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

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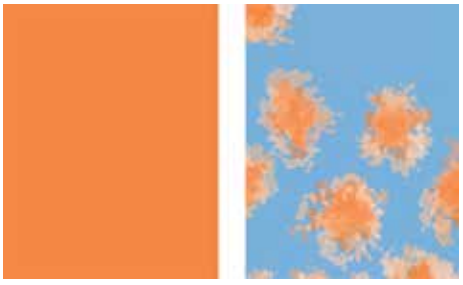
Chemokine signaling mechanisms underlying inflammation and infection control: insights from the zebrafish model

Frida Sommer



Cover: Illustration of migrating macrophages by Frida Sommer

Thesis layout: Frida Sommer

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

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Thesis outline

Inflammation is a critical defense response of the body to injury or infection. However, inflammation becomes harmful to our cells and tissues if it develops into a chronic condition. Inflammatory diseases comprise a large number of pathological conditions characterized by the persistence of inflammation within a specific tissue. Examples of diseases that are associated with chronic inflammation include autoimmune diseases, ulcerative colitis, asthma, allergies, and persistent viral and bacterial infections. Inflammation is also characteristic of the tumor environment and strongly influences cancer progression. The inflammatory response can be broadly divided into three phases: early inflammation, acute inflammation, and resolution. The recruitment and coordinated exchange of leukocyte populations to inflammatory foci are crucial in controlling the transit from one stage of the inflammatory response to the next, and in driving the resolution stage and the following tissue repair processes. The synchronized recruitment of immune cells to inflammatory sites is orchestrated by differentially expressed chemokine receptors on the membrane of leukocytes. Chemokine receptors are one of the largest subfamilies of class A of (rhodopsin-like) G-protein coupled receptors (GPCRs). They bind small proteins called chemokines to mediate the migration of leukocytes to inflammatory sites. Due to their complex and seemingly redundant nature, chemokine signaling networks require multiple regulatory mechanisms to orchestrate intercellular and tissue-specific functions during the immune response against pathogens and upon tissue damage. GPCRs account for approximately one-third of the proteins targeted by approved pharmacological therapies. Given the successful application of GPCRs as drug targets, unraveling the fundamental mechanistic principles underlying chemokine signaling and furthering our understanding of their interactions with other cellular processes could aid the development of novel clinical strategies to treat inflammatory disorders.

Macrophages are highly motile phagocytic cells that play pivotal roles in inflammation, pathogen clearance, tissue repair, and immunomodulation. The chemokine receptors CXCR3 and CCR2 and their ligands mediate macrophage trafficking in various homeostatic and pathological contexts. The expression of these receptors and the induction of their ligands have been implicated in inflammatory conditions characterized by increased monocyte infiltration into specific tissues, such as mycobacterial infection and atherosclerosis. Furthering our understanding of the role of these receptors in macrophage function and their contribution to macrophage recruitment during inflammatory responses and pathogen clearance will contribute to characterize inflammatory conditions in greater depth and offer therapeutic alternatives to several pathologies.

Zebrafish serve as a powerful vertebrate model to study chemokine signaling axes since most human chemokine receptors have at least one zebrafish ortholog. Its optical trans-

parency allows the real-time tracking of fluorescently labeled leukocytes in a whole organism at early embryonic and larval stages. Due to its high fecundity, short generation time, and external fertilization, it is possible to use large samples and easily implement a wide array of molecular tools to generate mutant, transgenic, and reporter zebrafish lines. This model has been successfully used to characterize chemokine signaling networks and their effects on macrophages and neutrophils during development, inflammation, and cancer. The CXCR3 gene is triplicated in zebrafish and genes are referred to as *cxcr3.1*, *cxcr3.2,* and *cxcr3.3.* The two latter genes are expressed on macrophages at early developmental stages and it has been determined that *cxcr3.2* is a functional homolog of human *CXCR3* as it is required for proper macrophage migration. The role of *cxcr3.3* in macrophage trafficking is explored in the present work. One CCR2 homolog has been identified in zebrafish (*ccr2*) and it is also required during macrophage recruitment to inflammatory sites. As in humans, the depletion of either *cxcr3.2* or *ccr2* leads to a decline in macrophage recruitment to injury and infection and an altered inflammatory state of these cells.

This work aimed to expand our understanding of the role of members of the Cxcr3 chemokine receptor family in zebrafish. We studied how Cxcr3 paralogs orchestrate the recruitment of macrophages to sites of infection and injury, how the Cxcr3 function links macrophage migration to lysosomal function and intracellular vesicular trafficking, and how Cxcr3 signaling works in concert with Ccr2 signaling during macrophage migration. Furthermore, we studied the potential use of the zebrafish model to screen inhibitors of human CCR2.

Chapter 1 provides an introduction to the principles of chemokine signaling and reviews recent work using the zebrafish model that has advanced our understanding of the functions of chemokine receptors in the biology of phagocytes. We discuss the evolutionary conservation of chemokine signaling axes between zebrafish and humans and provide an overview of the known roles of different chemokine receptors in zebrafish development, cancer-, wound-, and pathogen-induced inflammation. The chapter concludes with a discussion on potential therapeutic applications involving different chemokine signaling axes and future perspectives in the field of chemokine signaling and inflammation research.

In **Chapter 2** we propose a regulatory mechanism of fine-tuning chemokine receptor activity, which involves an interplay between conventional and atypical members of the Cxcr3 family. By comparing *cxcr3.3* and *cxcr3.2* mutant phenotypes, we could show the atypical receptor Cxcr3.3 antagonizes the function of the conventional receptor Cxcr3.2 in regulating macrophage recruitment in mycobacterial infection and during the inflammatory response to injury. We discuss the parallels with mechanisms for the regulation of mammalian CXCR axes.

In **Chapter 3** we explore the link between chemotactic signaling and lysosomal function since these two processes are closely intertwined. Macrophages lacking the Cxcr3.2 receptor display a marked increase in the expression of lysosomal genes and a rounded shape. We show that the disruption of the Cxcr3 signaling axis leads to the accumulation of lysosomal contents and better clearance of intercellular bacteria, arguably as a result of the upregulation of lysosomal genes. In the last part of the chapter, we suggest that the aberrant motility of Cxcr3.2-deficient macrophages is due to altered intracellular vesicle traffic dynamics that prevent them from acquiring the polarized phenotype characteristic of migrating cells.

Chapter 4 focuses on assessing the viability of using the zebrafish as an *in vivo* screening platform for CCR2 inhibitors aimed at treating multiple inflammatory diseases and characterizing the involvement of both Cxcr3.2 and Ccr2 in macrophage migration. We demonstrate that both chemokine receptors participate in the inflammatory response to injury by mediating macrophage recruitment and that the chemokine system in zebrafish in humans is conserved enough to allow interactions between human ligands and zebrafish receptors. In this chapter, we also develop a simple *in vivo* pre-clinical screening method to test human chemokine receptor inhibitors using the zebrafish model in a fast and robust manner.

Finally, in **Chapter 5** we discuss the challenges and perspectives of chemokine signaling research, the relevance of viewing chemokine signaling networks as parts of broader cellular processes and expand on the potential use of the zebrafish model to screen chemokine receptor inhibitors.

1 General introduction: Chemokine receptors and phagocyte biology in zebrafish

Frida Sommer, Annemarie H. Meijer and Vincenzo Torraca

F. Sommer, A. H. Meijer and V. Torraca, "Chemokine receptors and phagocyte biology in zebrafish," Frontiers in Immunology, vol. 11, p. 325, 2020.

Abstract

Phagocytes are highly motile immune cells that ingest and clear microbial invaders, harmful substances, and dying cells. Their function is critically dependent on the expression of chemokine receptors, a class of G-protein-coupled receptors (GPCRs). Chemokine receptors coordinate the recruitment of phagocytes and other immune cells to sites of infection and damage, modulate inflammatory and wound healing responses, and direct cell differentiation, proliferation, and polarization. Besides, a structurally diverse group of atypical chemokine receptors (ACKRs) are unable to signal in G-protein-dependent fashion themselves but can shape chemokine gradients by fine-tuning the activity of conventional chemokine receptors. The optically transparent zebrafish embryos and larvae provide a powerful in vivo system to visualize phagocytes during development and study them as key elements of the immune response in real-time. In this review, we discuss how the zebrafish model has furthered our understanding of the role of two main classes of chemokine receptors, the CC and CXC subtypes, in phagocyte biology. We address the roles of the receptors in the migratory properties of phagocytes in zebrafish models for cancer, infectious disease, and inflammation. We illustrate how studies in zebrafish enable visualizing the contribution of chemokine receptors and ACKRs in shaping self-generated chemokine gradients of migrating cells. Taking the functional antagonism between two paralogs of the CXCR3 family as an example, we discuss how the duplication of chemokine receptor genes in zebrafish poses challenges, but also provides opportunities to study sub-functionalization or loss-of-function events. We emphasize how the zebrafish model has been instrumental to prove that the major determinant for the functional outcome of a chemokine receptor-ligand interaction is the cell-type expressing the receptor. Finally, we highlight relevant homologies and analogies between mammalian and zebrafish phagocyte function and discuss the potential of zebrafish models to further advance our understanding of chemokine receptors in innate immunity and disease.

Introduction

Phagocytosis refers to the recognition and internalization of particles larger than 0.5 μm into a plasma membrane-derived vesicle called the phagosome. Phagocytes are cells

that can phagocytose harmful particles, pathogens, and dying cell debris. Phagocytes are broadly divided into professional and non-professional phagocytes [1]. In non-professional phagocytes like epithelial cells, endothelial cells, and fibroblasts, phagocytosis is a facultative function as these cells have other tissue-resident functions, although they can contribute to tissue homeostasis by phagocytosing apoptotic debris [2]. In contrast, professional phagocytes efficiently identify, engulf, and clear invading pathogens, harmful substances, and dying cells. This group includes highly motile cells such as neutrophils, monocytes, macrophages, eosinophils, mast cells, and dendritic cells as well as tissue-resident cells like osteoclasts [3]. Professional phagocytes express multiple specialized membrane-bound receptors that recognize target particles of different nature. Pattern recognition-receptors (PRRs) identify pathogen-associated molecular patterns (PAMPS) and damage-associated molecular patterns (DAMPS) and activate the immune response [1, 3]. The phagocytosis process is initiated by other surface receptors. Among these, scavenger receptors mediate the phagocytosis of endogenous ligands, like lipoproteins, as well as microbial invaders. Opsonic receptors recognize targets detected and bound by soluble host molecules, such as complement proteins and antibodies. Receptors for apoptotic cells recognize soluble cues secreted by dying cells (e.g. lysophosphatidylcholine and ATP) or characteristic molecules exposed on the surface of dying cells, such as phosphatidylserine [1, 2]. Professional phagocytes play pivotal roles in immunomodulation, development, pathogen clearance, and antigen presentation [2, 3].

In addition to pattern recognition and phagocytic receptors, phagocytes express various types of chemokine receptors that coordinate cell movement and confer certain functional properties to these cells [4, 5]. Chemokine receptors belong to the G-protein-coupled receptor (GPCR) family and transiently activate GTP-binding proteins that remodel actin structures of the cytoskeleton to control the contractile machinery of the cell and direct cell migration [mm]. Dynamic actin rearrangements control the formation of pseudopodia during cell migration towards a target as well as the formation of protrusions that surround harmful particles and pathogens before internalization within the phagosome during phagocytosis [5, 6, 7]. Chemokine receptors are essential for phagocyte function as they trigger the rearrangement of actin-containing structures required for cell motility, which is at the core of developmental and immunological processes and tissue maintenance and remodeling [8, 9, 10]. Likewise, chemokine receptor signaling contributes to the differentiation, proliferation, and polarization of phagocytes, which are determining factors in host-pathogen interactions, inflammatory responses, inflammation resolution, and wound healing [4, 5, 6, 11, 12].

Zebrafish are increasingly used as a model species to study development and disease owing to the accessibility of the early life stages (embryos and larvae) for genetic analyses, chemical screens, and intravital imaging [6, 13, 14, 15, 16, 17]. These useful features of the zebrafish have been exploited to study the roles of phagocytes in models of in-

fectious and inflammatory diseases and cancer. In this review, we will illustrate how the zebrafish model contributed to our understanding of the role of chemokine signaling axes in phagocyte biology and highlight its main contributions to the understanding of chemokine signaling axes in phagocytes by addressing relevant homologies and analogies between mammalian and zebrafish phagocyte function. We will focus on the two major structural subfamilies of chemokine receptors, CC and CXC, and on the migratory properties of macrophages and neutrophils in the context of development and disease. We will discuss the regulatory role of atypical chemokine receptors (ACKRs), in shaping chemokine gradients and how duplication of chemokine receptor genes in zebrafish allows assessing sub-functionalization or loss/gain of function events and the challenges that gene duplication poses. Finally, we will discuss the potential of zebrafish models to further our understanding of chemokine receptors in innate immunity and immune-related disease.

