# Chemokine signaling mechanisms underlying inflammation and infection control: Insights from the zebrafish model

Sommer, F.

# 4 Inhibition of macrophage migration in zebrafish demonstrates *in vivo* efficacy of human CCR2inhibitors

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## Abstract

The chemokine signaling axes CCR2-CCL2 and CXCR3-CXCL11 participate in the inflammatory response by recruiting macrophages to damaged tissue or sites of infection and are, therefore, potential pharmacological targets to treat inflammatory disorders. Although multiple CCR2 orthosteric and allosteric inhibitors have been developed, none of these compounds has been approved for clinical use, highlighting the need for a fast, simple and robust preclinical test system to determine the *in vivo* efficacy of CCR2 inhibitors. Herein we show that human CCL2 and CXCL11 drive macrophage recruitment in zebrafish and that CCR2 inhibitors designed for humans also limit macrophage recruitment in this model organism due to the high conservation of the chemokine system. We demonstrated anti-inflammatory activities of three orthosteric and two allosteric CCR2 inhibitors using macrophage recruitment to injury as a functional readout of their efficiency, while simultaneously evaluating toxicity. These results provide proof-of-principle for screening CCR2 inhibitors in the zebrafish model.

## Introduction

Chemokines and their receptors play central roles in several pathological processes by mediating the recruitment of leukocytes to sites of inflammation [1, 2, 3]. CCR2 (CC chemokine receptor 2) is constitutively expressed on monocytes and macrophages [4], while a small population of natural killer cells, T- cells, endothelial cells, and basophils express the receptor under inflammatory conditions [5, 6, 7]. CCR2 binds primarily to CCL2 (MCP-1), but also to CCL8 (MCP-2), CCL7 (MCP-3), CCL13 (MCP-4), and CCL16 to coordinate the recruitment of cells and orchestrate inflammatory processes essential for the immune response [8, 9, 10]. CCL2 expression is elevated in diseases characterized by chronic inflammation and by increased monocyte infiltration into specific tissues, such as atherosclerosis, multiple sclerosis, Alzheimer's disease and ischemic stroke [10, 11]. Recent work shows that the receptor is also linked to metabolic diseases, including diabetes [12, 13]. Besides CCR2, another chemokine receptor driving leukocyte recruitment to inflammatory sites is CXCR3 [14, 15]. Its function is well-characterized in T-cells but its relevance in macrophage biology and the innate immune response have been explored only recently [14, 15, 16, 17]. Like those of CCR2, the ligands of CXCR3, CXCL9/MIG, CXCL10/IP10, and CXCL11/I-TAC are induced upon several pathological conditions and tissue damage [18, 19, 20]. Therefore, inhibitors of CCR2 and CXCR3 are attractive anti-inflammatory drugs that reduce the

recruitment of monocytes and macrophages to inflammatory foci and serve to treat multiple pathological conditions [11, 16, 17].

The depletion of CCR2 in mice leads to a significant reduction in monocyte recruitment to sites of inflammation [21, 22, 23]. The homologous receptor in zebrafish has been shown to reduce the recruitment of primitive monocytes/macrophages (hereafter referred to as macrophages) upon infection and injury in developing embryos and larvae [18, 24, 25]. Similarly, we have shown that the depletion of Cxcr3.2, the zebrafish homolog of human CXCR3, also reduces macrophage recruitment to injured tissue and infectious foci [14]. Furthermore, we used a human CXCR3 inhibitor to efficiently block Cxcr3.2-mediated recruitment of macrophages in zebrafish [14, 15]. Based on evidence for the conservation of the chemokine signaling axes between human and zebrafish [26], we propose that the zebrafish model could serve as a robust *in vivo* screening platform for human CCR2 inhibitors.

The usefulness of zebrafish for anti-inflammatory drug screens has been demonstrated in several studies [27, 28]. Due to its optical transparency and the wide variety of available molecular tools, the zebrafish model allows the real-time tracking of fluorescently labeled leukocytes at the whole organism level [29]. Zebrafish are small in size, have high fecundity and short generation time, thereby allowing the screening of large and relatively homogeneous sample groups [29, 30, 31]. Compounds can be administered by immersion as zebrafish larvae are in principle permeable to most small molecules and toxicity can be easily accessed through survival curves and tracking of developmental and morphological abnormalities [30]. Large zebrafish families can be stored in relatively small spaces and their housekeeping requirements are cost-effective [29, 30]. Taking all the advantages of the model into consideration, we believe that taking advantage of the non-invasive imaging of live zebrafish after exposure to human CCR2 inhibitors provides a means to identify potential therapeutic compounds and assess their effect on leukocyte properties.

In the present work, we assess the usefulness of the zebrafish model to robustly screen Ccr2 inhibitors using a test panel of known orthosteric and allosteric inhibitors for human CCR2. We show that both zebrafish Ccr2 and Cxcr3.2 participate in the inflammatory response through the recruitment of macrophages, and the simultaneous ablation of both receptors leads to a further decrease in macrophage recruitment than the depletion of a single receptor. Local injection of human CCL2 and CXCL11 proteins into the hindbrain cavity of zebrafish larvae induced macrophage chemotaxis, suggesting that chemokine signaling axes in human and zebrafish are sufficiently conserved to enable interspecies crosstalk. In addition, we show that CCR2 inhibitors efficiently block macrophage recruitment and phenocopy *ccr2* knockdown. Therefore, we demonstrate the feasibility of screening CCR2 inhibitors in zebrafish larvae using macrophage recruitment to injury as a functional read-out of their efficiency.

