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Developmental and Comparative Immunology 116 (2021) 103932

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

Full Length Article

Inhibition of macrophage migration in zebrafish larvae demonstrates *in vivo* efficacy of human CCR2 inhibitors

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ARTICLE INFO

Keywords:
Chemokine receptor
Anti-inflammatory
Inhibitor
Macrophages
In vivo
Screening

ABSTRACT

The chemokine signaling axes CCR2-CCL2 and CXCR3-CXCL11 participate in the inflammatory response by recruiting leukocytes 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 larvae 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 read-out of their efficiency, while simultaneously evaluating toxicity. These results provide proof-of-principle for screening CCR2 inhibitors in the zebrafish model.

1. Introduction

Chemokines and their receptors play central roles in several pathological processes by mediating the recruitment of leukocytes to sites of inflammation (Charo and Ransohoff, 2006; Thelen and Stein, 2008; Zabel et al., 2015). CCR2 (CC chemokine receptor 2) is constitutively expressed on monocytes and macrophages (Fantuzzi et al., 1999), while a small population of natural killer cells, T-cells, endothelial cells, and basophils express the receptor under inflammatory conditions (Helden et al., 2012; Mack et al., 2005; Connor et al., 2004). 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 (Boring et al., 1997; Lu et al., 1998; Chu et al., 2014). 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 (Chu et al., 2014; Xia and Sui, 2009). Recent work shows that the receptor is also linked to metabolic diseases, including diabetes (Weisberg et al., 2006; Kolattukudy and Niu, 2012).

Besides CCR2, several other chemokine receptors are important for driving leukocyte recruitment to inflammatory sites (Charo and Ransohoff, 2006; Lacotte et al., 2009; López-Cotarelo et al., 2017). One of the key players is CXCR3, which is expressed on a variety of immune and non-immune cell types and has been studied widely in relation to cancer inflammation, chronic inflammatory disorders, and autoimmune diseases (Aloyouny et al., 2020; Altara et al., 2015; Karin, 2020) While CXCR3 is best known for its role in adaptive immunity, especially T-cell responses, this receptor also contributes to the functions of several innate immune cells types, such as mast cells, basophils, and macrophages (Torraca et al., 2015; Sommer et al., 2020; Groom and Luster, 2011; Lu et al., 2017; Brightling et al., 2005; Ruschpler et al., 2003). 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 (Cambier et al., 2017; Chung et al., 2016; Lee et al., 2015). Therefore, inhibitors of CCR2 and CXCR3 are attractive anti-inflammatory drugs that reduce the recruitment of leukocytes to inflammatory foci and serve to treat multiple pathological conditions (Xia and Sui, 2009; Groom and Luster, 2011; Lu et al., 2017).

In the present study, we focus on the role of CCR2 and its interaction with CXCR3 in the recruitment of monocytes/macrophages. The depletion of CCR2 in mice leads to a significant reduction in monocyte recruitment to sites of inflammation (Tanuma et al., 2006; Veillard et al., 2005; Tokuyama et al., 2005). Notwithstanding the evolutionary distance between human and zebrafish, it has been shown that the human

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https://doi.org/10.1016/j.dci.2020.103932
Received 7 September 2020; Received in revised form 3 November 2020; Accepted 17 November 2020
Available online 22 November 2020

Abbreviations

Cxcr3.2	zebrafish paralog of the human CXCR3 chemokine receptor
DMSO	Dimethylsulfoxid
I-TAC	interferon-inducible T-cell alpha chemoattractant (CXCL11)
IP10	Interferon gamma-induced protein 10 (CXCL10)
MCP1, 2, 3, 4	monocyte chemoattractant protein-1, 2, 3, 4 (CCL2, 8, 7, 13)
MIG	monokine induced by gamma interferon
MPEG	macrophage-expressed gene
MPX	myeloperoxidase
PBS	Phosphate-buffered saline