Fundamentals of chemokine signaling and regulation

Chemokines are small secretory and transmembrane cytokines that induce directed chemotaxis of macrophages and neutrophils through their specific receptors under pathological and homeostatic conditions [5, 7, 18]. Chemokine receptors belong to the chordate-restricted class A of (rhodopsin-like) heptahelical G-protein coupled receptors (GPCRs), which is grouped into four subclasses according to the pattern of highly conserved cysteine residues they display near their N-terminus (CC, CXC, CX3C, and XC) [5, 19]. The cysteine motif of a chemokine receptor is followed by an "R" for "receptor" or an "L" for ligands and a number indicating the chronological order in which the molecules were identified. [5, 20, 19]. A further subfamily containing the characteristic motif CX has been identified only in zebrafish at present [19]. Following nomenclature conventions, human chemokine receptors are written in capital letters, while those of other species use the lowercase to simplify the distinction between species. The structure of chemokine receptors consists of an intracellular COOH terminus, an extracellular NH2 terminus, and seven transmembrane domains linked by three extracellular and three intracellular loops [5, 12]. Chemokine receptors mediate leukocyte trafficking during cell migration processes such as infection, damage, development, cell proliferation and differentiation [21, 22, 23, 24]. GPCRs are the largest and most diverse family of membrane receptors in eukaryotes and the most common pharmaceutical target making chemokine receptors attractive targets to treat chronic inflammatory conditions [12, 25].

Inactive chemokine receptors are coupled to heterotrimeric G proteins. The Gα subunit is bound to GDP (guanosine diphosphate) in resting conditions and exchanges the GDP molecule for GTP (guanosine triphosphate) when the chemokine receptor binds a cognate ligand. The GTP-Gα subunit complex dissociates from the receptor and the Gβ-γ

heterodimer, which triggers the canonical downstream signal pathways that ultimately result in the intracellular mobilization of Ca^{+2} and the rearrangement of cytoskeletal components required by the vesicle trafficking machinery and for cell migration [5, 26, 27, 28]. Besides the conventional G protein-dependent signaling pathways, chemokine receptors can directly activate JAK/STAT (Janus kinase /Signal transducer and activator of transcription) signaling, a pathway shown to induce chemotaxis of progenitor germ cells (PGCs) in zebrafish [6, 29, 30, 31]. Furthermore, chemokine receptors can also signal through β-arrestin to mediate the internalization and intracellular degradation of chemokines and chemokine receptors [12, 30, 32, 33].

Chemokine networks are highly promiscuous and redundant and can result in antagonistic and synergistic interactions since different signaling pathways share signal transducing elements. Due to its complex nature, chemokine signaling axes build up tangled networks that need tight spatio-temporal regulation to evoke specific responses [34]. Some regulatory mechanisms of chemokine signaling include biased signaling, allosteric modulation of receptor activation, receptor internalization, receptor dimerization, ligand sequestration and ligand processing [5, 28, 35, 36]. Furthermore, the function of conventional chemokine receptors can be fine-tuned by ACKRs. These atypical chemokine receptors constitute a structurally diverse group unified by their shared function of shaping chemokine gradients. ACKRs cannot signal in the canonical G protein-mediated fashion, but most of them can signal through β-arrestins and mediate chemokine degradation [33, 37]. Several studies demonstrate that the ligand-scavenging function of AKCRs provides an important regulatory mechanism during cell migration and phagocyte recruitment [33, 37, 38, 39].

Zebrafish as a window to chemokine receptor functions

The zebrafish model has been successfully used to study how chemokine signaling networks determine macrophage and neutrophil functions and to ascribe these receptors a role in immunity, inflammation, and cancer models [4, 13, 16, 22, 40, 41, 42]. It is a powerful vertebrate model well suited for non-invasive *in vivo* imaging given its optical transparency at early embryonic and larval stages. Transgenic lines specifically labeling neutrophils and macrophages by linking fluorescent proteins to the *mpx* and *lyz* promoters for the former, and the *mpeg1.1* and *mfap4* promoters for the latter, allow us to visualize and track these phagocytes at a whole organism level. A wide variety of gene-editing methods like CRISPR-Cas9 and transitory gene knockdown (morpholinos) or RNA-based gene overexpression can be delivered by microinjecting eggs at the single-cell stage [16, 43]. The zebrafish model is ideal to assess developmental processes and since over 80% of all human disease genes identified so far have at least one functional homolog in zebrafish, it serves as a powerful animal model for human diseases too [22, 43].

Most human chemokine receptors and ACKRs have at least one (putative) zebrafish ortholog [6, 30, 44] as shown in **Table 1**. The last common ancestor of humans and zebrafish went through two rounds of whole-genome duplication during vertebrate evolution [19]. Subsequently, a series of intrachromosomal duplication events occurred in the taxon that led to zebrafish [4, 19, 44, 45]. These events resulted in the duplication of several chemokine receptor genes that either preserved their original function, lost their function, or acquired a new one [19, 44].

While most of the human chemokine receptor genes can be found as single or multi-copy genes in the zebrafish genomes, some cases remain unresolved (**Figure 1**). For example, no homologs of CCR1, CCR3, and CCR5 are currently annotated in the Zebrafish Information Network (ZFIN) database. Moreover, there are zebrafish chemokine receptors annotated without a human counterpart, such as Ccr11 and Ccr12. Also, a CX family of chemokine receptors has been identified that is restricted to (zebra)fish [6, 19, 44].

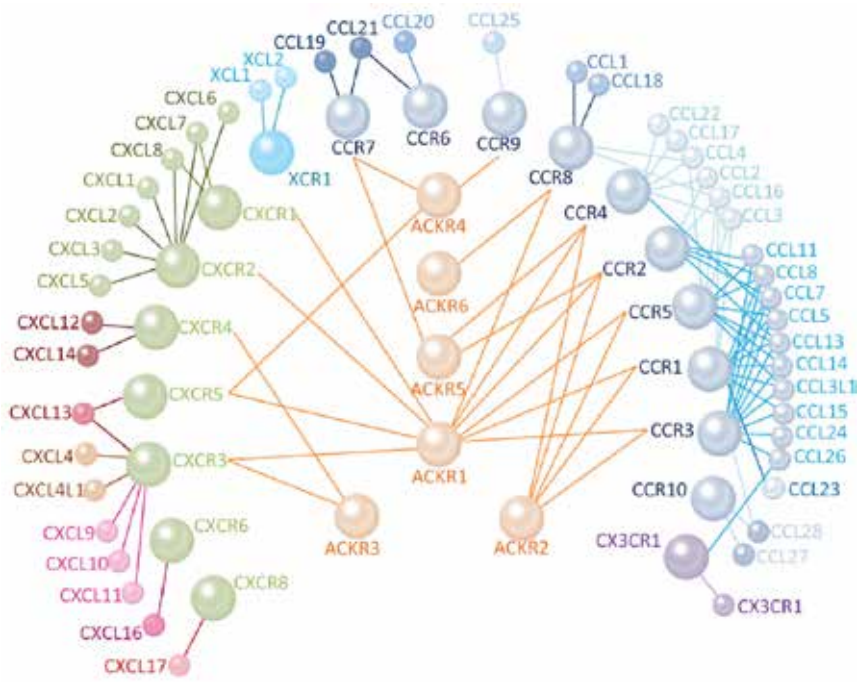

Figure 1. Human chemokine signaling networks are highly promiscuous. There are 25 receptors and 45 ligands in the human chemokine signaling network including seven members of the CXCR family (green), 1 XCR (cyan), 10 CCR (blue), and 1 CX3CR (violet). The CXCL chemokines are shown in shades of pink, XCL in cyan, CCL in shades of blue, and CX3CL in violet. The color intensity of the lines connecting receptors and ligands indicates the binding specificity. Darker colors indicate a higher binding affinity. There are six characterized AKCRs (orange) that antagonize the function of conventional chemokine receptors (connected with lines) by binding one or more of their ligands.

Table 1. Chemokine receptor genes, their ligands and their role in embryonic development, cancer progression, wound-induced inflammation and pathogen-driven inflammation

Chemokine receptor	Human	Ligands	Zebrafish	Ligands	embryonic development	Cancer progression	Wound-induced inflammation	Pathogen-driven inflammation
CXCR1	CXCR1	CXCL6, 8	Cxcr1	Cxcl8a Cxcl8b1, 3		Neutrophil recruitment [15,46]. Sustained inflammation [15,45,46, 48, 54]. Tumor growth and expansion [45,46, 48, 78].	Neutrophil recruitment, pro-inflammatory function [46, 48]	
CXCR2	CXCR2	CXCL, 2, 3, 5, 6, 7, 8	Cxcr2	Cxcl8a Cxcl8b.1,.2.3 Cxcl18b		Chronic inflammation [46,48, 78].	Neutrophil reverse migration, anti-inflammatory function [46,53, 89].	Neutrophil recruitment And bacterial clearance [49, 68, 89, 93, 97]
CXCR3	CXCR3A CXCR3B	CXCL4-B, 9-A/B, 10-A/B, 11A/B	Cxcr3.1, 2, 3.	Cxcl11-like chemokines aa, ac, ad, ae, af and ag		Cell proliferation Cell survival Tumor expansion Angiostatic effect Cxcr3.2 recruits macrophages and neutrophils to injury [48, 50, 51, 53].	Cxcr3.2 recruits macrophages and neutrophils to injury [48, 50, 51, 53]. Cxcl11aa is a pro-inflammatory marker (M1) [52,98].	Cxcr3.2: macrophage recruitment and motility [50, 51, 53], neutrophil recruitment [50, 51]. Cxcr3.3: ligand scavenger, a regulator of Cxcr3.2 function [53].
CXCR4	CXCR4	CXCL12	Cxcr4a Cxcr4b	Cxcl12b Cxcl12a	**Cxcr4a:** guidance of multicellular vessel growth and coordination of gastrulation movements [73, 74]. **Cxcr4b:** progenitor germ cells (PGCs) [6, 31, 57, 58, 60, 61, 63].	Macrophage and neutrophil recruitment [45, 46, 80, 81]. Tumor angiogenesis Tumor dissemination [80, 81].	Neutrophil recruitment and retention at the wounding site. Pro-inflammatory [85].	Neutrophil recruitment Bacterial clearance 97]. Granuloma vascularization [49].
CCR2	CCR2	CCL2	Ccr2	Ccl2 (mcp1)			Macrophage recruitment 67, 68]. Ccr2 is an anti-inflammatory marker (M2) [87, 88].	Recruitment of permissive macrophages [87, 88].
ACKR3 **(CXCR7)**	ACKR3	CXCL11 CXCL12	Ackr3b (Cxcr7a/b)	Cxcl12a	Scavenges Cxcl12a to shape chemokine gradients [6, 36, 61, 63, 75].	Tumor angiogenesis Chemotaxis [82].		

This review will focus on the zebrafish homologs of human of CXCR1/2, CXCR3, CXCR4, ACKR3, and CCR2 (**Supplementary Table 1**) since these receptors have a known function in phagocyte function during development and inflammatory processes. Below we discuss how the genes encoding these receptors are conserved, and in some cases, duplicated in zebrafish. In the subsequent sections, we review how studies in zebrafish contributed to understanding the roles of these receptors in developmental and disease processes.

The Cxcr1/2-Cxcl8 signaling axis: The CXCR1/2-CXCL8 signaling axis is one of the primary chemotactic pathways in neutrophils and of major interest to assess inflammatory processes [46]. Zebrafish chemokine receptors Cxcr1 (Il8ra) and Cxcr2 (Il8rb) are functionally homologous to their mammalian counterparts. Furthermore, chemokines of the CXCL8 (IL-8) family, which interact with these receptors, are conserved between humans and zebrafish, while not present in mice [47]. Cxcr1 and 2 are highly expressed on zebrafish neutrophils and mediate their recruitment by binding to their shared ligands Cxcl8a, Cxcl8b1, Cxcl8b2 and Cxcl8b3 (Cxcl8L2.1, .2 and .3, respectively) [6, 19, 48, 49]. Cxcl8a and the three Cxcl8b variants are all reported to act via Cxcr1 and Cxcr2 to induce neutrophil recruitment, whereby no specific binding patterns involving the three Cxcl8b variants have been reported so far [6, 48]. The Cxcl18b chemokine found in zebrafish and other teleost fish also attracts neutrophils via Cxcr2 [50]. Whether this chemokine activates Cxcr1 remains unknown.

The Cxcr3-Cxcl11 signaling axis: Human CXCR3 is predominantly expressed on T cells, but also multiple other leukocyte cell types, including macrophages [51, 52]. The *cxcr3* gene is triplicated in zebrafish and the copies are referred to as *cxcr3.1*, *cxcr3.2*, and *cxcr3.3*. In humans, CXCR3 binds to CXCL9 (MIG: monokine induced by gamma interferon), CXCL10 (IP-10: interferon-gamma induced protein 10) and CXCL11 (I-TAC: inflammatory-inducible T-cell alpha chemoattractant) [19, 53]. These chemokines are thought to be derived from a common CXCL11-like ancestral gene. In zebrafish seven *cxcl11*-like chemokine genes have been identified and are annotated as *cxcl11aa*, *ac*, *ad*, *ae*, *af, ag,* and *ah* [51]. The Cxcl11aa ligand has been functionally studied and was shown to mediate cell recruitment through Cxcr3.2 [51, 52, 54]. Studies in zebrafish larvae have focused on *cxcr3.2* and *cxcr3.3*, which are expressed on macrophages and neutrophils while *cxcr3.1* is not detectable at this stage [51]. While Cxcr3.2 appears to function as a conventional chemokine receptor, like human CXCR3, Cxcr3.3 has features of ACKRs such as a DCY motif instead of the highly conserved DRY motif that prevents classic G protein-mediated signaling [12, 54]. Supporting that Cxcr3.3 regulates Cxcr3.2 function, these paralogs have antagonistic effects on macrophage recruitment to sites of infection and injury in zebrafish [51, 54]. The functional antagonism between the zebrafish paralogs *cxcr3.2* and *cxcr3.3* can be viewed as a regulatory

mechanism analogous to the functional antagonism of human CXCR3 splice variants A and B [53, 55, 56, 57].