## Materials and Methods

### Zebrafish lines and husbandry

Zebrafish husbandry and experiments were conducted in compliance with guidelines from the Zebrafish Model Organism Database (http://zfin.org), the EU Animal Protection Directive 2010/63/EU, and the directives of the local animal welfare body of Leiden University (License number: 10612). All transgenic and mutant zebrafish lines used in the present study were generated in the AB/TL background. The homozygous mutant (*cxcr3.2-/-*) and homozygous wildtype (wt) siblings (*cxcr3.2+/+*) derived from the *cxcr3.2*$^{hu6044}$ zebrafish line were crossed into the Tg(*mpeg1:mCherryF*)$^{ump2}$ background to assess macrophage function. The double transgenic line Tg(*mpx:gfp/mpeg1:mCherry-F*) was used to visualize both neutrophils and macrophages, and homozygous mutant (*myd88-/-*) and their homozygous wildtype siblings (*myd88+/+*) of the *myd88*$^{hu3568}$ allele were used to assess *ccl2* and *cxcl11aa* induction upon injury and infection. Zebrafish eggs and larvae were kept at 28.5°C in egg water (60 µg/ml Instant Ocean sea salts and 0.0025% methylene blue) and anesthetized with 0.02% buffered tricaine, (3-aminobenzoic acid ethyl ester; Sigma Aldrich, St. Louis, MO, USA) before injections, tail-amputation, and imaging. To image the hindbrain, larvae were kept in egg water containing 0.003% PTU (1-phenyl-2-thiourea, Sigma Aldrich) to prevent pigmentation.

### Macrophage and neutrophil recruitment to the hindbrain cavity and injury

1 nL of commercially available (PeproTech) human CXCL11 and CCL2 proteins (100 nM) were injected into the hindbrain ventricle of Tg (*mpeg1:mCherryF cxcr3.2+/+)* and Tg (*mpx: gfp/mpeg1:mCherryF cxcr3.2+/+*) larvae at 48 hpf. 1 nL of PBS was injected as a control. For injections with zebrafish Cxcl11aa, the protein was purified as previously described [14]. After 3 hours, larvae were fixed with 4% paraformaldehyde (PFA), the samples were blinded and macrophages and neutrophils within the hindbrain ventricle were counted under Leica TCS SP8 MP confocal microscope (Leica Microsystems) by going through a z-stack of the whole hindbrain ventricle. For the tail-amputation recruitment assay, 10-20 anesthetized 3 dpf larvae were transferred to a 2% agarose covered petri-dish and, using a glass blade, the caudal fin was amputated without damaging the notochord. After amputated larvae were put back into egg water and fixed with 4% PFA 4 hours after amputation. The tail area was imaged with a Leica M165C stereo-florescence microscope and visualized with the LAS AF lite software. The macrophages localized within an area of 200 µm from the cut towards the trunk were considered recruited cells. For all recruitment assays a Kruskal-Wallis test was conducted to assess significance (*$p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$) and corrected with Bonferroni post-hoc test. Data are shown as mean ± SEM.

**Active site sequence homology analyses and functional assessment of ligand-binding specificity and allosteric modulation**

A BLASTp alignment was conducted to assess the overall protein identity on the NCBI public database [32]. Sequence similarity of the critical residues within the allosteric intracellular binding site of the human CCR2 receptor [33] was assessed after multiple sequence alignment of human CCR2, human CXCR3 (ENSG00000186810) and the zebrafish orthologs Ccr2 (ENSDARG00000079829 and ENSDARG00000105363) and Cxcr3.2 (ENSDARG00000041041) in UniProt (uniprot.org) with Clustal Omega version 1.2.4 [34]. Identity was reported as the percentage of identical residues. To assess macrophage recruitment, we injected 1 nL of CCL2 and CXCL11 on their own (100 nM) in the hindbrain of 2 dpf Tg (*mpeg1:mCherryF cxcr3.2+/+)* and a combination of both chemokines (100 nM). To assess allosteric modulation of both receptors, four batches of 10-20 2 dpf larvae were pre-incubated with the allosteric intracellular CCR2 inhibitor CCR2-RA-[R] (25 μM) or DMSO 0.05% (vehicle) for two hours before the chemokines were injected. Similarly, batches of 10-20 larvae were incubated with the allosteric inhibitor or vehicle for three hours following injection. Larvae were fixed with 4% PFA, the samples were blinded and macrophages in the hindbrain cavity were counted under a Leica TCS SP8 MP confocal microscope (Leica Microsystems) by going through a z-stack of the entire area. A Kruskal-Wallis test was conducted to assess significance (*$p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$) and data are shown as mean ± SEM.

**RNA extraction and purification, cDNA synthesis, and quantitative PCR analysis**

Three biological samples of 10 wt (*myd88+/+)* and *myd88* mutant *(myd88-/-)* 3 dpf larvae were collected at 4 hours post-tail-amputation in QIAzol lysis reagent (Qiagen). The same was done with *myd88*+/+ and *myd88-/-* 2 dpf larvae at 4 days post-systemic-infection with the *Mycobacterium marinum* M-strain. For infection, the M-strain was grown and freshly prepared for injection as described in [35]. RNA was extracted using the miRNeasy mini kit (Qiagen) following the manufacturer's instructions. cDNA was generated using the iScript™ cDNA Synthesis Kit (Bio-Rad) and qPCR reactions were run on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) using iTaq™ Universal SYBR® Green Supermix (Bio-Rad). Three technical replicates were done for every biological sample. The cycling conditions were: 3 min pre- denaturation at 95°C, 40 denaturation cycles for 15 sec at 95°C, annealing for 30 sec at 60°C (for all primers), and elongation for 30 sec at 72°C. We used the housekeeping gene *ppiab* (peptidylprolyl isomerase Ab) and analyzed the data with the $2–\Delta\Delta Ct$ method**.** A One-way ANOVA was used to test for significance and data are plotted as mean ± SEM (ns $p > 0.05$, * $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$).