ligand, CCL2, can recruit primitive monocytes/macrophages (hereafter referred to as macrophages) through a CCR2 receptor homolog in zebrafish embryos (Cambier et al., 2014). Furthermore, knockdown of this receptor has been shown to reduce the recruitment of primitive macrophages upon infection and injury in developing zebrafish embryos and larvae, showing that the role of CRR2 in the inflammatory response is preserved from fish to mammals (Cambier et al., 2017; Cambier et al., 2014; Xie et al., 2019). Cxcr3 occurs in three variants in zebrafish, among which Cxcr3.2 can be considered the functional homolog of human CXCR3 in zebrafish larval macrophages and neutrophils, whereas Cxcr3.1 is not detectably expressed at this developmental stage and Cxcr3.3 is an atypical receptor antagonizing Cxcr3.2 function (Torraca et al., 2015; Sommer et al., 2020). Similar to Ccr2, we have shown that the depletion of Cxcr3.2 also reduces macrophage recruitment to injured tissue and infectious foci (Torraca et al., 2015). Furthermore, we used a human CXCR3 inhibitor to efficiently block Cxcr3.2-mediated recruitment of macrophages in zebrafish embryos (Torraca et al., 2015; Sommer et al., 2020). These and other studies have demonstrated a significant degree of functional conservation of chemokine signaling axes between human and zebrafish, supporting the growing use of zebrafish larvae as a model for human immune-related diseases such as inflammatory disorders, infectious diseases, and cancer (Sommer et al., 2020; Oliveira et al., 2013; Powell et al., 2018; Patton and Tobin, 2019; Brugman et al., 2014; Bussmann and Raz, 2015; Gabellini et al., 2018). Furthermore, exploiting this evolutionary functional conservation, we propose that the zebrafish larval model could complement cell-based screens and serve as a robust *in vivo* platform to evaluate the efficacy of human CCR2 inhibitors in a tissue context.

The usefulness of zebrafish larvae for anti-inflammatory drug screens has been demonstrated in several studies (Loynes et al., 2010; Isles et al., 2019). Due to its optical transparency and the wide variety of available molecular tools, zebrafish embryo/larval models allow the real-time tracking of fluorescently labeled leukocytes at the whole organism level (Mathias et al., 2012). Zebrafish are small in size, have high fecundity, and short generation time, thereby allowing the screening of large and relatively homogeneous sample groups (Mathias et al., 2012; Tan and Zon, 2011; Yang et al., 2014). 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 (Tan and Zon, 2011). Large zebrafish families can be stored in relatively small spaces and their housekeeping requirements are cost-effective (Mathias et al., 2012; Tan and Zon, 2011). Taking all the advantages of the model into consideration, we believe that taking advantage of the non-invasive imaging of live zebrafish larvae 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 larval 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 ventricle 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.

2. Materials and methods

2.1. 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 embryos 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.

2.2. Macrophage and neutrophil recruitment to the hindbrain ventricle 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 [Vincenzo]. After 3 h, 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 h after amputation. The tail area was imaged with a Leica M165C stereofluorescence 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 data are shown as mean ± SEM.

2.3. 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 (Wheeler et al., 2007). Sequence similarity of the critical residues within the allosteric intracellular binding site of the human CCR2 receptor (Zheng et al., 2016) 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 (Sievers et al., 2011). 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 2 h before the chemokines were injected. Similarly, batches of 10–20 larvae were incubated with the allosteric inhibitor or vehicle for 3 h following injection. Larvae were fixed with 4% PFA, the samples were blinded and macrophages in the hindbrain ventricle 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.

2.4. 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 h 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 (Benard et al., 2012). 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 s at 95 °C, annealing for 30 s at 60 °C (for all primers), and elongation for 30 s at 72 °C. We used the housekeeping gene *ppiab* (peptidylprolyl isomerase Ab) and analyzed the data with the 2–ΔΔCt 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′-ACTCAACATGGTGAAGCCAGT GCT-3′, *and cxcl11aa Rv*: 5′-CTTCAGCGTGGCTATGACTTCCAT-3'.

2.5. ccr2 morpholino injections

1 nL (0.5 mM) of a previously described *ccr2* morpholino (5′-AACTACTGTTTTGTGTCGCCGAC-3′) (Cambier et al., 2017) 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.