The Cxcr4a/b-Ackr3b/Cxcl12-signaling axis: CXCR4 signaling mediates functions of a variety of cell types, within and beyond the immune system [58, 59]. The CXCR4-CXCL12 (SDF1: stromal cell-derived factor) axis is remarkably conserved between zebrafish and humans although both the receptor and ligand genes are duplicated in zebrafish and annotated as *cxcr4a/b* and *cxcl12a/b*, respectively [6, 30, 60]. Both Cxcr4 receptors can bind both ligands, although Cxcr4a preferentially binds to Cxcl12b and Cxcr4b binds Cxcl12a with a higher affinity [29]. The duplication of the *cxcr4* gene in zebrafish is a representative example of gene sub-functionalization. Cxcr4a is primarily associated with cell proliferation and vessel extension, while Cxcr4b regulates neutrophil and macrophage interactions with other cell types and has been implicated in the modulation of inflammation, neutrophil and macrophage migration, metastatic and angiogenic events, and tissue regeneration, [29, 60, 61, 62]. In mammals, CXCR4-CXCL12 is subject to modulation by an atypical chemokine receptor ACKR3, which binds the CXCR4 ligand CXCL12 but also the CXCR3 ligand CXCL11 [58, 63]. The zebrafish *ackr3b* (*cxcr7b*) gene is on the same chromosome as *cxcr4a/b* and it has been shown that the Ackr3b protein binds both Cxcl12 and Cxcl11 but cannot induce cell migration [62, 64, 65, 66]. By competing with Cxcr4b for the shared Cxcl12a ligand, Ackr3b helps to maintain chemokine gradients during chemotaxis [62, 64]. The potential interaction between Ackr3b and Cxcr3.2-Cxcl11aa signaling has not been characterized yet [67]. As discussed below, Ackr3b has been implicated in several pathological conditions as well as in zebrafish development [6, 62, 63, 64, 66].

The Ccr2-Ccl2 signaling axis: CCR2 is the receptor for monocyte chemoattractant protein -1 (MCP-1/CCL2) [68]. Identifying zebrafish orthologs of human CC chemokine receptors has been challenging since multiple zebrafish cc- receptor genes have a remarkably high similarity to a single human CC chemokine receptor gene. However, a *ccr2* orthologue could be identified in zebrafish, supported by functional evidence, as human CCL2 was shown to trigger macrophage recruitment in zebrafish embryos in a *ccr2*-dependent manner [68, 69].

The duplication of several chemokine receptor genes in zebrafish poses a challenge for the identification of homologies and at the same time, it provides an experimental platform to assess both loss of function and sub-functionalization events to further our understanding of chemokine signaling in phagocyte function as exemplified by the Cxcr4 and Cxcr3 paralogs [29, 54]. In the following sections, we will illustrate how zebrafish embryonic development helped to unravel fundamental chemokine signaling mechanisms and discuss in detail the roles of zebrafish chemokine receptors Cxcr1/2, Cxcr3.2/3.3, Cxcr4b, Ackr3b and Ccr2 in macrophage and neutrophil biology in the

context of cancer and wound and pathogen-driven inflammation.

Dissecting chemokine signaling principles using developing zebrafish

The chemokine signaling axes involved in phagocyte biology are also functional in other cell types of the developing zebrafish embryo [70]. This model brought fundamental new insight into the principles of chemokine signaling. It was a long-held idea that the membrane-spanning domains and the extracellular portions of a chemokine receptor conferred signal specificity [71]. However, recent work on zebrafish showed that cell identity and chemokine receptor signal interpretation modules (CRIM) are the major determinants for the functional specificity of a chemokine receptor-ligand interaction [70, 71]. The directed expression of chemokine receptors that were not naturally expressed by a cell through mRNA injections of zebrafish eggs showed that the foreign receptor could overtake the function of the original receptor in the presence of its ligand. Even receptors that do not share high sequence similarities, like CC and CXC receptors, were found to evoke the same response if expressed on the same cell-type showing that CRIM process a generic signal into a discrete response that is dictated by the cell type. Consistent with the fact that cell identity and CRIM determine the functional specificity of chemokine receptors, the same chemokine receptor can elicit very different biological responses depending on the cell that expresses it [70]. For example, when Cxcr4a is expressed on hematopoietic progenitor cells, it modulates chemotaxis, yet in neuronal progenitor cells, it inhibits proliferation [72].

Studies in zebrafish embryos also contributed to elucidate regulatory mechanisms of chemokine signaling. One such process is the cleavage of certain chemokines (like Cxcl8) by matrix metalloproteinases (MMPs) to activate and confer them enhanced chemotactic properties. The use of a broad-spectrum MMP inhibitor showed reduced neutrophil and macrophage recruitment to sterile heart injury in zebrafish showing that MMPs are key mediators of inflammation and tissue regeneration [36]. An outstanding example of ACKR-mediated regulation of chemotaxis comes from the characterization of the paralogs *cxcr4a* and *cxcr4b* and the interaction of the latter with Ackr3b to fine-tune single-cell migration during development. The Cxcl12b-scavenging function of Ackr3b is required for shaping a self-generated chemokine gradient that guides the migration of the lateral line cell primordium [6, 62, 64, 73]. An analogous Cxcr4/Ackr3b/Cxcl12 system indispensable to form an endogenous chemokine gradient within the mouse lymph node was described later, confirming the observation made in zebrafish [74]. In fact, the identity of Ackr3b as a scavenger receptor that signals via β-arrestins was first described in zebrafish and later confirmed in human cells and mice [38]. Similarly, Cxcr1/2-Cxcl8 driven migration of neutrophils along immobilized gradients within tissue was first described in zebrafish [75]. During this process, tissue-bound chemokine gradients form through the binding of chemokine and heparan sulfate pro-

teoglycans (HSPGg) resulting in a process called haptotaxis. This type of cell movement coordinates both directional guidance of cells (orthotaxis) and motility restriction in the proximity of the source of the chemotactic signal [75]. Haptotaxis was later confirmed in murine dendritic cell recruitment via Ccl21 [76].

Among the chemokine receptors of phagocytes, it is especially the interacting Cxcr4/Ackr3b pair that has much broader roles in developmental processes. We briefly summarize the zebrafish studies that revealed these developmental roles below, which are important to take into account also when studying immune cell functions.

The Cxcr4a/b-Ackr3b-Cxcl12 axis in development: Cxcr4a is mainly involved in guiding multicellular vessel growth [77] and in controlling proper gastrulation movements by ensuring adhesion between cell-matrix and endodermal cells [78]. The Cxcr4b-Cxcl12a signaling axis regulates the migration of a wide range of cell types including neuronal cells, axons, neutrophils, neural crest cells, endothelial cells, and muscle cell precursors [6, 64, 77, 78, 33]. Primordial germ cells express Cxcr4b and migrate towards Cxcl12a gradients tracing their migration route. These cells specifically respond to Cxcl12a and neglect the Cxcl12b ligand, involved in other developmental processes, which can be found along their migration path. Ackr3b, expressed mostly by somatic cells, plays a fundamental role in removing Cxcl12b from the extracellular space and clearing the path for PGC migration [31, 58, 59, 61]. It scavenges chemokines to shape time and tissue-specific gradients to tightly regulate developmental processes involving cell migration [6, 62, 64]. The Cxcr4a/b- Ackr3b-Cxcl12 interaction was first observed *in vivo* during zebrafish PGCs migration [33]. Ackr3b orchestrates the lysosomal degradation of Cxcl12a in a β-arrestin-dependent process while the receptor itself is recycled back to the plasma membrane [37]. Moreover, the scavenging activity of Ackr3b is crucial for the maintenance of a self-generated chemokine gradient that directs the migration of the lateral line primordium during the development of the zebrafish posterior lateral line (PLL) [62, 64, 73].

Chemokine receptors in cancer progression

Cancer progression is strongly influenced by chemokine-dependent leukocyte recruitment and infiltration into primary tumors as well as by the subsequent dissemination of cancer cells from primary tumors into adjacent and distant tissues [15, 55, 79]. Live visualization of fluorescently labeled tumor cells in zebrafish larvae enables early assessment of vascular remodeling events, tumor dissemination, and metastasis at the organismal level [24, 60]. Zebrafish cancer models are also suitable to image early tumor-initiation events and the crucial interplay between the tumor cells and the microenvironment [46]. In particular, xenotransplantation models, in which human invasive cells are systemically inoculated into zebrafish larvae, are useful to assess the interactions between

human tumor cells and host leukocytes that underlie early metastatic onset [80]. Aditionally, the larval zebrafish system offers a simple and robust screening platform for anti-tumor compounds targeting different stages (angiogenesis, metastasis, etc.), further emphasizing its translational value [24, 60].

The tumor environment is a highly inflammatory focus that attracts leukocytes through secretion of cytokines of different natures, including chemokines [46]. Chemokine receptors CXCR1, 2, 3, 4, and 7 have been implicated in tumor angiogenesis, sustaining tumor growth and expansion both in zebrafish and humans, as discussed below. The role of CCR chemokine receptors in cancer using the zebrafish model has not been addressed yet.

The Cxcr1/Cxcr2-Cxcl8 axis in cancer: Neutrophils are the first responders to acute inflammation, infection, and damage. These cells exhibit remarkable phenotypic plasticity that is determined by the integration of extracellular cues [46]. In zebrafish, cancer cells recruit neutrophils through chemokine receptors Cxcr1 and 2 and their Cxcl8 ligands [15, 47]. Neutrophil populations have a dual role in the development of different cancers. Tumor-associated neutrophils (TANs) directly engage with tumor cells and are reported to support tumor growth, tissue invasion, and angiogenesis mimicking sites of chronic inflammation. In contrast, anti-tumor neutrophils undergo apoptosis and reverse migration back into the vasculature, thereby favoring the resolution of inflammation [46, 47]. Using the zebrafish model, it became clear that TANs are recruited to tumor-initiating sites through the Cxcr1-Cxcl8a pathway and that in this context, Cxcr2 is not required for efficient neutrophil recruitment. Fewer neutrophils are recruited to tumor-initiating foci in *cxcr1* mutant zebrafish larvae and proliferation of tumor cells is restricted, suggesting that TANs are critical for early stages of neoplasia and tumorigenesis [47]. In agreement with these observations, Cxcr1 expression is lower in anti-tumor neutrophils that display a predominantly anti-inflammatory phenotype [49, 81].

The Cxcr4a/b-Ackr3--Cxcl12 axis in cancer: A vast body of literature associates the chemokine receptor CXCR4 with the development of cancer pathogenesis in humans, mice, and zebrafish [6, 15, 24, 53, 82]. Cxcr4b is highly expressed on zebrafish neutrophils and together with its ligand Cxcl12a, it facilitates tumor angiogenesis and dissemination into different tissues by attracting malignant Cxcr4-expressing cells into healthy organs and tissues where ligand can be found [55, 58, 82]. Zebrafish larvae lacking *cxcr4b* (ody mutants) fail to induce micrometastases and to sustain human cancer cells after xenotransplantation. Basal neutrophil motility is attenuated and whole-body neutrophil counts are lower in *cxcr4b* mutants than in wild type (wt) larvae [80]. Accordingly, tumors in *cxcl12a* mutant zebrafish cannot metastasize, further supporting that Cxcr4b signaling promotes tumor expansion [60].

While neutrophils are important cellular mediators of inflammation and play a central role in tumor initiation and expansion; macrophages represent a significant amount of the leukocytes that infiltrate tumors. Macrophages phagocytose cancer cells and dying neutrophils whilst secreting immunomodulatory cytokines. Macrophages also express Cxcr4b and respond to Cxcl12a [11, 83]. A study focused on glioblastoma progression used the zebrafish model to show that tumor cells secrete Cxcl12a to recruit macrophages to the tumor site [83]. Cxcr4b-Cxcl12a signaling in macrophages is also linked to tumor-promoting functions by enhancing proliferation and invasiveness, modifying the extracellular matrix, and favoring tumor neovascularization [15, 28, 61]. Interestingly, live visualization of zebrafish macrophages and microglia showed dynamic interactions with cancer cells which did not result in phagocytosis of the malignant cells, thereby avoiding an anti-tumor function of macrophages [80]. *cxcr4b* mutant larvae had a lower tumor burden in this context too and depletion of macrophages and microglia significantly reduced oncogenic cell proliferation, suggesting that Cxcr4b signaling promotes macrophage infiltration during initial stages of brain cancer [83].

As discussed above, Cxcr4b signaling can be fine-tuned through ligand scavenging by the atypical Ackr3b receptor. Human ACKR3 is linked to tumor growth, invasion, and metastasis [11]. Tumor cells and vascular endothelial cells of different tissues show an increased expression of Ackr3b and it has been suggested to include this receptor as a marker for cancer [58]. A study by van Rechem et al. [84] found that Ackr3b is a direct target of the tumor suppressor HIC1 (Hypermethylated in Cancer 1) which is inactive in many human tumors. The role of Ackr3b in cancer pathogenesis is still unknown in zebrafish and as multiple studies found that Ackr3b depletion results in severe developmental abnormalities [6, 29, 30, 37], a gene knockout/down approach to assessing its role in cancer progression would require the development of cell-specific or conditional knockout systems.

