neutrophil velocity (μm/s). The p values were calculated using one-way ANOVA and a Bonferroni multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001.
FIGURE 6. Resolution of the cellular component of inflammation is decreased in both cxcl8 morphant larvae. (A) Representative maximum intensity projections from wide-field fluorescence microscope micrographs of 3 dpf Tg(mpx:gfp)i114 control and morphants larvae tail fins at 6, 24, and 48 h pw. Scale bar, 100 μm. (B) Counts of fluorescent neutrophils at the wound were made at 6, 24, and 48 h pw. Data are shown as means ± SEM (n = 40 performed as three independent experiments). The p values were calculated using two-way ANOVA and a Bonferroni multiple comparison test. For both cxcl8 morphants, significant differences were observed at 6 hpw in comparison with control larvae (p < 0.001) but not at 24 or 48 hpw (p > 0.05).
trophil subpopulations may respond differentially to CXCL8 chemokines and other chemoattractants expressed locally at the site of inflammation and could thus display distinct migratory behaviors. Under normal inflammatory conditions, these neutrophil subpopulations could be mobilized via sensing distinct chemotactic cues to inflamed areas where they could perhaps exert different functions. In the absence of either Cxcl8 signal, only the Cxcl8-unresponsive neutrophils would then migrate toward the inflamed area, possibly with an increased velocity by responding to other chemoattractants, such as, for example, LTB4, which induces neutrophil recruitment as demonstrated by us and others (21, 47). In agreement with a scenario of distinct functional populations, a recent study has proposed that zebrafish neutrophils can be functionally classified either as consumers or producers of H2O2 (48). The idea that neutrophils have distinct functional populations is taking shape (49, 50), and the acceptance of different functional subpopulations of neutrophils will require additional work. In this respect, a differential neutrophil response to CXCL8 may actually constitute a pivotal criterion to discriminate between the identified neutrophil populations.

To restore the normal tissue function and homeostasis, it is crucial that an acute inflammatory process should be well resolved (51). Among other markers, inflammation resolution is characterized by a significant decrease in the number of neutrophils at the inflamed sites (17, 52–55). Given the impact of both Cxcl8s in the recruitment of zebrafish neutrophils, we have also addressed whether and how resolution was affected in cxcl8-l1 and cxcl8-l2 morphants. As expected for a normal resolution, our results showed that at 24 hpf, control larvae presented a significantly reduced number of neutrophils when compared to 6 hpf. Such a scenario was not observed for both morphants that were not able to reduce their neutrophil counts at the wound at 24 or even at 48 hpf. In fact, the number of neutrophils at wounds remained fairly constant in cxcl8 morphants from 6 to 48 hpf. These results, together with the impaired induction of the proinflammatory mediators IL-1β and PTGS2b in Cxcl8-deficient larvae after wounding, suggest that inflammation resolution was compromised in these larvae. Additionally, these results also demonstrate that CXCL8 chemokines can modulate the expression of other inflammatory mediators such as factors with a proresolution function that might be enrolled in neutrophil apoptosis signaling pathways, in macrophage-mediated phagocytosis, or even in neutrophil reverse migration, among other events critical for inflammation resolution (17, 23, 56, 57). As such, the unavailability of

**FIGURE 7.** Pharmacological inhibition of Cxcr2 impaired neutrophil recruitment. (A) Representative wide-field fluorescence microscope micrographs of 3 dpf Tg(mpx:gfp)i114 control and morphant larvae, pretreated or not with 5 μM SB225002, followed by tail fin wounding. Scale bar, 100 μm. (B) Counts of fluorescent neutrophils at the wound were made at 6 hpf. Data are presented as means ± SEM (n = 25 performed as three independent experiments). The p values were calculated using a two-tailed Mann–Whitney U test. **p < 0.01. (C) SDS-PAGE analysis of Cxcl8-I2 recombinant protein. Lane 1, BSA (2 μg); lane 2, Cxcl8-I2 (2 μg). (D) Representative wide-field fluorescence microscope micrographs of 3 dpf Tg(mpx:gfp)i114 pretreated with 5 μM SB225002 followed by otic vesicle injection of PBS, 30 μM Cxcl8-I2, and/or 30 nM LTB4. Images were taken at 1 h after infection. Scale bar, 100 μm. (E) Counts of fluorescent neutrophils recruited to the ear (encircled) were made 1 h after infection. Data indicate means ± SEM (n = 32 performed as three independent experiments). The p values were calculated using one-way ANOVA and a Bonferroni multiple comparison. ***p < 0.001.
these chemokines during acute inflammation would necessarily affect the resolution of the process. Nevertheless, it is surprising that Cxcl8-deficient larvae showed faster healing than control larvae, despite being unable to resolve the inflammation. Although the mechanisms involved in this process deserve further investigation, our results are in agreement with a recent study that reported the absence of neutrophils to contribute to a faster and more successful repair of the tail fin (58).