2.6. Functional screening of Ccr2 inhibitors

To use zebrafish larvae as a screening platform for CCR2 inhibitors, we designed a simple workflow consisting of three steps: a 2-h pre-incubation of 3 dpf larvae with the compound of interest at a given concentration, followed by tail-amputation, and a 4-h 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-fluorescence 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 (Cherney et al., 2008), INCB3344 (Brodmerkel et al., 2005; Xue et al., 2010), and RS504393 (Mirzadegan et al., 2000)) and three allosteric intracellular CCR2 inhibitors (CCR2-RA-[R] (Zheng et al., 2016; Dasse et al., 2007; Zou et al., 2007), JNJ27141491 (Buntinx et al., 2008), and SD-24 (Peace et al., 2010) 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 (Doyon et al., 2008; Zou et al., 2007; Xue et al., 2010; Peace et al., 2010). 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.

3. Results

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

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 (Burns et al., 1998; Kawai et al., 1999). Our data show that *ccl2* (Fig. 1 A, B) and *cxcl11aa* (Fig. 1C and 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* (Torraca et al., 2015; Cambier et al., 2014; Xie et al., 2019). We injected wt and *cxcr3.2* mutants with *ccr2* morpholino (Cambier et al., 2017) 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 (Fig. 1E and F), confirming that the lack of both receptors has a bigger impact on the inflammatory response than the absence of a single receptor.

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 larvae 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