The primers used were:
*ppiab* Fw: 5'-ACACTGAAACACGGAGGCAAAG-3',
*ppiab* Rv: 5'-CATCCACAACCTTCCCGAACAC-3',
*ccl2 Fw:* 5'-GTCTGGTGCTCTTCGCTTTC-3',
*ccl2: Rv:* 5'-TGCAGAGAAGATGCGTCGTA-3',
*cxcl11aa Fw*: 5'-ACTCAACATGGTGAAGCCAGTGCT-3', *and*
*cxcl11aa Rv*: 5'-CTTCAGCGTGGCTATGACTTCCAT-3'.

**_ccr2_ morpholino injections**

1 nL (0.5 mM) of a previously described *ccr2* morpholino (5'-AACTACTGTTTTGT-GTCGCCGAC-3') [18] targeting the beginning of the translational site of the gene (ENSDARG00000079829) was injected into the yolk of fertilized zebrafish eggs at the 1-2 cell stage.

**Functional screening of Ccr2 inhibitors**

To use zebrafish as a screening platform for CCR2 inhibitors, we designed a simple workflow consisting of three steps: a two-hour pre-incubation of 3 dpf larvae with the compound of interest at a given concentration, followed by tail-amputation, and a 4-hour incubation with the compound at the same concentration as in the pre-incubation step. Amputated larvae were fixed using 4% PFA and imaged with a Leica M165C stereo-florescence microscope. The macrophages localized within an area of 200 μm from the cut towards the trunk were considered recruited cells. All samples were blinded before imaging. Incubation with DMSO 0.05% was used as a negative control. We assessed the effect of three orthosteric (BMS22 [36], INCB3344 [37, 38], and RS504393 [39]) and three allosteric intracellular CCR2 inhibitors (CCR2-RA-[R] [33, 40, 41], JNJ27141491 [42], and SD-24 [43] on macrophage migration to the injury at an initial concentration of 100 μM. Both BMS22 and RS504393 were purchased from Tocris Bioscience (Abingdon, UK), while the other antagonists were synthesized in-house according to published methods [44, 41, 38, 43]. The compounds that effectively reduced macrophage migration to the injury in tail-amputated larvae were tested using half the concentration in subsequent steps until the compounds were no longer effective. Toxicity was reported as the percentage of larvae that survive after each step in the procedure. When the compounds were toxic, the concentration was halved until toxicity was low and macrophage migration was still reduced. Fine-tuning efficiency and toxicity yielded the optimal concentration for each compound. A Kruskal-Wallis test was conducted to assess significance (*$p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$) in macrophage recruitment data and data are shown as mean ± SEM. Survival tests were conducted to estimate toxicity.

## Results

### The Ccr2-Ccl2 and Cxcr3.2-Cxcl11aa chemokine axes contribute to inflammation in zebrafish.

To determine the contribution of the Ccr2-Ccl2 and Cxcr3.2-Cxcl11aa chemokine axes to different inflammatory stimuli, we analyzed *ccl2* and *cxcl11aa* expression in response to infection or wounding, and asked if the induction of these genes is dependent on Myd88 (myeloid differentiation response gene 88), known as a universal TLR-adaptor molecule implicated in the inflammatory response towards pathogens and damage [45, 46]. Our data show that *ccl2* (**Figure 1 A, B**) and *cxcl11aa* (**Figure 1C, D**) are both induced in wt zebrafish larvae upon infection with a mycobacterial pathogen, *M. marinum* (*Mm*), and upon injury by means of tail amputation. In contrast, the induction of these genes is abolished in *myd88* mutant larvae. These data show that *myd88* is required for *ccl2* and *cxcl11aa* induction and suggest that both the Ccr2-Ccl2 and Cxcr3-Cxcl11 axes are implicated in the response to wounding and *Mm* infection. We and others previously showed that macrophage recruitment is reduced in *cxcr3.2* mutant larvae and under knockdown conditions of *ccr2* [14, 24, 25]. We injected wt and *cxcr3.2* mutants with *ccr2* morpholino [18] to examine whether the absence of both chemokine receptors would result in a further reduction in macrophage recruitment than the absence of a single receptor. Macrophage recruitment following tail amputation was reduced in morpholino-injected wt and *cxcr3.2* mutants compared to PBS-injected controls (**Figure 1E-F**), confirming that the lack of both receptors has a bigger impact on the inflammatory response than the absence of a single receptor.

### Human CCL2 and CXCL11 chemokines specifically attract macrophages in zebrafish larvae.

To functionally assess whether human chemokines exert their chemoattractant activity in zebrafish, we locally injected the macrophage-specific attractants CCL2 and CXCL11 into the hindbrain of zebrafish and quantified the macrophages within the cavity after 3 hours. Both CCL2 and CXCL11 efficiently recruited macrophages as compared to vehicle (PBS) controls (**Figure 2-A-B**). To rule out that macrophage recruitment was triggered in a non-specific manner due to the injection of heterologous chemokine proteins, we injected the neutrophil-specific chemokine CXCL8 and observed that neutrophils, but not macrophages, were recruited to the hindbrain (**Figure 2-C-D**), confirming that human chemokines induce cell-specific chemotaxis of macrophages or neutrophils in zebrafish. We previously described the purification of a zebrafish CXCL11 homolog, named Cxcl11aa [14]. There was no significant difference in the chemoattractant properties of human CXCL11 and zebrafish Cxcl11aa in macrophage recruitment to the hindbrain (**Figure 2-E**), therefore, these chemokines were used indistinctively through-

out this study.