Chemokine receptors in wound-induced inflammation

The zebrafish model is well suited to assess aseptic wound-induced inflammation and tissue regeneration either by amputating the ventral or tail fin or by pinching tissue with sterile needles [81, 85, 86]. Recruitment of neutrophils first, and macrophages in a later phase, is key during the inflammatory response, which is broadly divided into three phases: early leukocyte recruitment, amplification or acute inflammation, and resolution [85]. Neutrophils recruited shortly after damage secrete chemokines that activate tissue-resident cells and recruit more leukocytes to the injury, thereby amplifying inflammation. As described in the previous section, Cxcl8a is a strong neutrophil attractant and therefore, a central element at all stages of the inflammatory process [81, 85, 87]. Neutrophils are known to be short-lived and to undergo apoptosis shortly after activation [40]. However, a recently characterized subpopulation of neutrophils returns

to the circulation after activation, has a longer lifespan and an anti-inflammatory effect [81, 85]. The tail-amputation model using larval zebrafish is well-suited for tracking neutrophil reverse migration since it enables in-vivo tracking of these cells at different stages of the inflammatory response [86, 88]. It helped to establish that neutrophils recruited upon injury emerge from hematopoietic tissue in the proximity of the affected area, that they shuttle between the vasculature and the injury during acute inflammation and redistribute in a proximal direction to different sites of the body during the resolution phase [88]. A detailed assessment of the transition from neutrophil recruitment and clustering during acute inflammation and neutrophil redistribution during the resolution phase showed to be regulated through Cxcl8a-induced trafficking and turnover of Cxcr1 and Cxcr2 on the membrane of neutrophils [87].

Two distinct subtypes of macrophages, pro-inflammatory and anti-inflammatory, drive the formation of a mass of highly proliferative stromal cells called blastema and subsequent tissue remodeling during epimorphic regeneration [89, 90]. Using the zebrafish tail-amputation model with fluorescently labeled macrophages (mCherry) and Tnfa (GFP), Nguyen-Chi et al. showed that shortly after tail amputation both pro-inflammatory (GFP+) and ant-inflammatory macrophages (GFP-) accumulated in damaged tissue and that anti-inflammatory macrophages remained associated to the injury until regeneration was completed unlike pro-inflammatory macrophages, which retracted from the area. Chemical depletion of macrophages showed that the initial interaction between TNFa-expressing macrophages and the damaged area is required for blastema formation. Knockdown of the Tnfa receptor *tnfar1* confirmed that Tnfa is fundamental for fin regeneration as it primes blastema cells to undergo regeneration in zebrafish [90]. This phenotypic polarization dynamics in macrophages had been reported in cell culture but it had not been confirmed in a live system. Below we discuss the chemokine receptors implicated in the wound-induced macrophage and neutrophil migration and polarization responses.

The Cxcr1/2-Cxcl8 axis in wound-induced inflammation: Both Cxcr1 and Cxcr2 are required for efficient recruitment of neutrophils to damaged areas at the initial stage of the inflammatory response [49]. Cxcr2, Cxcl8a (Cxcl8L1) and Cxcl8b (Cxcl8L2) are transcriptionally upregulated after tail amputation in zebrafish. However, Cxcl8a and Cxcl8b have differential roles in neutrophil migration during inflammatory responses. Cxcl8a mainly orchestrates neutrophil recruitment to sites on injury whereas Cxcl8b redirects neutrophils back into the bloodstream [91]. Work in zebrafish also showed that the bidirectional movement of neutrophils between the injury and vasculature during acute inflammation is coordinated by distinct roles of Cxcr1 and Cxcr2 [47, 92]. Neutrophils that undergo reverse migration express lower levels of Cxcr1 relative to Cxcr2, suggesting that Cxcr2 is involved in recruiting neutrophils back into the vasculature. Further research showed that the Cxcr1-Cxcl8a axis recruits neutrophils to the inflammatory focus while Cxcr2-Cxcl8a orchestrates reverse migration and resolution

of inflammation [79]. Recently, Coombs et al. showed that both Cxcr1 and Cxcr2 mediate the initial recruitment of neutrophils to damaged tissue but that these receptors exert different functions during the transition from acute inflammation to the resolution phase. Cxcr1 shows a strong initial response towards Cxcl8a but undergoes gradual desensitization followed by receptor internalization, whereas Cxcr2 remains stably expressed on the plasma membrane with sustained responsiveness toward Cxcl8b, and orchestrates neutrophil dispersal during the resolution phase [87].

Cxcr3 and Ccr2 axes in wound-induced inflammation: Macrophages are crucial players of the inflammatory response triggered by tissue damage and exhibit remarkable phenotypic plasticity [89, 93]. Live tracking of fluorescently labeled macrophages in zebrafish showed that these cells are recruited to injury shortly after neutrophils at early stages. Cxcr3.2, a functional CXCR3 ortholog in zebrafish, and Ccr2 both mediate the recruitment of macrophages to injury [51, 52, 54, 68, 69]. Mutation of *cxcr3.2* and knockdown of *ccr2* result in attenuated recruitment of macrophages to the wound [51, 52]. Cxcr3.2 depletion also reduced neutrophil recruitment, unlike Ccr2 knockdown which affected macrophages only [52, 54, 69]. At the beginning of the inflammatory response, macrophages acquire a pro-inflammatory phenotype characterized by the secretion of inflammatory markers (M1) like Tnfa, Il1-b, and the Cxcr3.2 ligand Cxcl11aa. As the inflammatory process develops, they transit towards an anti-inflammatory phenotype (M2) characterized by the expression of chemokine receptor Ccr2 and Cxcr4b [89]. Ccr2 is thought to mediate the transition from acute inflammation (M1) to tissue regeneration processes (M2) as phagocytosis of necrotic and apoptotic neutrophils by macrophages is associated with the beginning of tissue regeneration [85, 90].

The Cxcr4a/b-Ackr3b-Cxcl12 axis in wound-induced inflammation: The chemokine signaling axis Cxcr4b-Cxcl12a is required for the proper development and distribution of neutrophils at early developmental stages and sustains inflammation by recruiting and retaining neutrophils at sites of injury [40, 94]. CRISPR-Cas9-mediated knockdown of Cxcr4b and Cxcl12b significantly increased the clearance of apoptotic neutrophils by macrophages and enhanced reverse migration of neutrophils thereby ameliorating inflammation. Chemical inhibition of the Cxcr4b-Cxcl12a axis leads to a faster resolution of inflammation by hindering the retention of neutrophils at the inflammatory site [81, 95]. Dominant gain-of-function truncations of CXCR4 are associated with warts, hypo-gammaglobulinemia, infections, and myelokathexis (WHIM) syndrome, a primary immunodeficiency disorder characterized by neutropenia [94]. The expression of homologous Cxcr4 WHIM truncations in zebrafish showed that neutrophil release into the blood was impaired and recruitment to injury after fin amputation was diminished. Larvae with the WHIM-truncated Cxcr4b displayed aberrant neutrophil development and distribution due to reduced chemotaxis, which could be reverted upon Cxcl12a depletion, suggesting that WHIM truncation increases Cxcr4b

sensitivity toward Cxcl12a [94].

The possible interaction between Cxcr4b and Ackr3b during inflammation has not yet been addressed.

Chemokine receptors in pathogen-induced inflammation

Chemokine receptors play a fundamental role in the immune response against invading pathogens by mediating leukocyte trafficking to sites of infection [4, 3, 96]. Bacterial infections can be followed from very early stages and with great detail using cell-specific fluorescent transgenic zebrafish lines and fluorescent bacteria. The optically clear larvae facilitate live visualization of complex host-pathogen interactions at the whole organism level and at the same time, it provides a reasonably simplified setting to assess chemokine signaling when used before adaptive immunity develops [96, 94, 97]. Most of the studies on chemokine receptor function in the context of infection were performed with the zebrafish-*Mycobacterium marinum* (*Mm*) model for tuberculosis. This model provides a surrogate system that strongly resembles *Mycobacterium tuberculosis* (*Mtb*) pathogenesis in humans, including the formation of granulomas, the histological hallmark of tuberculosis. *Mm* is a natural pathogen of teleost fish and a close genetic relative of *Mtb* which permits assessing co-evolution between host and pathogen [97]. Both *Mm* and *Mtb* can survive intracellularly in macrophages. Macrophages are the primary components of granulomas and play a dual role in mycobacterial pathogenesis. Macrophage recruitment to infection sites is crucial for neutralizing mycobacteria but it also provides them with a niche for replication and a vector for dissemination into host tissues [98].

The Cxcr2-Cxcl8 axis in pathogen-induced inflammation: Cxcr2 (but not Cxcr1) mediates infection-induced neutrophil mobilization from the caudal hematopoietic tissue (CHT) to infectious foci [99]. Neutrophils are very efficient at killing pathogens through degranulation and the rapid release of reactive oxygen species (ROS) [100]. Mycobacteria primarily infect macrophages to replicate and expand at initial stages of infection [69]. At later stages, when the infection is well established, neutrophils are recruited primarily through Cxcr2 and Cxcl8a secreted by macrophages and epithelial cells [101, 102]. Unlike Cxcl8a, Cxcl18b is secreted by non-phagocytic cells of the stroma within granulomatous lesions during *Mm* infection [50]. Neutrophils contribute to the phagocytosis and destruction of infected macrophages and are therefore crucial to control mycobacterial infection [101, 103].

The Cxcr3-Cxcl11 and Ccr2-Ccl2 signaling axis in pathogen-induced inflammation: Chemokine receptors direct the course of mycobacterial infection by controlling leukocyte recruitment with distinctive microbicidal properties [68, 89, 90]. *Mm* recruits macrophages at the early stages of infection through the Cxcr3.2 and Ccr2 chemokine

receptors [51, 54, 69]. Cambier et al. 2014 proposed that phenolic glycolipid in the bacterial cell wall induces *ccl2* transcription and recruits blood circulating monocytes via Ccr2 in a toll-like receptor-independent way. The monocytes recruited via Ccr2 are permissive to mycobacterial replication and are less efficient in clearing the pathogen because they contain less inducible nitric oxide synthases [69]. On the other hand, the authors suggest that toll-like receptor-mediated recruitment of tissue-resident macrophages primes cells to adopt a microbicidal phenotype and that mycobacteria evolved different mechanisms to evade detection by these cells. Once Ccr2-expressing monocytes are recruited, mycobacteria can transfer from the microbicidal tissue-resident macrophages to the Ccr2-expressing permissive monocytes. This permissive monocyte recruitment driven by mycobacteria will amplify the infection as infected macrophages that egress from the granuloma seed secondary granulomas away from the initial infection site [68]. Interestingly, Cxcl11aa (the main ligand of Cxcr3.2) is induced in a manner dependent on the myeloid differentiation response gene 88 (Myd88) [104]. Myd88 serves as an adaptor molecule for the majority of toll-like receptors suggesting that macrophages recruited through Cxcr3.2 might have different microbicidal properties than those recruited through Ccr2 [104, 105].

The depletion of either Ccr2 or Cxcr3.2 results in a reduced recruitment of macrophages to sites of infection [51, 52, 68]. However, *cxcr3.2* knockout limits *Mm* dissemination as fewer macrophages are recruited to sites of infection due to aberrant macrophage motility that prevents macrophage-mediated seeding of secondary infectious foci [51]. Cxcr3.3 restricts Cxcr3.2 function in macrophages through its Cxcl11aa-scavenging function. Macrophages of *cxcr3.3* mutant zebrafish larvae are more mobile than wt controls, and recruitment to sites of infection and injury is, therefore, more efficient. Cxcr3.3 depleted larvae, show exacerbated Cxcr3.2 signaling due to higher ligand bioavailability and enhanced bacterial dissemination resulting from higher macrophage motility [54] (**Figure 2**).

The Cxcr4a/b-Ackr3b-Cxcl12 axis in pathogen-induced inflammation: As mentioned in previous sections, neutrophils are recruited through Cxcr4b and the chemokine Cxcl12a [81, 95]. The depletion of Cxcr4b in zebrafish led to a significant reduction in neutrophil recruitment to infectious foci and a higher bacterial burden further emphasizing the relevance of neutrophils in the control of mycobacterial infection [101]. Macrophages expressing Cxcr4b have been implicated in the delivery proangiogenic signaling within the granulomatous structures although the mechanism is unknown. Granulomas in *cxcr4b* depleted zebrafish larvae were poorly vascularized, bacterial growth was restricted and dissemination reduced [106].