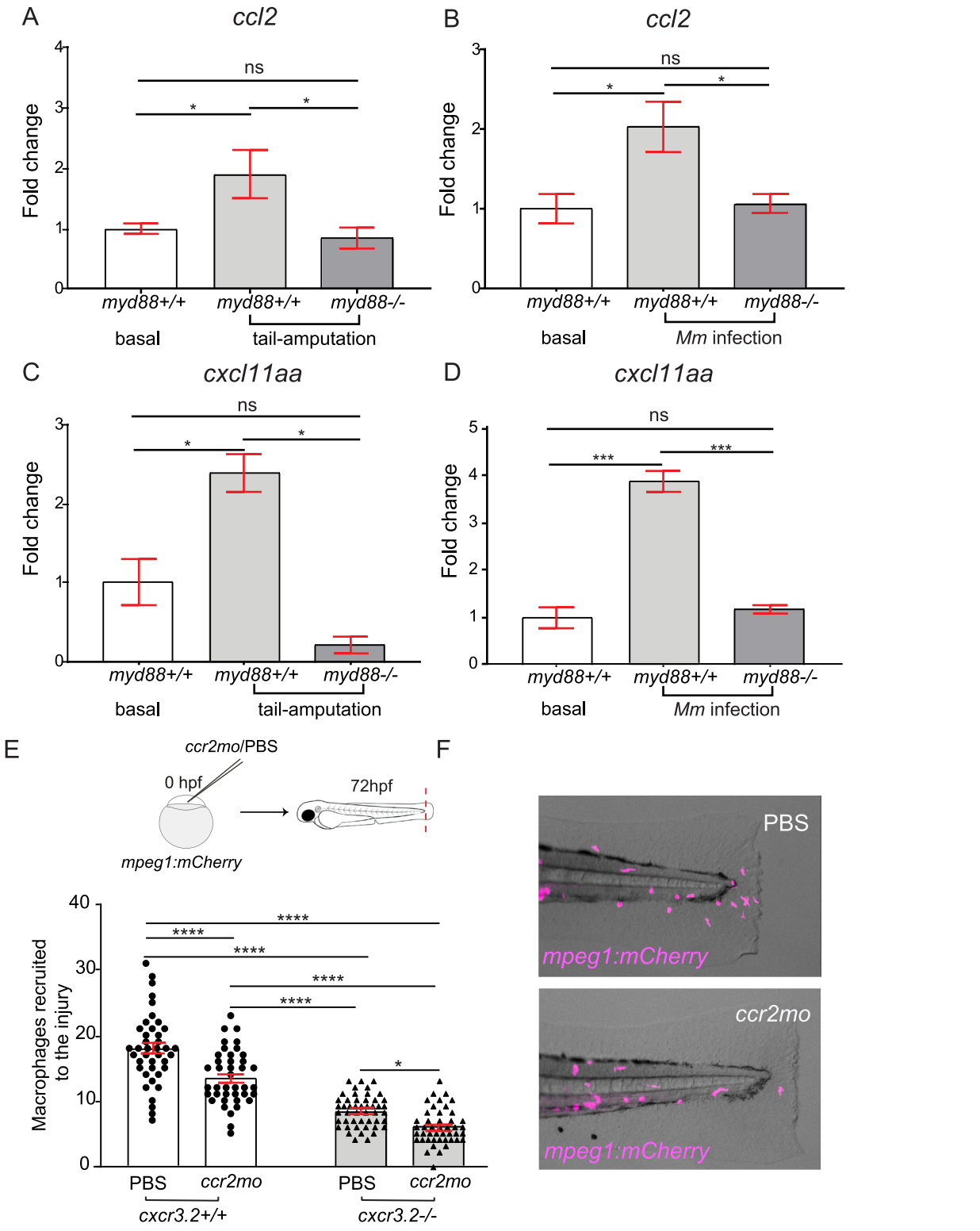

Fig. 1. The Ccl2/Ccr2 and Cxcl11aa/Cxcr3.2 chemokine axes contribute to inflammation in zebrafish larvae.

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 $p > 0.05$, *$p \leq 0.05$,***$p \leq 0.001$).

3.2. 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 embryos and quantified the macrophages within the ventricle after 3 h. As previously reported (Cambier et al., 2014), CCL2 efficiently recruited

macrophages as compared to vehicle (PBS) controls (Fig. 2-A-B). A similar level of macrophage recruitment was triggered by CXCL11 (Fig. 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 human chemokine CXCL8, which is a neutrophil-chemoattractant, and observed that neutrophils, but not macrophages, were recruited to the hindbrain (Fig. 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 (Torraca et al., 2015). There was no significant difference in the chemoattractant properties of human CXCL11 and zebrafish Cxcl11aa in macrophage recruitment to the hindbrain (Fig. 2-E-F), therefore, these chemokines were used indistinctively throughout this study.

Macrophages (*mpeg1:mCherry*) are recruited to hindbrain of zebrafish larvae 3 h after injection of human CCL2 and CXCL11 proteins into the hindbrain ventricle as compared to PBS control injection (**A**), with the area of quantification (hindbrain ventricle) 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 (hindbrain ventricle) outlined in representative images (**D**). Human CXCL11 and zebrafish Cxcl11aa showed no difference in their macrophage chemoattractant properties in the hindbrain recruitment assay (**E**), with the area of quantification (hindbrain ventricle) outlined in representative images (**F**). Statistical analyses were done with pooled data of three independent replicates (10–15 larvae each). A Kruskal-Wallis (**A,C**) and a Mann Whitney (**E**) test were used to assess