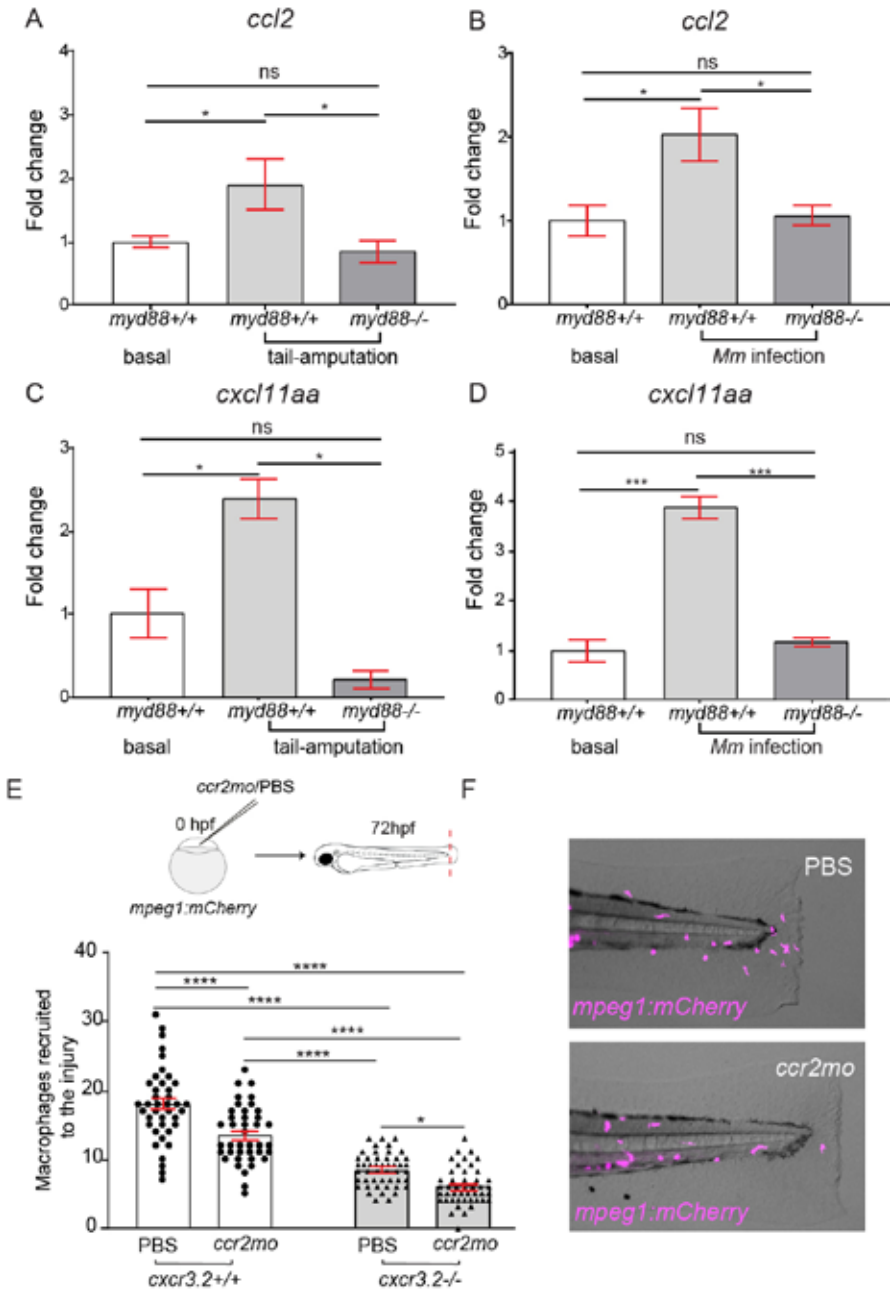


**Figure 1. The Ccl2/Ccr2 and Cxcl11aa/Cxcr3.2 chemokine axes contribute to inflammation in zebrafish.** Quantitative PCR data on whole larvae show that both *ccl2* **(A-B)** and *cxcl11aa* **(C-**

**D)** are induced upon injury (tail amputation) and infection in wt zebrafish but not in *myd88* mutant larvae. Knockdown of *ccr2* and mutation of *cxcr3.2* results in reduced macrophage recruitment upon injury and the depletion of both receptors further decreases recruitment (**E**), with representative images showing the areas of macrophage (*mpeg1:mCherry*) recruitment quantification in the tail amputation assay (**F**). The graphs show the pooled data of three independent replicates. The qPCR results were analyzed with the 2–ΔΔCt method and a two-way ANOVA. A Kruskal-Wallis test was used to test for significance in the recruitment assays. Results are plotted as mean ± SEM (ns $p>0.05$, * $p\leq0.05$,*** $p \leq 0.001$).

## The human CCR2 inhibitor CCR2-RA-[R] inhibits macrophage recruitment in zebrafish

We assessed whole protein identity shared between CCR2 and CXCR3 as well as with the two zebrafish Ccr2 genes and Cxcr3.2 through a BLASTp analysis [32]. Human CCR2 shares 44% (ENSDARG00000079829) and 43% (ENSDARG00000105363) identity with the two zebrafish Ccr2 genes, and 34% and 30% with human CXCR3 and zebrafish Cxcr3.2, respectively (**Figure 3A** top). Based on the binding mode of the human CCR2 allosteric inhibitor CCR2-RA-[R] shown in the crystal structure of human CCR2 [33], we assessed the similarity between the intracellular binding site of the CCR2 and CXCR3 receptors in humans and zebrafish. The key residues for CCR2-RA-[R]-binding in human CCR2 are highly conserved in zebrafish Ccr2 (70.5% identity in both genes), but also in human CXCR3 (65% identity) and zebrafish Cxcr3.2 (59% identity) (**Figure 3A** bottom).