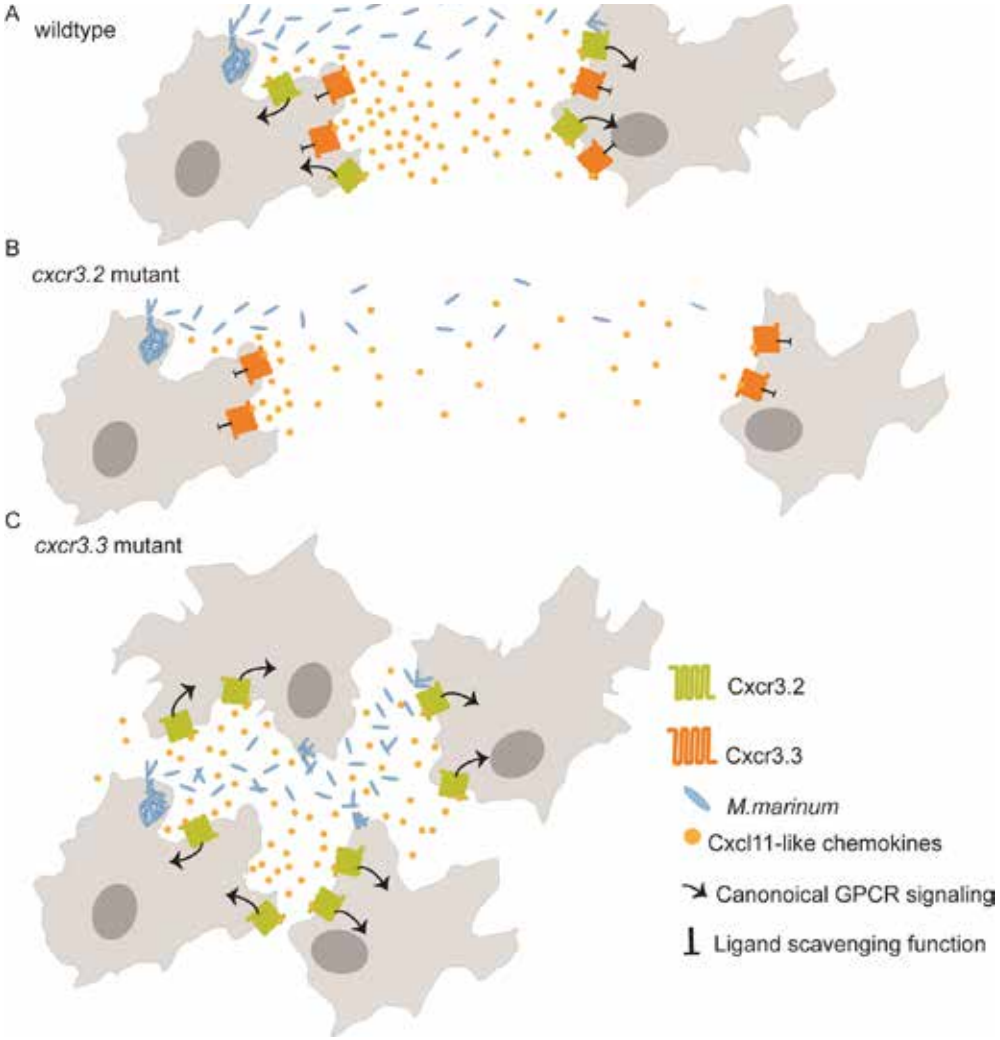

Figure 2. The paralogs *cxcr3.2* and *cxcr3.3* have antagonistic functions that regulate macrophage recruitment to sites of infection. Cxcr3.2 (green) is a functional homolog of human Cxcr3 required for macrophage recruitment to sites of infection and other inflammatory settings. Cxcr3.3 (orange) displays the structural of Ackrs such as the substitution of the central Arginine (R) of the highly conserved E/DRY-motif for a Cysteine (DCY) that prevents canonical GPCR signaling (arrow). Cxcr3.3 regulates Cxcr3.2-mediated macrophage recruitment through its scavenging function (blunt arrow) of Cxcl11-like chemokines (yellow dots). (**A**) Shows how macrophages infected with *M. marinum* (blue rods) recruit non-infected macrophages through the secretion of Cxcl11-like chemokines to contain the bacterial infection and to clear dying macrophages in wt zebrafish larvae. (**B**) shows how macrophage recruitment is reduced in *cxcr3.2* mutants (as an actively signaling chemokine is depleted) and how fewer macrophages become infected with *M. marinum* due to reduced macrophage motility, favoring the contention of mycobacterial infection. (**C**) shows enhanced recruitment of macrophages to sites of infection due to an exacerbated Cxcr3.2 signaling because of higher ligand avail-

ability in absence of the scavenging function of Cxcr3.3. The dissemination of mycobacteria into these newly recruited macrophages will later seed secondary granulomas, supporting the dissemination of the infection.

Concluding remaks

The zebrafish model significantly contributed to the expansion of our knowledge on phagocyte behavior, function, and properties in the context of development, cancer progression, and sterile and pathogen-driven inflammation. Due to its genetic accessibility, zebrafish can be exploited to model congenital syndromes involving chemokine receptors implicated in leukocyte function, such as the WHIM syndrome [94].
zebrafish can be exploited to model congenital syndromes involving chemokine receptors implicated in leukocyte function, such as the WHIM syndrome [94]. It has been of great value to unveil fundamental principles underlying chemokine signaling regulation, signal integration and to explore receptor sub-functionalization events [6, 17, 96]. Furthermore, the functional diversification of duplicated chemokine receptor genes in zebrafish might reveal core mechanisms of chemokine signaling, like the ligand processing function of MMPs and the Cxcr3.2-Cxcr3.3 functional antagonism, and expand our knowledge on the function and interaction of ACKRs as well as to identify and explore analogous regulatory systems in humans [54, 36].

The tight connection between chemokine receptors and macrophage and neutrophil recruitment posits them as interesting therapeutic targets to treat chronic inflammation, a condition that can be induced by persistent infections like mycobacterial infections and precedes pathologies like cancer, autoimmune diseases and tissue damage [81, 85]. The development of antibodies targeting chemokine receptors or chemokines that mediate neutrophil recruitment like Cxcr1/2-Cxcl8 and Cxcr4/ Ackr3b-Cxcl12 could be used as an alternative anti-inflammatory and anti-oncogenic treatment to modulate neutrophil recruitment to inflammatory foci and tumor-initiating niches, respectively [47]. Promoting neutrophil reverse migration to accelerate the resolution of inflammation by pharmacologically inhibiting Cxcr1-Cxcl8a signaling presents another approach to counteract inflammation and to restrict tumor progression [46, 95]. While pharmaceutical targeting of the Cxcr4/ Ackr3b-Cxcl12 signaling axis to inflammatory conditions remains plausible, it should be noted that this pathway is central for embryonic development and therefore, a developing organism like zebrafish larvae, might not be an optimal model for screening compounds targeting these axes [6, 30].

CXCR3 signaling in cancer also presents a therapeutic target. Unlike the mutation of *ackr3b*, *cxcr3.2* and *cxcr3.3* mutant larvae showed no major effects on embryonic development. Therefore, in future work zebrafish larvae can be used to screen chemical

inhibitors targeting the CXCR3 axis. Studies show that disrupting CXCR3 signaling using chemical antagonists results in lower tumor burden in human lung cancer due to reduced cell proliferation and survival as well as increased caspase-independent cell death [107]. However, CXCR3 has also been ascribed an angiostatic effect that blocks tumor neovascularization and some of its platelet-derived ligands work as anti-tumor agents by inhibiting lymphangiogenesis [108]. The role of Cxcr3 and Cxcr4 signaling axes and their interaction with Ackr3b in cancer progression have not been explored using the zebrafish model in the context of cancer, but it could contribute to clarify the discrepant observations made so far. Also, the disruption of Cxcr3.2 signaling in mycobacterial infection resulted in reduced granuloma formation in zebrafish, similar to CXCR3 knockout in mice [109]. Fine-tuning CXCR3 signaling could, therefore, serve the development of host-directed antibacterial therapies to circumvent the treatment limitations imposed by the ever-growing multi-drug resistance of bacterial strains.

Considering that chemokine receptors mediate interactions between macrophages and their extracellular environment, it would be interesting to unravel the chemotactic cues underlying macrophage polarization and their localization during infectious, inflammatory and tissue regeneration processes. Therapies aimed at enhancing macrophage efferocytosis (clearance of apoptotic cells by phagocytes) of neutrophils during inflammation or biasing macrophage polarization towards an anti-inflammatory and regenerative phenotype could serve as novel targets of regenerative drugs [90]. Zebrafish stands out as a powerful model to study macrophage functional plasticity during inflammation in real-time and within a whole organism mostly because of the availability of several M1 transgenic lines. The generation of fluorescent transgenic zebrafish lines for M2 markers, such as *cxcr4b* and *ccr2*, would be helpful to further dissect the role of chemokine receptor signaling in macrophage polarization [89, 90]. Fine-tuning macrophage polarization could enable us to prime macrophages to adopt an inflammatory phenotype that favors pathogen clearance or a tissue-regenerative phenotype to reduce inflammation as a therapy against multiple pathogens and conditions.

Due to its accessibility and its many advantages, the zebrafish model keeps up with state-of-the-art technologies, such as genome editing techniques like CRISPR/Cas9, the application of cell/tissue-specific RNA-sequencing and proteomics analyses [16, 96, 43]. Together with cutting-edge microscopy techniques like super-resolution microscopy and lattice light-sheet microscopy, which can provide information about dynamic intracellular processes, the identity of chemokine receptors' downstream effectors and signal integration events can be further investigated. The link between chemokine signaling and relevant intracellular processes, like autophagy, in several contexts, could be assessed in homeostasis and disease to reveal fundamental signaling and physiological mechanisms within phagocytes.

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Supplementary table 1. Zebrafish chemokine receptor genesene accession numbers

Receptor	Accession number	Ligands	Accession number
		Human	
CXCR1	ENSG00000163464	**CXCL6** **CXCL8**	ENSG00000124875 ENSG00000169429
CXCR2	ENSG0000018087	**CXCL1** **CXCL2** **CXCL3** **CXCL5** **CXCL7**	ENSG00000163739 ENSG00000081041 ENSG00000163734 ENSG00000163735 ENSG00000163736
CXCR3	ENSG00000186810	**CXCL4** **CXCL9** **CXCL10** **CXCL11**	ENSG00000109272 ENSG00000138755 ENSG00000169245 ENSG00000169248
CXCR4	ENSG00000121966	**CXCL12**	ENSG00000107562
CXCR7 (ACKR3)	ENSG00000144476		
CCR2	ENSG00000121807	**CCL2**	ENSG00000108691
		Zebrafish	
Cxcr1	ENSDARG00000052088	**Cxcl8a**	ENSDARG00000104795
		Cxcl8b.1	ENSDARG00000102299
		Cxcl8b.3	ENSDARG00000099169
Cxcr2	ENSG00000180871	**Cxcl19**	ENSDARG00000102776
		Cxcl18b	ENSDARG00000075045
Cxcr3.1 **Cxcr3.2** **Cxcr3.3**	ENSDARG00000078177 ENSDARG00000041041 ENSDARG00000070669	**Cxcl11-like chemokines aa, ac, ad, ae, af, ag, ah**	ENSDARG00000100662 ENSDARG00000092423 ENSDARG00000093779 ENSDARG00000116337 ENSDARG00000094706 ENSDARG00000113389 ENSDARG00000095747

Receptor	Accession number	Ligands	Accession number
		Zebrafish	
Cxcr4a	ENSDARG00000057633	**Cxcl12a**	ENSDARG00000037116
Cxcr4b	ENSDARG00000041959	**Cxcl12b**	ENSDARG00000055100
Cxcr7a	ENSDARG00000062478		
Cxcr7b	ENSDARG00000058179		
Ccr2	ENSDARG00000105363	**Ccl2**	ENSDARG00000098460

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2 Antagonism between regular and atypical Cxcr3 receptors regulates macrophage migration during infection and injury in zebrafish

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Abstract

The CXCR3-CXCL11 chemokine-signaling axis plays an essential role in infection and inflammation by orchestrating leukocyte trafficking in human and animal models, including zebrafish. Atypical chemokine receptors (ACKRs) play a fundamental regulatory function in signaling networks by shaping chemokine gradients through their ligand scavenging function, while being unable to signal in the classic G-protein-dependent manner. Two copies of the *CXCR3* gene in zebrafish, *cxcr3.2 and cxcr3.3,* are expressed on macrophages and share a highly conserved ligand-binding site. However, Cxcr3.3 has structural characteristics of ACKRs indicative of a ligand-scavenging role. In contrast, we previously showed that Cxcr3.2 is an active CXCR3 receptor since it is required for macrophage motility and recruitment to sites of mycobacterial infection. In this study, we generated a *cxcr3.3* CRISPR-mutant to functionally dissect the antagonistic interplay between the *cxcr3* paralogs in the immune response. We observed that *cxcr3.3* mutants are more susceptible to mycobacterial infection, while *cxcr3.2* mutants are more resistant. Furthermore, macrophages in the *cxcr3.3* mutant are more motile, show higher activation status, and are recruited more efficiently to sites of infection or injury. Our results suggest that Cxcr3.3 is an ACKR that regulates the activity of Cxcr3.2 by scavenging common ligands and that silencing the scavenging function of Cxcr3.3 results in an exacerbated Cxcr3.2 signaling. In human, splice variants of CXCR3 have antagonistic functions and CXCR3 ligands also interact with ACKRs. Therefore, in zebrafish, an analogous regulatory mechanism appears to have evolved after the *cxcr3* gene duplication event, through diversification of conventional and atypical receptor variants.