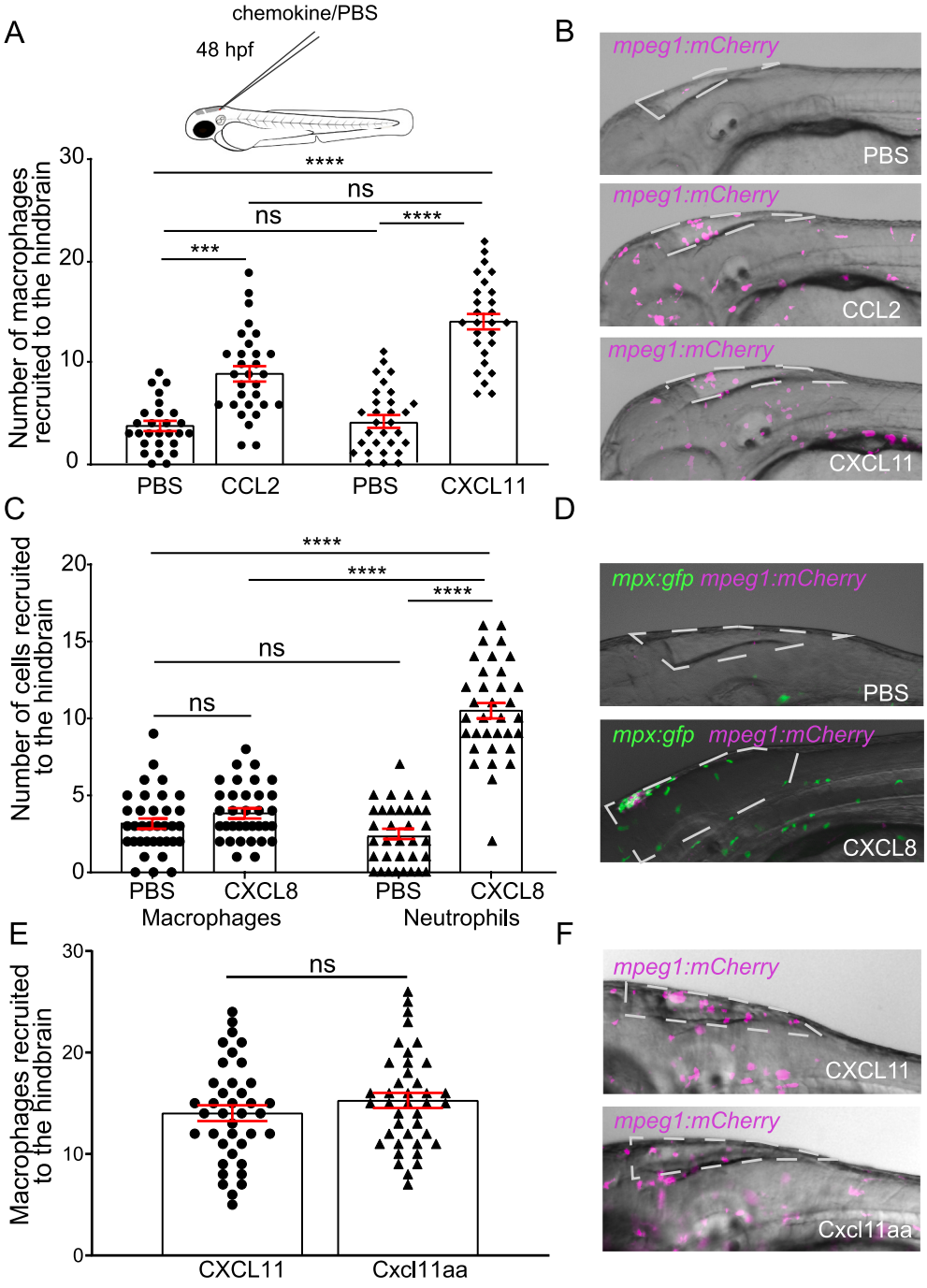

Fig. 2. Locally injected human CCL2 and CXCL11 proteins attract macrophages to the hindbrain ventricle of zebrafish larvae.

significance (*p ≤ 0.05, ***p ≤ 0.001, ****p ≤ 0.0001) and data are shown as mean ± SEM.

3.3. The human CCR2 inhibitor CCR2-RA-[R] inhibits macrophage recruitment in zebrafish larvae

CCR2-RA-[R] is an CCR2 antagonist that binds to an allosteric, intracellular site of the receptor that is linked with G-protein-binding (Zheng et al., 2016). We aimed to use the hindbrain macrophage recruitment assay in zebrafish larvae to evaluate the efficacy of CCR2-RA-[R] *in vivo*. To predict if this inhibitor could function across species, we assessed whole protein identity and similarity within the CCR2-RA-[R] binding site shared between the human and zebrafish receptors. The Zv11 zebrafish genome reference sequence contains two genes that are annotated as Ccr2. The protein that is referred to as Ccr2 by us and others (encoded by ENSDARG00000079829 (Cambier et al., 2014; Xie et al., 2019), shares 44% identity with human CCR2, while the protein encoded by the second gene (ENSDARG00000105363) shares 43% identity (Fig. 3A top). In addition, human CCR2 shares 34% and 30% with human CXCR3 and zebrafish Cxcr3.2, respectively (Fig. 3A top). Next, we assessed the similarity between the predicted CCR2-RA-[R] binding sites in the CCR2 and CXCR3 receptors in humans and zebrafish, based on the binding mode of CCR2-RA-[R] shown in the crystal structure of human CCR2 (Zheng et al., 2016). We found that the

A

CCR2	Ccr2	CXCR3	Cxcr3.2
Overall protein identity (%)	44.38 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