Due to the high similarity between the allosteric intracellular binding sites, we used CCR2-RA-[R] to test inhibition of chemokine-induced macrophage recruitment by both Ccr2 and Cxcr3.2 . We observed reduced CCL2-mediated macrophage recruitment when larvae were incubated with CCR2-RA-[R], whereas CXCL11-mediated recruitment remained unaffected upon CCR2-RA-[R] treatment. The co-injection of CCL2 and CXCL11 did not detectably enhance macrophage recruitment compared to CCL2 alone, and CCR2-RA-[R] incubation reduced recruitment to a similar level as that elicited by CXCL11, consistent with only Ccr2-mediated recruitment being affected by the inhibitor (**Figure 3B**). The inhibition of Ccr2-mediated macrophage recruitment by CCR2-RA-[R] phenocopied knockdown of *ccr2* (**Figure 3C**). Taken together, these data demonstrate that zebrafish Ccr2 is inhibited by an allosteric inhibitor designed for human CCR2, and suggest that CCR2-RA-[R] does not inhibit Cxcr3.2 at the concentration tested.

## Zebrafish is a powerful screening platform for human CCR2 inhibitors *in vivo*.

To further assess the suitability of the zebrafish model for screening CCR2 inhibitors, we developed a work-flow to screen a test panel of compounds using macrophage re-

cruitment to injury as a functional read-out of their efficiency. We pre-incubated a batch of 50 zebrafish larvae with each of the compounds of interest for two hours after which we proceeded to amputate the tail fin and incubated the amputated larvae in the compound for another 4 hours. We used DMSO (vehicle) as a control for all incubations. Thereafter, we fixed the larvae and imaged and quantified macrophages recruited to the damaged area.

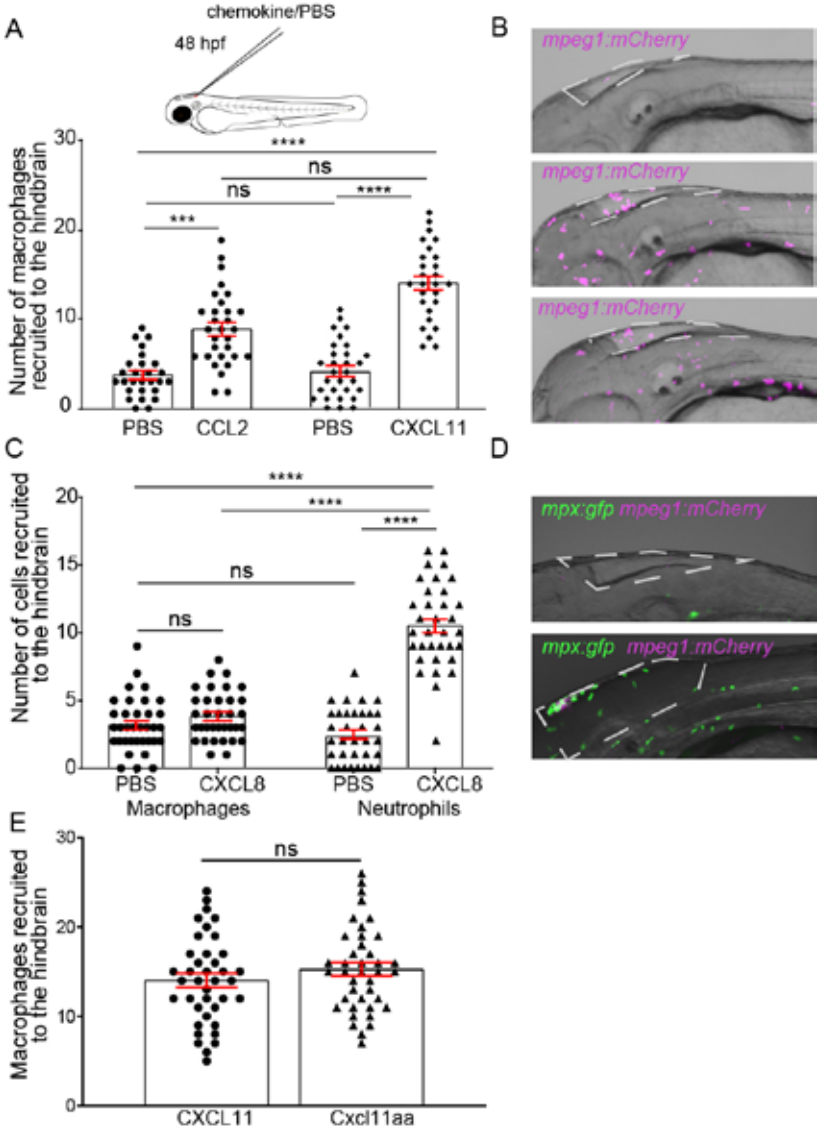


**Figure 2. Locally injected human CCL2 and CXCL11 proteins attract macrophages to the hindbrain ventricle of zebrafish larvae.** Macrophages (*mpeg1:mCherry*) are recruited to the hind-

brain ventricle of zebrafish larvae 3 hours after injection with human CCL2 and CXCL11 proteins as compared to PBS control injection (**A**), The area of quantification is outlined in representative images (**B**). Neutrophils (*mpx:gfp*) but not macrophages (*mpeg1:mCherry*) are recruited to the hindbrain after injection of Cxcl8 as compared to PBS control injection (**C**), with the area of quantification outlined in representative images. Human CXCL11 and zebrafish Cxcl11aa showed no difference in their macrophage chemoattractant properties in the hindbrain recruitment assay (**E**). Statistical analyses were done with pooled data of three independent replicates (10-15 larvae each). A Kruskal-Wallis (A-D) and a Mann Whitney (E) test were used to assess significance (* $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$) and data are shown as mean ± SEM.