Introduction

Chemokine signaling is essential for the proper functioning of the immune system. Leukocyte populations differentially express chemokine receptors that participate in processes such as development, differentiation, cell proliferation, leukocyte trafficking, and immune responses [1, 2, 3, 4]. Chemokine receptors are a type of G-protein-coupled

receptors (GPCRs) that belong to the class A (rhodopsin-like) family. They have the prototypal GPCR structure consisting of an extracellular NH_2 terminus, an intercellular COOH terminus, and 7 transmembrane domains (TM) interconnected by 3 extracellular (EC) and 3 intracellular (IC) loops [5, 6]. This receptor class has been divided into 5 subclasses based on the pattern of highly conserved cysteine residues they display (C, CC, CXC, CX3C and, XC) and on the chemokines that they bind (CCL, CXCL, XCL, CX3CL) [6, 7]. A distinctive feature of chemokine signaling is its pleiotropic nature. Most chemokine receptors can bind multiple chemokines, and chemokines can also bind to numerous receptors [5, 2]. The redundancy of the interactions and the diversity of processes involving chemokine receptors require tightly regulated mechanisms to confer specificity to the response resulting from a receptor-ligand interaction [8, 6, 9]. Therefore, chemokine signaling-axes regulation and signal integration occur at different levels (genetic, functional, spatial and temporal) and engage a wide variety of mechanisms to evoke specific responses [10, 11, 12].

One kind of mechanism for regulating chemokine receptor activities involves atypical chemokine receptors (ACKRs), a heterogeneous group of proteins [13, 14]. Despite their structural diversity and distant evolutionary relationships, all ACKRs are unified by their inability to signal in the classic G-protein-dependent fashion and by their shared capacity to shape chemokine gradients [13, 15]. These receptors display characteristic features such as amino acid substitutions within the central activation E/DRY-motif (aspartic/glutamic acid- arginine- tyrosine- motif) [13, 16], which is crucial for G-protein coupling and further downstream signaling [16]. The central Arginine (R) of the E/DRY-motif is highly conserved (96%) among functional GPCRs as it is critical for locking and unlocking the receptor and substitutions of this residue usually result in loss of function [17, 16]. In addition, ACKRs show alterations in amino acid residues within the TM domains that function as microswitches by stabilizing the active conformation of a GPCR. ACKRs have been shown to exert their function by scavenging or sequestering chemokines or by altering the activity or membrane expression of conventional chemokine receptors [10, 13]. The functional read-out of ACKRs is that they fail to induce cell migration, contrary to the well-characterized chemotactic function of conventional chemokine receptors [13, 18].

The zebrafish model has been successfully used to functionally unravel mechanistic processes underlying chemokine networks involving ACKRs [19, 20]. The optical transparency of larvae facilitates live visualization of immunological processes and provides a reasonably simplified in-vivo model for chemokine signaling if used before adaptive immunity arises [21, 22, 23, 24]. Besides, due to the extensive duplication of chemokine receptor genes in teleost fish, the zebrafish provides a useful experimental system to address sub-functionalization or loss of function events. The sub-functionalization of two *CXCR4* genes, *cxcr4a* and *cxcr4b*, was determined using the zebrafish model. In

several studies, *cxcr4a* was associated primarily with cell proliferation [19, 11], whereas *cxcr4b* was related to the retention of hematopoietic stem cells in hematopoietic tissue, recruitment of leukocytes to sites of infection and damage, modulation of inflammation, neutrophil migration, primordial cell and tissue migration, and tissue regeneration [25]. Cxcr4b interacts with Cxcl12a and it was shown that this chemokine is also a ligand for the scavenger receptor Cxcr7 (ACKR3) [26, 27]. Interacting with both receptors, Cxcl12a has been shown to control the migration of a tissue primordium, in which expression of *cxcr4b* and *cxcr7* is spatially restricted to the leading and trailing edge, respectively [19, 11]. The scavenging role of CXCR7 (ACKR3) in the regulation of the CXCL12-CXCR4 axis was later confirmed in human cells [26]. Moreover, the zebrafish model allowed to visualize the contribution of endogenous chemokine receptors in shaping self-generated gradients of migrating cells [20], and revealed how the cell-type expressing a given chemokine receptor is the major determinant for the functional specificity of a chemokine receptor-ligand interaction, and not the receptor-ligand pair itself [28].

The human CXCR3 chemokine receptor and its ligands (CXCL9-11) have been proven instrumental for T-cell functioning as well as for macrophage recruitment to sites of infection and injury, and are therefore implicated in several infectious and pathological conditions, including tuberculosis [29, 30]. CXCR3 ligands have been proposed as clinical markers for the diagnosis of this infectious disease and the response to treatment [31, 32]. In a previous study, we assessed the role of CXCR3 in mycobacterial infection using the zebrafish-*Mycobacterium marinum* model and observed that CXCR3 ligands were induced upon infection in this model, like in human patients [29, 33]. *Mycobacterium marinum* is a close relative of *Mycobacterium tuberculosis* and a natural pathogen of various ectotherms, such as zebrafish, which has become widely used to unravel early innate immune responses against mycobacterial infections [21, 33, 34]. In zebrafish there are three copies of the CXCR3 gene: *cxcr3.1*, *cxcr3.2*, and *cxc3.3*. We determined that the latter two are expressed on macrophages at early developmental stages as well as at 5 and 6 days post-fertilization (dpf) [35] and that *cxcr3.2* is a functional homolog of human *CXCR3* [29]. Macrophages play a pivotal role in mycobacterial infections since they are motile and phagocytic cells as well as a constituent cell-type of the characteristic granulomas that represent inflammatory infection foci [30, 33]. The efferocytosis of infected macrophages in granulomas contributes to the amplification of the infection and is a crucial process to consider to design new therapeutic strategies [21, 29]. In a previous study, we showed that Cxcr3.2 is required for the proper migration of macrophages to infectious foci [29]. However, in agreement with studies in *cxcr3* mutant mice, mutation of *cxcr3.2* is beneficial to the host in the context of mycobacterial infection [30]. We showed that *cxcr3.2* mutation favors bacterial contention, since it results in a reduced macrophage motility, thereby preventing macrophage-mediated dissemination of bacteria and limiting the expansion of granulomas.

While Cxcr3.2 is required for macrophage migration in zebrafish, the function of its paralog, Cxcr3.3, which is also expressed on macrophages, remains unknown. In the present study, we investigated the regulatory interplay between Cxcr3.2 and Cxcr3.3 in the context of *M. marinum* infection and in the response to injury, using a tail-amputation model. Opposite to *cxcr3.2* mutants, functional assays showed that *cxcr3.3* mutation leads to poor control of the infection and that *cxcr3.3* mutant macrophages are more motile and, consequently, display an enhanced recruitment to sites of infection and damage. As a result of an enhanced macrophage recruitment and an increased cell motility, bacterial dissemination is facilitated in the *cxcr3.3* mutants. Structural predictions suggest that the Cxcr3.3 receptor can bind the same ligands as Cxcr3.*2* because of the high conservation of the ligand-binding sites, but also that it cannot signal using classic G-protein-dependent pathways. Taking both our structural and functional data together, we posit that the two CXCR3 zebrafish paralogs *cxcr3.2* and *cxcr3.3* function antagonistically. We propose that Cxcr3.3 is an ACKR that functionally regulates the activity of Cxcr3.2 by scavenging common ligands and that knocking out *cxcr3.3* results in an exacerbated Cxcr3.2 signaling due to an excess of available chemokines.

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 committee of Leiden University (License number: 10612). All WT, mutant and transgenic lines used in this study were generated in the AB/TL background. The zebrafish lines used were: WT-AB/TL, homozygous mutant (*cxcr3.2-/-*) and WT siblings (*cxcr3.2*+/+) of *cxcr3.2*^{hu6044}, homozygous mutant (*cxcr3.3-/-*) and WT siblings (*cxcr3.3*+/+) of *cxcr3.3*^{ibl50}, and the same lines crossed into *Tg (mpeg1: mCherry-F)*^{ump2} background and Tg *(mpx: eGFP)*^{i114} [36], and homozygous mutants (*dram1-/-*)and wild type siblings (*dram1*+/+) of *dram1*^{ibl53} [37]. Eggs and larvae were kept at 28.5°C in egg water (60 µg/ml Instant Ocean sea salts and 0.0025% methylene blue). All larvae were anesthetized with 0.02% buffered tricaine, (3-aminobenzoic acid ethyl ester; Sigma Aldrich, St. Louis, MO, USA) before infection, tail-amputation, and imaging. Larvae were kept in egg water containing 0.003% PTU (1-phenyl-2-thiourea, Sigma Aldrich) to prevent pigmentation before confocal imaging.

Generation and characterization of the *cxcr3.3* mutant zebrafish line

A *cxcr3.3-/-* (*cxcr3.3*^{ibl50}) zebrafish line was generated using CRISPR-Cas9 technology. Short guide RNAs (sgRNAs) targeting the proximal region of the *cxcr3.3* gene (ENSDARG00000070669) were designed using the chop-chop web-server [38, 39]. The 122

bp DNA template was generated by annealing and amplifying semi-complementary oligonucleotides using the following PCR program: initial denaturation 3 min at 95°C, 5 denaturation cycles at 95°C for 30 s, annealing for 60 s at 55°C, elongation phase for 30 s at 72°C and final extension step at 72°C for 15 min. The reaction volume was 50 µL, 200uM dNTPs and 1 unit of Dream Taq polymerase (EP0703, ThermoFisher). The oligonucleotides were purchased from Sigma-Aldrich using the default synthesis specifications (25 nmol concentration, purified by desalting). The sequences of the oligonucleotides used were:

Fw:5'GCGTAATACGACTCACTATAGG ACTGGTTCTGGCAGTATTGG TTTTAGAGCTAGAAA TAGCAAGTTAAAATAAGGCTAGTC 3'

Rv:5'GATCCGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACG-GACTAGCCTT ATTTTAACTTGCTATTTCTAGCTCTAAAAC 3'

The amplicon was subsequently amplified using the primers: Fw: 5' ATCCGCAC-CGACTCGGT 3' and Rv: 5' GCGTAATACGACTCACTATAG 3' and purified using the Quick gel extraction and PCR purification combo kit (00505495, ThermoFisher). The PCR products were confirmed by an agarose gel electrophoresis and by Sanger sequencing (Base Clear, Netherlands). The sgRNA was generated using the MEGA short script ®T7 kit (AM1354, ThermoFisher) and the mRNA for a zebrafish optimized NLS-Cas9-NLS was transcribed using the mMACHINE® SP6 Transcription Kit (AM1340, Thermo Fisher) from a Cas9 plasmid (39312, Addgene) in both cases, the RNeasy Mini Elute Clean up kit (74204, QIAGEN Benelux B.V., Venlo, Netherlands) was used to purify the products. AB/TL embryos were injected with a mixture of 150 pg sgRNA /150 pg/Cas9 mRNA at 0 hpf and CRISPR injections were confirmed by PCR and Sanger sequencing. Five founders (F0) were outcrossed with AB/TL fish and efficiently transmitted the mutated allele. The chosen mutation consists of a 46 bp deletion directly after the TM1 domain and a stable line was generated by incrossing heterozygous F1 carriers. The stable homozygous *cxcr3.3* mutant line was later outcrossed with *Tg (mpeg1: mCherry-F)* and *Tg (mpx: eGFP)* transgenic lines to visualize macrophages and neutrophils, respectively.

The offspring of a Tg *(mpeg1:mCherry-F cxcr3.3+/-)* family cross was genotyped to assess the segregation pattern of the *cxcr3.3* gene. To assess macrophage and neutrophil development, a 25-30 larvae from 5 single crosses of *Tg (mpeg1: mCherry-F* WT, *cxcr3.3-/- and cxcr3.2-/-)* and *Tg (mpx: eGFP* WT, *cxcr3.3-/- and cxcr3.2-/-)* fish were pooled together and observed under a Leica M165C stereo-fluorescence microscope from 1 dpf- 5 dpf to quantify the total number of macrophages and neutrophils, respectively, in the head and tail areas. The same batch of fish was observed under the stereomicroscope from 1 dpf- 5 dpf to determine if there were morphological aberrations.

Transient *cxcr3.3* overexpression

An expression construct pcDNA™3.1/V5-His TOPO-*CMV:cxcr3.3* was generated and injected into the yolk at 0 hpf to overexpress the gene in AB/TL (**Figure 3C**) and *cxc3.3* mutant larvae (**Figure 3E**). Overexpression levels were verified by qPCR analysis.

Phylogenetic analysis and protein-ligand binding site prediction

Amino acid sequences of *CXCR3* genes and *ACKR*s from 13 species (**supplementary table 1.**) were aligned and trimmed using the free-access server gBlocks [40] and the protein evolution analysis method was fitted using ProtTest3 [41]. Evolutionary analyses were conducted in MEGA7 [42]. The evolutionary history was inferred by using the Maximum Likelihood method based on the Dayhoff matrix-based model. The tree with the highest log likelihood (-27586.19) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log-likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+*G*, parameter = 1.6611)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 48 amino acid sequences. There was a total of 529 positions in the final dataset. Protein-ligand site prediction was done using the COACH server [43, 44] and protein structure was visualized using UGENE [45, 46, 47].

Systemic infection with *Mycobacterium marinum* and determination of bacterial burden

M. marinum M-strain, expressing the fluorescent marker wasabi, was grown and prepared freshly for injection as described in [48], and embryos were systemically infected with 300 CFU of *M. marinum*-wasabi by microinjection at 28 hpf in the blood island (BI) [48, 49]. Infected larvae were imaged under a Leica M165C stereo-florescence microscope and the bacterial burden was determined using a dedicated pixel counting program at 4 days post-infection (4 dpi) [50]. Data were analyzed using a two-tailed t-test and a One-way ANOVA when more than two groups were compared. Results are shown as mean ± SEM (ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$) and combine data of 3 independent replicates of 20-30 larvae each.