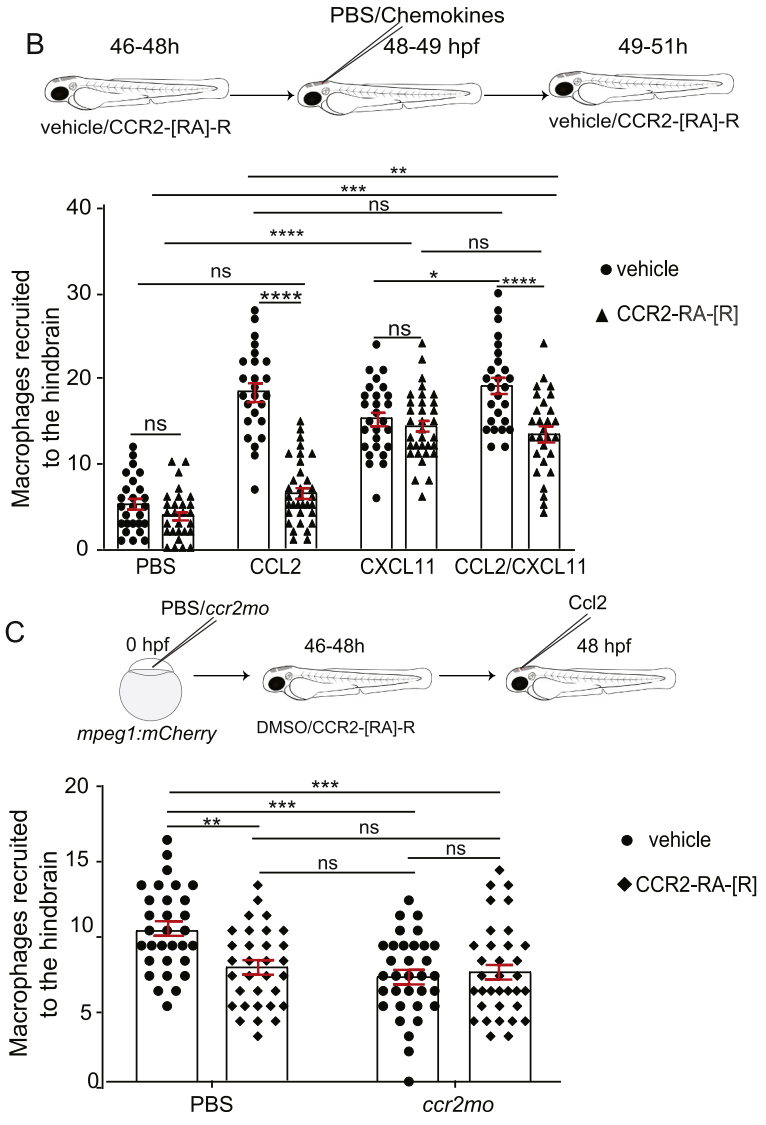

Fig. 3. The human CCR2 inhibitor CCR2-RA-[R] inhibits macrophage recruitment in zebrafish larvae.

key residues for CCR2-RA-[R]-binding in human CCR2 are highly conserved in zebrafish Ccr2, showing 70.5% identity in both variants, but also in human CXCR3 (65% identity) and zebrafish Cxcr3.2 (59% identity) (Fig. 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 (Fig. 3B). The inhibition of Ccr2-mediated macrophage recruitment by CCR2-RA-[R] phenocopied knockdown of *ccr2* with an antisense morpholino targeted at Ccr2 (ENSDARG00000079829), suggesting that this is the predominant Ccr2 variant active under our assay conditions (Fig. 3C). Furthermore, CCR2-RA-[R] reduced macrophage recruitment only in wt larvae but not in *ccr2* knockdown larvae, supporting that the inhibitor exerts it effect through the referred Ccr2 variant. 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.

Comparison between whole protein sequences (top) and key residues involved in the intracellular binding of CCR2-RA-[R] in human CCR2 (bottom). Critical residues of the CCR2-RA-[R] binding site 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 2 h 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 3 h. CCL2-induced macrophage recruitment was ablated in larvae incubated with CCR2-RA-[R] (triangles) compared with the vehicle incubation (dots). 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 reduced 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 ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001) and data are shown as mean ± SEM.