To determine test concentrations, we performed toxicity evaluations. We found allosteric compounds to be more toxic than orthosteric inhibitors (**Supplementary Figure 1C-F and Supplementary Table 1)**. The allosteric inhibitors JNJ-27141491 and SD-24 were toxic at concentrations above 10 μM (15 μM and 20 μM) and killed most larvae after the 2-hour pre-incubation step (**Supplementary Figure 2C**). CCR2-[RA]-R had no toxic effects (**Supplementary Figure 1C-F and Supplementary Table 1)** at any stage of the procedure and showed optimal results at 25 μM (**Figure 5 A).** SD-24 was still toxic at 10 μM (**Supplementary Figure 1D and Supplementary Table 1)** but efficiently reduced macrophage recruitment at 5 μM and 1 μM, while JNJ-27141491 was not toxic at concentrations under 10 μM (**Supplementary Table 1)** but failed to reduce macrophage recruitment (**Figure 5 B**). Three orthosteric inhibitors, BMS22, INCB3344, and RS504393, all showed only low toxicity (**Supplementary Figure 1A-B and Supplementary Table 1**) and effectively reduced macrophage recruitment to injury at 100 μM (**Figure 5C**). BMS22 and INCB3344 were still effective at 50 μM but RS504393 no longer affected recruitment at that concentration (**Figure 5D**). None of the orthosteric inhibitors reduced macrophage recruitment at concentrations <25 μM. Despite that testing of some compounds was limited by toxicity, our data show that the zebrafish model serves as a robust *in vivo* screening platform for human CCR2 inhibitors.

## Discussion

The CCR2-CCL2 chemokine signaling axis is associated with a wide variety of inflammatory diseases and is therefore considered an attractive target for anti-inflammatory drugs [10, 7, 47]. Both orthosteric and allosteric CCR2 inhibitors have been developed, but none of these have demonstrated sufficient efficacy for clinical use [11, 48, 49]. This illustrates the need for efficient preclinical test systems to determine the *in vivo* efficacy of CCR2 inhibitors. Here we present the zebrafish model as an *in vivo* screening platform for CCR2 inhibitors, which enables tracking leukocyte recruitment in a live organism while simultaneously assessing toxicity. We show that two primary signaling axes, Ccr2-Ccl2 and Cxcr3-Cxcl11, contribute to the inflammatory response in zebrafish larvae by mediating macrophage recruitment. We present evidence for cross-species conservation of these chemokine signaling axes, as shown by the compatibility of human chemokines and zebrafish receptors in eliciting macrophage recruitment.

A

| CCR2 | Ccr2 | CXCR3 | Cxcr3.2 |
|---|---|---|---|
| Overall protein identity (%) | 44.38<br>43.36 | 30 | 34.71 |

| Residue number | 63 | 67 | 77 | 81 | 134 | 138 | 237 | 240 | 241 | 244 | 245 | 305 | 309 | 310 | 311 | 312 | 315 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human CCR2 | V | L | T | L | L | R | K | R | A | V | I | Y | G | E | K | F | Y |
| Zebrafish Ccr2 | V | L | T | L | M | R | K | K | V | L | I | Y | G | E | K | F | A |
| Human CXCR3 | V | L | T | L | I | R | R | R | A | L | V | Y | G | V | K | F | R |
| Zebrafish Cxcr3.2 | V | I | T | L | I | R | Q | R | S | L | I | Y | G | V | K | F | E |

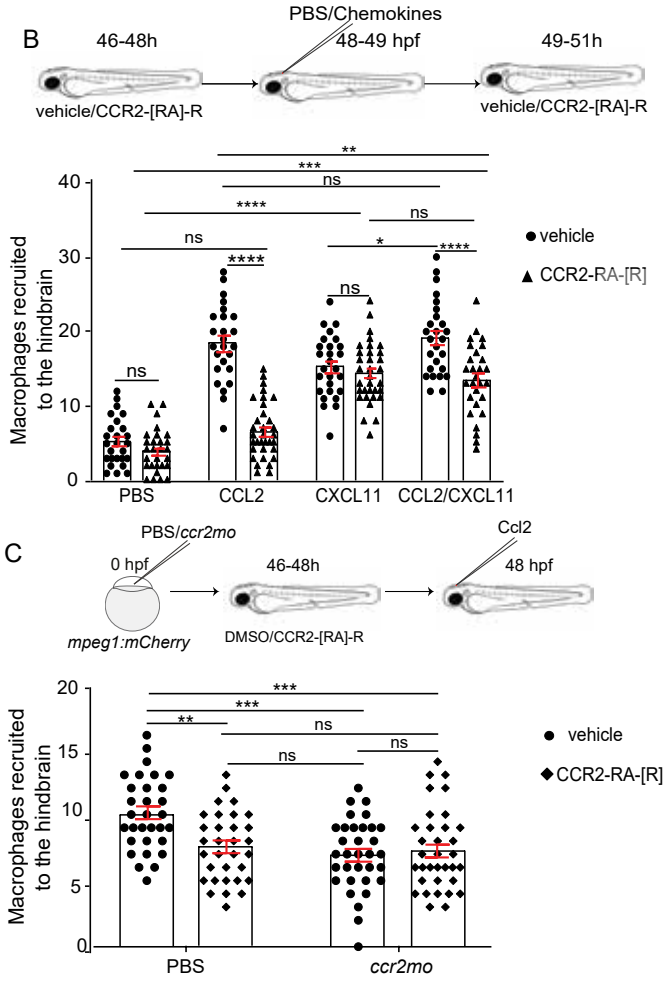


**Figure 3. The human CCR2 inhibitor CCR2-RA-[R] inhibits macrophage recruitment in zebrafish.** Comparison between whole protein sequences (top) and key residues involved in the intracellular binding of CCR2-RA-[R] in human CCR2 (bottom). Critical residues for intracellular ligand-binding in human CCR2 are highly conserved in CXCR3, zebrafish Ccr2 (identical in both variants) and zebrafish Cxcr3.2 (**A**). Zebrafish larvae were pre-incubated for two hours in DMSO 0.05% (vehicle) or CCR2-RA-[R]. Following incubation, the human chemokines CCL2 and CXCL11 or a PBS