In mammals CXCL8 binds to CXCR1 and CXCR2 with different affinities and thus activates different cascade pathways (3, 7–9). These receptors have been proposed to exert different functions in the neutrophil inflammatory response (7, 59). Owing to its intrinsic higher affinity toward its ligand chemokines, CXCR2 is thought to play a more active role in neutrophil recruitment and degranulation (7, 36, 59). As for CXCR1, it is thought to be more important for regulating cytotoxic events such as ROS formation and protease release at the injury site (7, 59, 60). As zebrafish also have CXCR1 and CXCR2 homologs (35), in this study we made use of a human selective non-peptide CXCR2 inhibitor, SB225002, to establish that 1) Cxcr2 plays a prominent role in neutrophil recruitment to wounds, and 2) neutrophil recruitment mediated by Cxcl8-12 mainly depends on Cxcr2 signaling.

In summary, we report that zebrafish Cxcl8-l1 and Cxcl8-l2 are both important in vivo for the recruitment of neutrophils in the wound-elicited acute inflammation. In particular, we have demonstrated that the presence of these chemokines is required for the maintenance of a normal neutrophil migratory behavior upon wounding. As evidenced in this study, there is still much work to be done to understand in detail how this small cytokine affects and modulates the neutrophil inflammatory response. In this respect, we think that the zebrafish will undoubtedly stand as the optimal experimental animal model to pursue these issues and to develop new therapeutic approaches strategically targeting CXCL8.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplemental legends:

**Supplemental Figure 1**: Knock-down analysis of *cxcl8-l1* and *cxcl8-l2* using splice-blocking morpholinos. Other two Cxcl8 morpholinos also reduces zebrafish neutrophil recruitment in acute inflammation. (A) Exon/Intron scheme of *cxcl8* genes, MOs and Primers targeting sites. (B) RT-PCR of *cxcl8-l1* and *cxcl8-l2* transcripts detection, in 3 dpf *Tg(mpx:gfp)i114* control and morphant larvae. Insertion of intron 1 in MOs targeting E1/I1 boundary resulted in several premature stop codons and early truncated Cxcl8 proteins (22 of 98 amino acid for Cxcl8-l1 and 24 of 118 amino acids for Cxcl8-l2). (C) Tailfins of *Tg(mpx:gfp)i114* morphants previously microinjected with MO StdC, MO *cxcl8-l1* E2/I2 and MO *cxcl8-l2* I2/E3 were transected at 3 dpf. Representative maximum intensity projections from *widefield fluorescence microscope* micrographs of 3dpf *Tg(mpx:eGFP)i114* control and morphant larvae. Scale bar = 100μm (D) Counts of fluorescent neutrophils at the wound were made at 1, 2, 4 and 6 hpw. Data indicate means ± SEM (n= 30 performed as 3 independent experiments). P values were calculated using two-way ANOVA and Bonferroni multiple comparison test. There were no significant differences in neutrophil number at 1 and 2 hpw for both morphants (P>0.05), but for 4 and 6 hpw a significant decrease were observed (P<0.01) both for MO *cxcl8-l1* E2/I2 and MO *cxcl8-l2* I2/E3 (E) DIC and GFP *widefield fluorescence microscope* micrographs from 3 dpf control and morphants larvae. (F) Total neutrophil counts in whole-larvae for each condition. Each bar represent means ± SEM (n= 20 performed as 2 independent experiments). P values were calculated using one-way ANOVA and Bonferroni multiple comparison test (P>0.05, no significant differences were observed)

**Supplemental Figure 2**: PhagoSight analysis reveals that CXCL8s morphants have increased zebrafish neutrophil recruitment velocities in acute inflammation. Tailfins of *Tg(mpx:gfp)i114* previously microinjected with MO StdC, MO *cxcl8-l1* and MO *cxcl8-l2*
were transacted or not at 3 dpf and 2 min time-lapse movies were made in widefield fluorescence microscope (n=10 larvae as 3 independent experiments for wound conditions (A-F), and n=5 larvae as 2 independent experiments for un-wound conditions (G-L)) followed by Phagotrack analysis, several distinct parameters were analyzed in order to study neutrophil migratory behavior in wound conditions: (A) Oriented Velocity; (B) Oriented Velocity in Translation; (C) Oriented Velocity in Exploration; (D) Lateral Velocity; (E) Lateral Velocity in Translation; (F) Lateral Velocity in Exploration; in un-wound conditions: (G) Meandering Index; (H) Total Number of Tracks; (I) Mean Speed; (J) Lateral Velocity; (K) Oriented Velocity; (L) 3D-Photomicrograph of a typical tracking experiment, with lines indicating the path of neutrophil movement over 6-hour time lapse in un-wound larvae tail fins, colors indicate neutrophil velocity in μm/s. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test, *P<0.05, **P<0.01, and ***P<0.001.

**Supplemental Movie 1:** Representative movie showing neutrophils recruitment to wound in a standard morphant larvae.

**Supplemental Movie 2:** Representative movie showing neutrophils recruitment to wound in a cxcl8-l1 morphant larvae.

**Supplemental Movie 3:** Representative movie showing neutrophils recruitment to wound in a cxcl8-l2 morphant larvae.

**Supplemental Movie 4:** Representative movie showing neutrophil tracks in a wounded standard morphant larvae.

**Supplemental Movie 5:** Representative movie showing neutrophil tracks in a wounded cxcl8-l1 morphant larvae.

**Supplemental Movie 6:** Representative movie showing neutrophil tracks in a wounded cxcl8-l2 morphant larvae.