3.4. Zebrafish larvae are a powerful screening platform for human CCR2 inhibitors in vivo

To further assess the suitability of the zebrafish larval model for screening CCR2 inhibitors, we developed a work-flow to screen a test panel of compounds using macrophage recruitment 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 2 h after which we proceeded to amputate the tail fin and incubated the

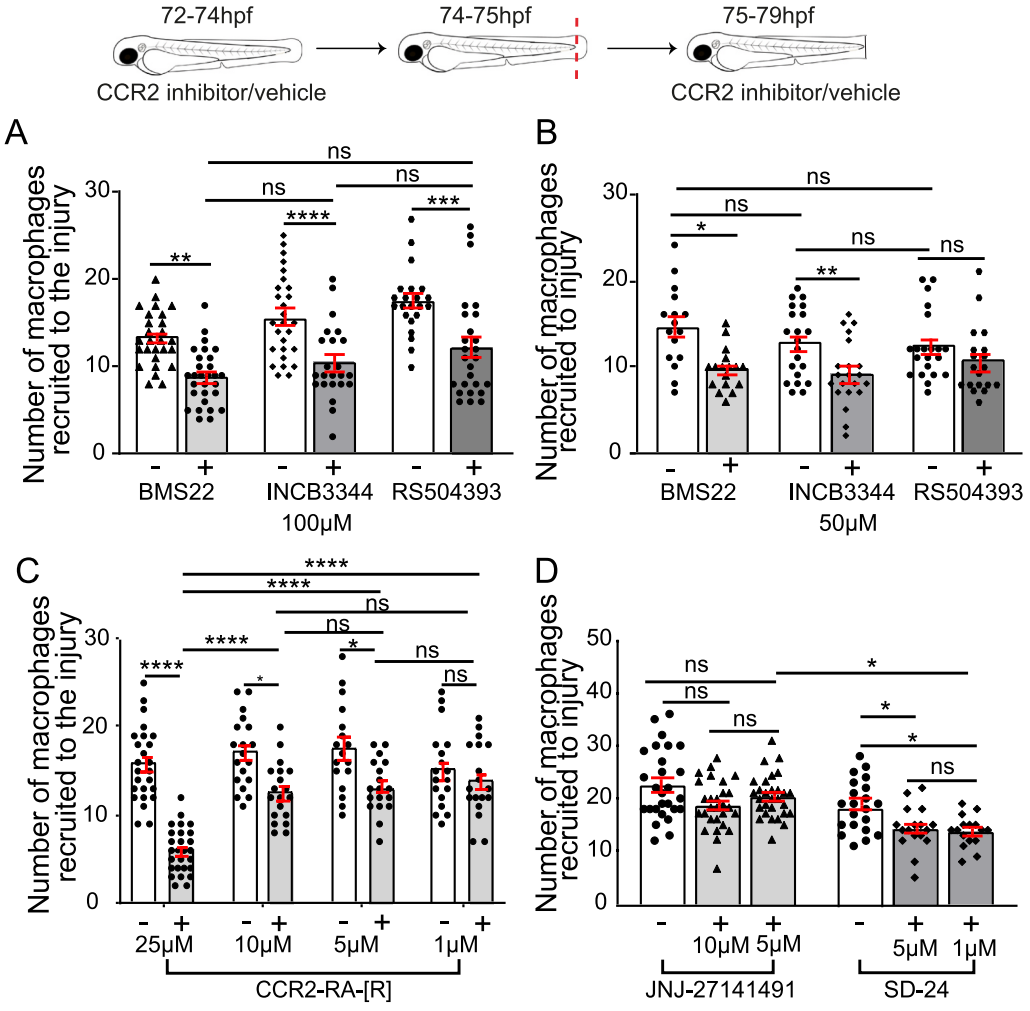

Fig. 4. Zebrafish larvae are a powerful screening platform for CCR2 inhibitors.