control were injected into the hindbrain ventricle and the larvae were immediately incubated in vehicle/CCR2-RA-[R] for another three hours. CCL2-induced macrophage recruitment was ablated in larvae incubated with CCR2-RA-[R] (triangles) compared with the vehicle incubation (dots). All chemokine-injected larvae recruited macrophages to the hindbrain (not shown) and only relevant differences are shown in the image. CXCL11-induced macrophage recruitment was unaffected by CCR2-RA-[R] treatment. CCR2-RA-[R] treatment reduced macrophage recruitment induced by CCL2/CXCL11 co-injection, but not to the same extent as in CCL2 injection alone (**B**). Macrophage recruitment was reducen in PBS- injected larvae incubated in CCR2-RA-[R] to similar levels as *ccr2mo*-injected larvae incubated either in PBS or in the inhibitor (**C**). Three independent replicates (10-12 larvae each) were pooled to conduct a Kruskal-Wallis test (* $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$) and data are shown as mean ± SEM.

Due to the high degree of conservation, we were able to demonstrate anti-inflammatory effects in zebrafish larvae using both orthosteric and allosteric CCR2 inhibitors designed for humans. These results show that the zebrafish can be implemented as a fast, robust, and efficient screening platform and contribute to speeding up the assessment of novel CCR2 inhibitory drug candidates and their *in vivo* efficacy.

The zebrafish homologs of CCR2 and CXCR3, named Ccr2 and Cxcr3.2, have previously been implicated in the recruitment of macrophages to injury and infection [14, 15, 24, 25], but the interaction between these receptors has not been addressed. Here, we show that knockdown of Ccr2 reduces macrophage recruitment not only in wt larvae but also in Cxcr3.2-deficient mutants, indicating the presence of a Ccr2-expressing macrophage population that functions independently of Cxcr3.2 in wound-induced migration. This is consistent with previous work suggesting that these receptors might recruit different macrophage populations [47, 18]. Studies in zebrafish reported that Ccr2 mediates the recruitment of circulating monocytes but not tissue-resident macrophages in the context of mycobacterial infection [18, 24]. In mammals, CCR2-CCL2/7 interactions are considered essential mediators of the egress of macrophages from the bone marrow into the peripheral circulation [47]. It has also been described that the expression levels of the CCR2 receptor change in the course of macrophage differentiation, where monocytes constitutively express CCR2 but the receptor is downregulated in fully differentiated macrophages [4]. Therefore, functionally distinct monocyte/macrophage populations that differentially express Ccr2 may also be present in the developing zebrafish larvae, but these remain to be characterized. Although different populations may be present, Ccr2 knockdown significantly reduced the overall migration of monocytes/macrophages, which makes it possible to use zebrafish larvae as a simple *in vivo* model to evaluate CCR2 inhibitors.

Based on previous observations showing that an allosteric human CXCR3 inhibitor works in zebrafish [14, 15], we set out to test human CCR2 inhibitors in this model. We showed that both orthosteric and allosteric CCR2 inhibitors efficiently reduce Ccr2-mediated macrophage recruitment in zebrafish and that this inhibitory effect phe-