Microbicidal capacity assessment

For determining the microbicidal capacity of zebrafish larval macrophages, embryos were infected with 200 CFU of an attenuated strain, ΔERP-*M. marinum*-wasabi [51]. Bacteria were taken from a glycerol stock and microinjected at 28 hpf into the BI. Infected larvae were fixed with 4% paraformaldehyde PFA at 44 hpi, mounted in 1.5% low-melting-point agarose (SphaeroQ, Burgos, Spain) and bacterial clusters were quantified under a Zeiss Observer 6.5.32 laser scanning confocal microscope (Carl Zeiss,

Sliedrecht, The Netherlands). A Mann-Whitney test was used to analyze the overall bacterial burden of the pooled data of 3 independent replicates of 9 fish each, where data are shown as mean ± SEM. A Kolmogorov-Smirnov test was used to analyze the distribution of bacterial cluster sizes (ns $p > 0.05$).

RNA extraction, cDNA synthesis, and qPCR analysis

For every qPCR assay a total of 3 biological samples (12 larvae each) were collected in QIAzol lysis reagent (Qiagen) and RNA was extracted using the miRNeasy mini kit (Qiagen) according to manufacturer's instructions. cDNA was generated using the iScript™ cDNA Synthesis Kit (Bio-Rad) and qPCR reactions were done using a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) and iTaq™ Universal SYBR® Green Supermix (Bio-Rad). For every biological sample, 3 technical replicates were performed. The cycling conditions we used 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. All data were normalized to the housekeeping gene *ppiab* (*peptidylprolyl isomerase Ab)* and were analyzed with the $2^{-\Delta\Delta Ct}$ method. The following primers were used for our analyses:
ppiab Fw: 5' ACACTGAAACACGGAGGCAAAG 3', *ppiab* Rv: 5' CATCCACAACCTTCCCGAACAC 3', *cxcr3.2* Fw: 5' CTGGAGCTTTGTTCTCGCTGAATG 3', *cxcr3.2* Rv: 5' CACGATGACTAAGGAGATGATGAGCC 3', *cxcr3.3* Fw: 5' GCTCTCAATGCCTCTCTGGG 3', *cxcr3.3* Rv: 5' GACAGGTAGCAGTCCACACT 3', *cxcl11aa* Fw: 5' GCTCTGCTTCTTGTCAGTTTAGCTG 3', *cxcl11aa* Rv: 5' CCACTTCATCCATTTTACCGAGCG 3'.
A One-way ANOVA was used to test for significance and data are plotted as mean ± SEM (ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Macrophage and neutrophil recruitment assays

100 CFU of *M. marinum*-wasabi (**Figure 5A-B**) or 1nL of purified Cxcl11aa protein (10 ng/mL, [29]) (**Figure 5C-D**) were injected into the hindbrain ventricle of *Tg (mpeg1: mCherry-F WT, cxcr3.2-/-* and *cxcr3.3-/-)* and *Tg (mpx: eGFP* WT, *cxcr3.3-/- and cxcr3.2-/-)* larvae at 48 hpf. PBS-injected larvae from each group were pooled before quantification to serve as a control group for the three genotypes. Samples were fixed with 4% PFA at 3 hpi, and macrophages within the hindbrain ventricle were counted under a Zeiss Observer 6.5.32 laser scanning confocal microscope (Carl Zeiss, Sliedrecht, The Netherlands) by going through a z-stack comprising the whole hindbrain ventricle. For the tail-amputation model, > 50 anesthetized 3 dpf larvae were put on a 2% agarose covered petri-dish and the caudal fin was cut with a glass blade avoiding to damage the notochord. Amputated larvae were put back into egg water and fixed with 4% PFA 4hours after amputation. The tail area was imaged with a Leica M165C stereo-florescence microscope and images were visualized using the LAS AF lite software. The macrophages localized within an area of 500 μm from the cut towards the trunk

were counted as recruited cells (**Figure 5F**). For both the hindbrain injection and the tail-amputation 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.

Tracking of migrating macrophages

Time-lapse images of migrating macrophages from two independent replicates (5 larvae per genotype each) near the caudal hematopoietic tissue (CHT) were acquired every 2 min for 3h under basal conditions (**Figure 6A**). To prevent imaging artifacts due to tail regeneration processes, time-lapse images of macrophages migrating towards the injury (3 independent replicates of 4 larvae per group each) using the tail-amputation model were acquired every 60 sec for 1.5 h (**Figure 6C**). 4-5 larvae of each group and for each condition (basal/wound-induced migration) were mounted in 1.5 % low-melting-point agarose and microscopy was done using a Nikon Eclipse Ti-E microscope (Nikon Instruments Europe B.V.) with a Plan Apo 20X/0.75 NA objective. Data were saved as maximum projection images and were further analyzed using the Fiji/ImageJ [52] plugin TrackMate v3.4.2 [53]. The tracking setting used were: Log detector, estimated blob diameter=20 microns, threshold diameter= 15 microns, no further initial thresholding method was applied. The chosen view was hyperstack displayer and the tracking algorithm chosen was the simple LAP tracker, keeping the default settings. Tracks were later filtered according to the numbers of spots on track (> 40 spots / track) and spots, links, and track statistics were used to estimate the mean speed of moving macrophages and the total displacement. The total displacement was manually calculated in Excel by adding all the links of a given track and a filter was applied to classify tracks with a maximum displacement < 20microns as static cells (mean speed = 0 and total displacement = 0). Data were analyzed with a One-way ANOVA (ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$) and are shown as mean ± SEM.

Macrophage circularity assessment

The cell circularity indexes were calculated using the "analyze particle" option in the Fiji/ImageJ software [52]. The maximum projection images of migrating macrophages of the three genotypes were processed in Fiji/ImageJ by using the "despeckle" filter and by generating a binary image. In total, 30 macrophages per larvae were manually selected and the circularity of the cell in every frame was determined using the "analyze particle" option. A frequency histogram (%) for each group was plotted using cell circularity index (CI) bins as follows: 0-0.2, 0.2-0.4, 0.4-0.6, 0.6-0.8 and 0.8-1. The frequency distributions were analyzed using a Kolmogorov-Smirnov test taking the WT distribution as reference distribution (** $p \leq 0.01$, **** $p \leq 0.0001$).

Bacterial dissemination assessment

200 CFU of *M.marinum-mCherry* were injected into the hindbrain ventricle of >30 WT, *cxcr3.2* and *cxcr3.3* mutants at 28 hpf. Whole larvae and tail areas were imaged

with a Leica M165C stereo-fluorescence microscope and visualized with the LAS AF lite software. Images were cropped in such way that the area encompassing the tail was always the same size (4 in/10.16cm x 11 in/27.94cm). The number and size of distal granulomas were analyzed with the "analyze particle" function in Fiji/ImageJ [52]. Particles with a total area >0.002 were considered as granulomas, smaller particles were excluded from our analysis. The percentage of infected larvae that developed distal granulomas was manually calculated and a χ^2 test was used to assess significance. A One-way ANOVA was used to assess cluster number and size (ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Data are shown as mean ± SEM.

Chemical inhibition of Cxcr3.2 and Cxcr3.3

Approximately 30 3-day old larvae of each genotype (WT, *cxcr3.2-/-* and *cxcr3.3-/-)* were pre-incubated in 2 mL egg water containing either DMSO (0.01%) or NBI 74330 (50 μM) for 2 hours before tail-amputation. Larvae were put back into 2mL egg water containing either DMSO or NBI 74330 after the amputation for 4h followed by fixation with 4% PFA. Imaging of the tail region and quantification of macrophage recruitment to the tail-amputation area was done as described above. For the bacterial burden assay, approximately 30 larvae of each group were pre-incubated either with 25 μM NBI74330 or 0.01% DMSO for 3 hours before infection (24 hpf-27 hpf). Larvae were infected with 300 CFU *M. marinum*-wasabi at 28 hpf in the BI and NBI74330 and DMSO treatments were refreshed at 48 hpi. Imaging and bacterial pixel quantification were performed as described above.

Results

Cxcr3.3 has features of both conventional Cxcr3 receptors and ACKRs

We have previously shown that zebrafish Cxcr3.2 is a functional homolog of human CXCR3, required for macrophage migration towards the infection-inducible Cxcl11aa chemokine ligand [29]. Since macrophages also express the paralog Cxcr3.3, we set out to investigate the interaction between these two Cxcr3 family receptors. Our phylogenetic analysis revealed that Cxcr3.3 clusters in the same branch as conventional Cxcr3 chemokine receptors (**Figure 1A**) despite having an altered E/DRY-motif (DCY) and distinctive microswitch features of ACKRs, which are unable to conventional signaling through G-proteins (**Figure 1B**). A protein-ligand binding site prediction algorithm [43, 44] showed that Cxcr3.2 and Cxcr3.3 share relevant structural features, such as a well-conserved main ligand-binding site (**Figure 1C and D**). While classical CXCR3 ligands (CXCL9, 10, 11) were not found, possibly due to the evolutionary distance between human and zebrafish, the top 4 hits for predicted ligands by this algorithm were shared by both Cxcr3 paralogs further confirming the well-preserved protein structure (**Supplementary Table 1**). Since the conventional and atypical Cxcr3 paralogs cluster

together, the alterations in the E/DRY-motif and in microswitches cannot be regarded as phylogenetic diagnostic features, yet these characteristics are known to be functionally determinant for GPCR activation [13, 54, 16]. Based on these observations, we hypothesize that Cxrc3.3 might antagonize the function of Cxcr3.2 since both receptors are predicted to bind the same ligands but Cxcr3.3 lacks the E/DRY-motif that is required for activation of downstream G-protein signaling and might, therefore, function as a scavenger.

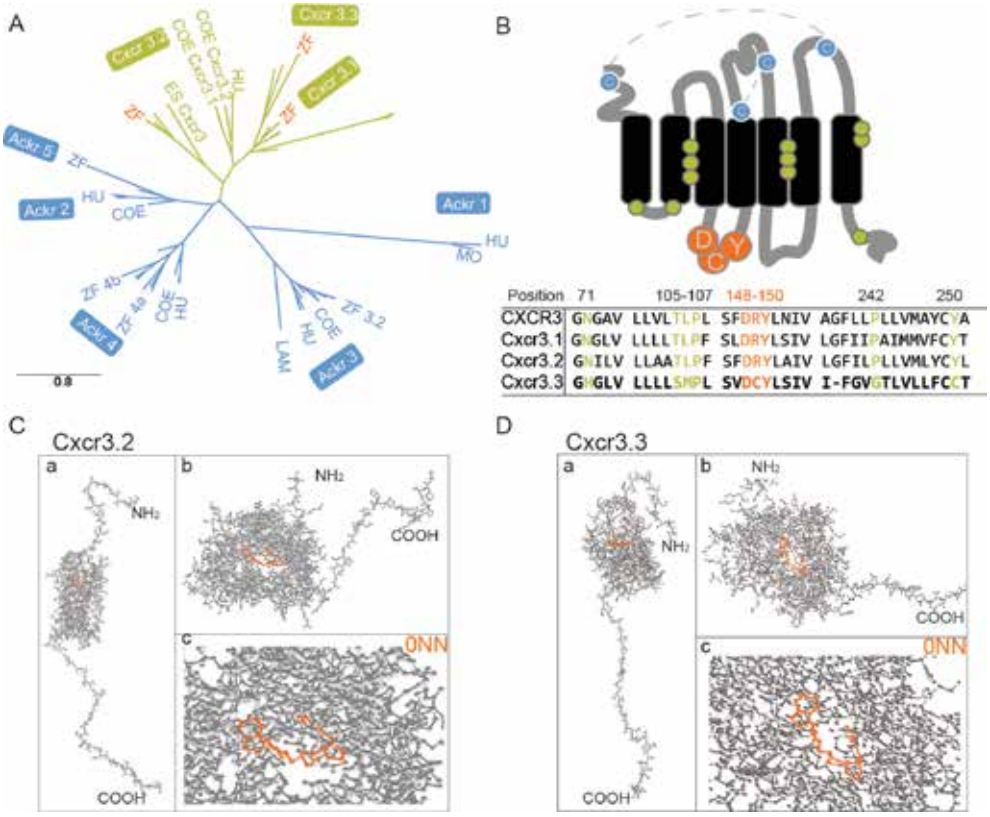

Figure 1. Cxcr3.3 has features of both conventional Cxcr3 receptors and ACKRs. Phylogenetic analyses including CXCR3 (green) and ACKR sequences (blue) of relevant species revealed that Cxcr3.3 is closely related to its paralogs Cxcr3.1 and Cxcr3.2 (**A**) (ZF=zebrafish, COE=coelacanth, HU=human, MO=mouse, ES= elephant shark, LAM= lamprey) despite having structural features of ACKRs (**B**), such as an altered E/DRY-motif (orange) and microswitches (green). The predicted primary ligand-binding site of both Cxcr3.2 and Cxcr3.3 is highly conserved and structural predictions suggest that they share several ligands (**Supplementary table 2**). **C** and **D** show the whole predicted structure of the Cxcr3.2 and Cxcr3.3 receptors (**a**), the ligand binding site of both proteins (**b**) and the

binding of one of the shared predicted ligands (0NN) by each receptor (**c**).

cxcr3.3 mutant larvae do not show morphological aberrations but transient differences in macrophage development

Using CRISPR-Cas9 technology we generated a *cxcr3.3* mutant zebrafish line. The mutation consists of a 46 bp deletion in the first exon, directly after the first transmembrane domain which guarantees that the GPCR is entirely dysfunctional (**Figure 2A, B**). The mutated gene did not affect survival since it segregated following Mendelian proportions (**Figure 2C**). The development of mutant embryos was tracked from 24 hpf-5 dpf and no evident morphological aberrations were observed (**Figure 2D**). Macrophages of *cxcr3.3* mutant and WT siblings in Tg *(mpeg1: mCherry-F)* reporter background embryos were quantified from 24 hpf-5 dpf. We also included the previously described *cxcr3.2* mutant [29] in this analysis. The total number of macrophages (**Figure 2E**) in *cxcr3.3* mutant larvae was higher at day 2. However, this minor increase was short-lived since by day 3 there was no difference among the groups. We also quantified macrophages in the head and tail since these were relevant areas for our experimental setups. We observed that at day 4, *cxcr3.2-/-* larvae had transiently fewer cells in the head area (**Figure 2F**). On the other hand, *cxcr3.3* mutant embryos had more macrophages during the first 2 days but leveled off after this time point (**Figure 2G**). Neutrophils were quantified in the same fashion as macrophages, using a Tg *(mpx: eGFP)* reporter line, but we did not detect any difference between the groups at any time point (**Supplementary figure 1).** Taking these observations into account, we performed all our experiments avoiding the time points at which macrophage development was inconsistent to prevent biased observations.