amputated larvae in the compound for another 4 h. 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. To determine test concentrations, we performed toxicity evaluations. We found allosteric compounds to be more toxic than orthosteric inhibitors (Supplementary Fig. 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-h pre-incubation step (Supplementary Fig. 2C). CCR2-[RA]-R had no toxic effects (Supplementary Fig. 1C- F and Supplementary Table 1) at any stage of the procedure and showed optimal results at 25 μM (Fig. 4 A). SD-24 was still toxic at 10 μM (Supplementary Fig. 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 (Fig. 4B). Three orthosteric inhibitors, BMS22, INCB3344, and RS504393, all showed only low toxicity (Supplementary Fig. 1A and B and Supplementary Table 1) and effectively reduced macrophage recruitment to injury at 100 μM (Fig. 4C). BMS22 and INCB3344 were still effective at 50 μM but RS504393 no longer affected recruitment at that concentration (Fig. 4D). 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 larval model serves as a robust *in vivo* screening platform for human 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 to 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 Fig. 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 Fig. 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.

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 to 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).

4. 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 drug development (Chu et al., 2014; Connor et al., 2004; Tsou et al., 2007). Both orthosteric and allosteric CCR2 inhibitors have been developed, but none of these have demonstrated sufficient efficacy for clinical use (Xia and Sui, 2009; Horuk, 2009; Struthers and Pasternak, 2010). This illustrates the need for efficient preclinical test systems to determine the *in vivo* efficacy of CCR2 inhibitors. Here we present the zebrafish larval model as an *in vivo* screening platform for CCR2 inhibitors, which enables tracking leukocyte recruitment in a live organism while simultaneously assessing toxicity.

Supported by our results and previous studies, two primary signaling axes, Ccr2-Ccl2 and Cxcr3-Cxcl11, contribute to the inflammatory response in zebrafish larvae by mediating macrophage recruitment (Cambier et al., 2014; Sommer et al., 2020; Torraca et al., 2015; Xie et al., 2019). Inhibiting either of these axes, genetically or chemically, leads to a major reduction in macrophage recruitment, indicating that the role of other chemokine receptors in macrophage recruitment in zebrafish larvae is limited. Chemokine networks in adult zebrafish are likely to have a much larger complexity, similar as in mammals. The less complicated chemokine system of zebrafish larvae has practical advantages for the drug screening approach in this study. We present evidence for cross-species conservation of the Ccr2-Ccl2 and Cxcr3-Cxcl11 chemokine signaling axes, as shown by the compatibility of human chemokines and zebrafish receptors in eliciting macrophage recruitment. Due to a substantial degree of conservation, we were able to demonstrate anti-inflammatory effects in zebrafish larvae using both orthosteric and allosteric CCR2 inhibitors designed for humans. The currently available allosteric CCR2 inhibitors cross-react with other CCR receptors, including CCR1 and CCR5 (Zheng et al., 2016). The zebrafish larval model could serve as a useful addition to cell-based screens to identify next generation inhibitors with improved specificity and help evaluating their *in vivo* efficacy prior to further preclinical assessment in rodent models. In future work, controlling for off target effects and systematic assessment of drug specificity would be facilitated by generating CRISPR/Cas9 mutants of Ccr2 and the closely related Ccr receptors or transgenic lines expressing different Ccr receptors in macrophages.

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 (Torraca et al., 2015; Sommer et al., 2020; Cambier et al., 2014; Xie et al., 2019), 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 (Tsou et al., 2007; Cambier et al., 2017). Studies in zebrafish embryos reported that Ccr2 mediates the recruitment of circulating monocytes but not tissue-resident macrophages in the context of mycobacterial infection (Cambier et al., 2017; Cambier et al., 2014). In mammals, CCR2-CCL2/7 interactions are considered essential mediators of the egress of macrophages from the bone marrow into the peripheral circulation (Tsou et al., 2007). 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 (Fantuzzi et al., 1999). 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 (Torraca et al., 2015; Sommer et al., 2020), 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 larvae and that this inhibitory effect phenocopies *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. 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 (Tanuma et al., 2006; Veillard et al., 2005; Tokuyama et al., 2005) 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.

Acknowledgments

We would like to thank Georges Lutfalla (University of Montpellier) Steve Renshaw (University of Sheffield) for the macrophage- and neutrophil specific zebrafish reporter lines and all members of the cell culture and fish facility teams for taking care of our zebrafish lines and cell cultures.

Funding

This work was supported by the National Council for Science and Technology (CONACyT), grant no. 410804.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2020.103932.

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