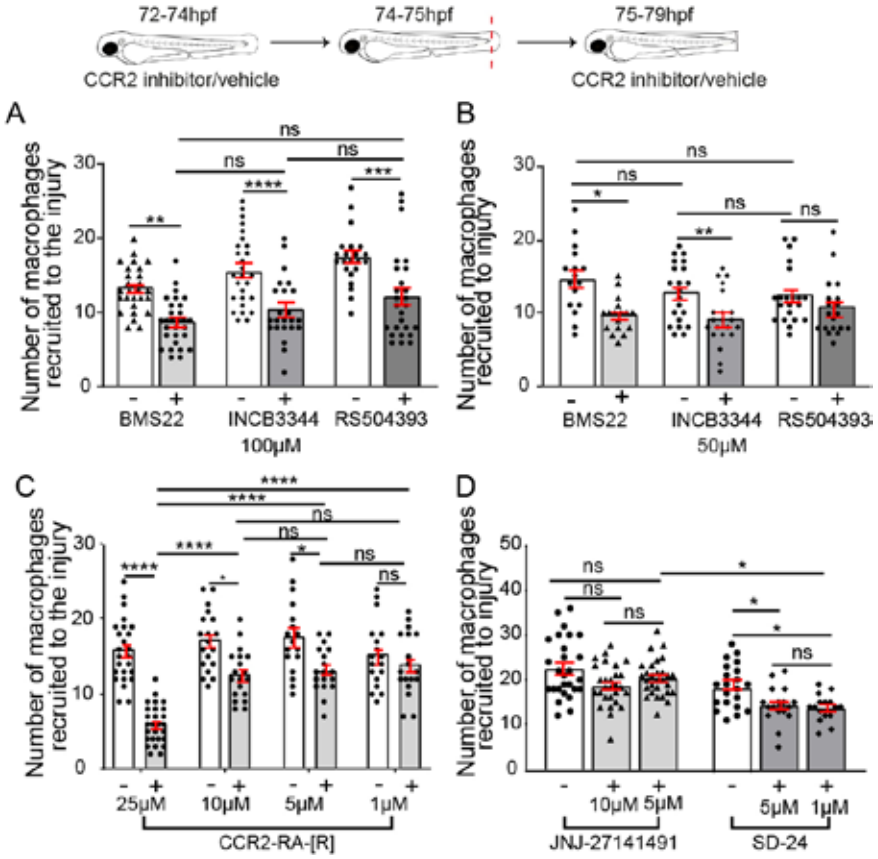


**Figure 4. Zebrafish is a powerful screening platform for CCR2 inhibitors.** Orthosteric CCR2 inhibitors BMS22, INCB3344, and RS504393 reduce macrophage recruitment to injury at a concentration of 100 µM (**A**). BMS22 and INCB3344 also reduce macrophage recruitment at 50 µM but RS504393 no longer exerts an inhibitory effect (**B**). The allosteric inhibitor CCR2-RA-[R] reduces macrophage recruitment at concentrations ranging from 5-25 µM without toxic effects at any stage. The compound is not effective at 1 µM (**C**). The allosteric inhibitors JNJ-27141491 and SD-24 showed high toxicity at concentrations >10 µM and >5 µM, respectively (**Supplementary Figure 1 and Supplementary Table 1**). SD-24 efficiently reduced macrophage recruitment in concentrations ranging from 1-5µM- while JNJ-27141491 is not effective at <10 µM (**D**). Survival was assessed after every stage of the process to assess toxicity (**Supplementary Figure 1, Supplementary Table 1**). Statistical analyses were done with pooled data of three independent replicates (10-15 larvae each). A Kruskal-Wallis test was used to assess significance (* $p \leq 0.05$, ** $p \leq 0.01$ *** $p \leq 0.001$, **** $p \leq 0.0001$) and data are shown as mean ± SEM.

nocopies *ccr2* downregulation. All orthosteric inhibitors tested (BMS22, INCB3344, and RS504393) blocked wound-induced macrophage recruitment effectively, as expected considering the high degree of conservation of the ligand-binding pockets of the human and zebrafish receptors. The allosteric CCR2-inhibitor CCR2-RA-[R] specifically

reduced Ccr2-mediated recruitment of macrophages and did not affect Cxcr3.2-mediated recruitment, indicating that CCR2-RA-[R] does not bind to Cxcr3.2 We could also demonstrate inhibitory activity for another allosteric inhibitor (SD-24), but not for a third one (JNJ-27141491), probably due to major differences in key amino acids between the human and zebrafish receptors. On the other hand, the concentration range at which these inhibitors could be tested was narrow due to their toxic effects. The sensitivity of developing zebrafish larvae to toxicity is a limiting factor in all compound screens. However, the simultaneous assessment of drug efficacy and toxicity in zebrafish assays also provides useful information for drug development, which can directly lead to optimizing the production of low toxicity derivatives.

Considering that CCR2 and CXCR3 are often thought to contribute together to inflammatory disease pathologies [21, 22, 23] and that the zebrafish homologs of both receptors drive wound-induced macrophage recruitment, zebrafish larvae could also provide a screening platform to test combinations of inhibitors specific for these receptors.

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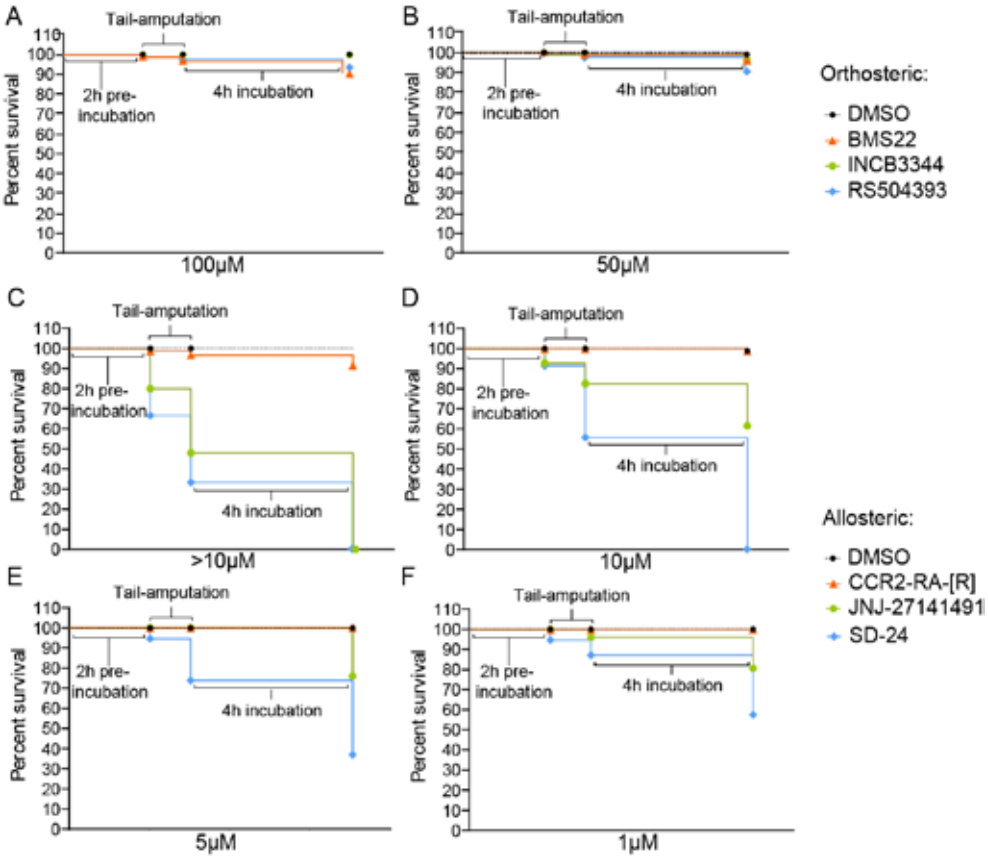
## Supplementary materials




**Supplementary Figure 1. Orthosteric CCR2 inhibitors have no toxic effects on zebrafish larvae.** All the orthosteric CCR2 inhibitors were safe and had no toxic effects on 3-day-old zebrafish larvae at concentrations of 100 µM and 50 µM (**A-B**). The allosteric inhibitor CCR2-RA-[R] had no toxic effects at concentrations ranging from 5-25µM- (**C-F**). The allosteric compound JNJ-27141491 was toxic at concentrations >10 µM (**C**) and SD-24 at >5 µM (**C-D**). The former was safe when used at concentrations <10 µM (**E**) and the latter at <5 µM (**F**).

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