Deficiency of *cxcr3.3* results in a higher *M. marinum* infection burden while overexpressing the gene lowers bacterial burden

We previously showed that mutation of *cxcr3.2* enabled zebrafish larvae to better control *M. marinum* infection, a phenotype that could be explained by a reduction of macrophage migration in the absence of Cxcr3.2, which limits the dissemination of infection [29]. To investigate our hypothesis that Cxcr3.2 and Cxcr3.3 might have opposing functions, we started by determining if Cxcr3.3 was also involved in the immune response to *M. marinum*. In contrast to the effect of the *cxcr3.2* mutation, systemically infected *cxcr3.3* mutant larvae had a higher bacterial burden than WT 4 days after infection with *M. marinum* (**Figure 3A, B**). We transiently overexpressed *cxcr3.3* by injecting a *CMV*: *cxcr3.3* construct into AB/TL fish at 0 hpf and observed that larvae overexpressing *cxcr3.3* had a lower bacterial burden than non-injected controls (**Figure 3C, D**).To rescue the mutant phenotype, we injected the *CMV*: *cxcr3.3* construct into *cxcr3.3* mu-

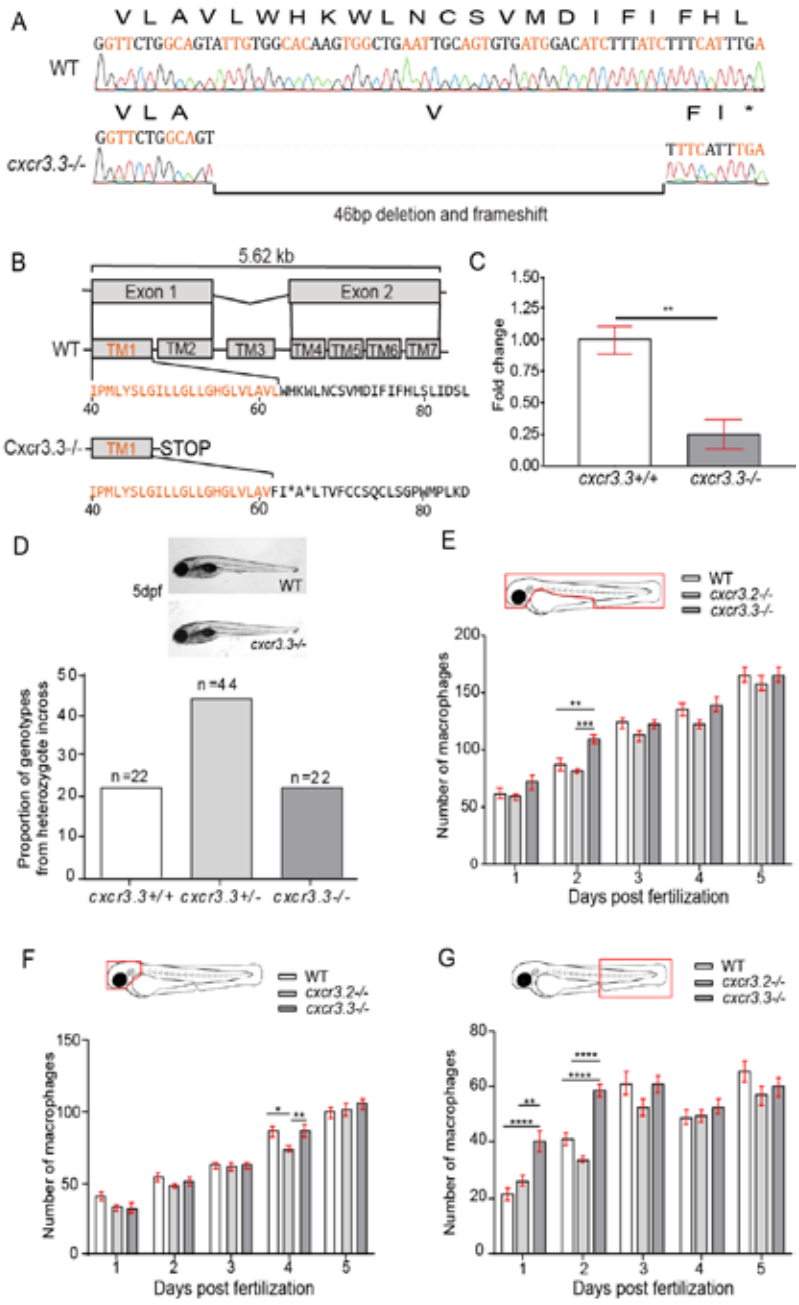

Figure 2. *cxcr3.3* mutant larvae do not show morphological aberrations or major differences in macrophage development. A 46 bp deletion was induced in the *cxcr3.3* gene using

CRISPR-Cas9 technology (**A**). The deletion is located in the first exon (orange) at the very end of the first transmembrane domain (TM1). The mutation shifts the reading frame and results in a premature stop codon (**B**). Nonsense-mediated decay assessment suggests that the *cxcr3.3* mutant gene codes a truncated Cxcr3.3 protein (**C**). No evident morphological aberrations were observed in *cxcr3.3*-/- larvae within the first 5 dpf and the mutant allele segregated following Mendelian proportions **(D)**. Macrophage development was faster in *cxcr3.3*-/- embryos at 2 dpf but reverted to WT and *cxcr3.2*-/- pace after day 3 (**E**). Fewer macrophages were found in the head area of *cxcr3.2*-/- larvae only at day 4 (**F**), while there were more macrophages in the tail region in *cxcr3.3*-/- (**G**). The cell numbers corresponding to each day are the average of 35 larvae of each of the 3 groups (genotypes). Data were analyzed using a two-way ANOVA and are shown as mean ± SEM (ns $p > 0.05$, * $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

tant larvae. We observed that the bacterial burden of the rescued mutants (*cxcr3.3 mutants + CMV-cxcr3.3*) was similar to WT and significantly lower than in non-injected *cxcr3.3* mutants (**Figure 3E, F**). For this assay, we used non-injected larvae as controls since there was no significant difference in bacterial burden of larvae injected with the empty *CMV*:vector and non-injected larvae (**Supplementary figure 2**). To assess whether there was genetic compensation when one of the *cxcr3* paralogs was depleted, we assessed the gene expression of *cxcr3.2* in *cxcr3.3* mutants and *cxcr3.3* in *cxcr3.2* mutants under basal conditions and upon infection with *M. marinum*. The expression of *cxcr3.2* remained unaffected in the absence of *cxcr3.3* and was induced upon infection with *M. marinum* (**Figure 3G**). On the other hand, *cxcr3.3* expression was lower in *cxcr3.2* mutant larvae and it was moderately induced upon infection (**Figure 3H**). We also assessed the expression of the Cxcl11aa ligand, as it is the most upregulated one out of the 7 Cxcl11-like ligands during *M. marinum* infection, in both *cxcr3* mutants [29, 31]. The gene was induced upon infection independently of the expression on *cxcr3.2* and *cxcr3.3* (**Figure 3I**). Thus, the expression of *cxcr3.3* is partially dependent on *cxcr3.2*, but it is not strongly induced upon infection like *cxcr3.2* and *cxcl11aa.* Furthermore, the expression data indicate that the increased bacterial burden of *cxcr3.3* mutants is not due to altered *cxcr3.2* expression. Together with our previous study on *cxcr3.2* [29], we conclude that mutation of *cxcr3.2* and *cxcr3.3* results in opposite infection outcomes and that *cxcr3.3* overexpression phenocopies the host-protective effect of the *cxcr3.2* mutation.

Macrophages lacking Cxcr3.3 efficiently clear IC bacteria

Lysosomal degradation of intracellular bacteria by macrophages is crucial for the containment of mycobacterial infections. The *ERP* (exported repetitive protein) virulence factor is required for bacteria to survive and replicate inside acidic compartments. Mycobacteria lacking *ERP* are easily eliminated by macrophages and can be used as an indicator of bacterial clearance efficiency since the initial infection dose (200 CFU) remains unchanged in the absence of bacterial replication [51]. To evaluate if the poor containment of the infection in *cxcr3.3 mutants* was associated to a deficient clearance of

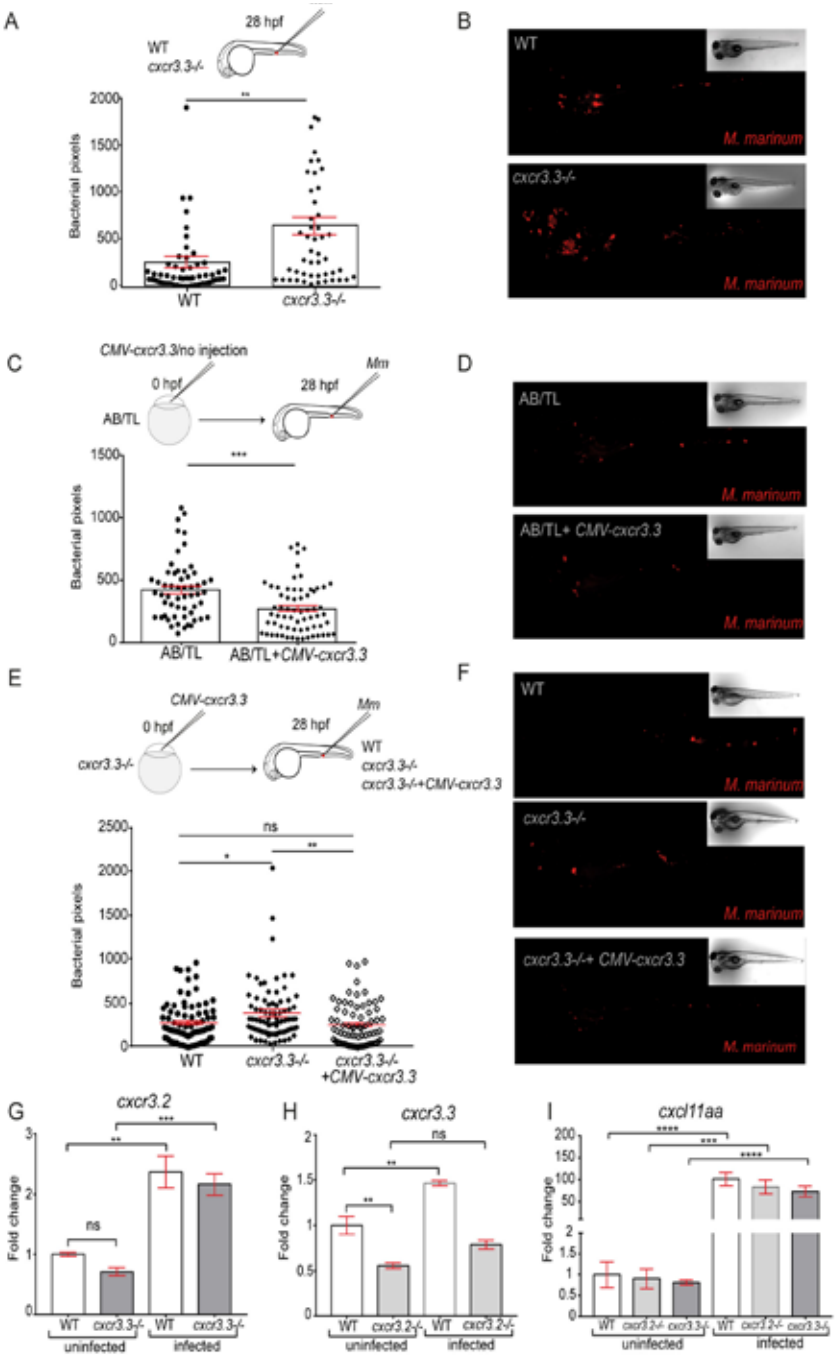

Figure 3. Depletion and overexpression of *cxcr3.3* result in opposite *M. marinum* infection outcomes. Cxcr3.3 deficient larvae had a higher bacterial burden than their WT siblings at 4 days

following blood island (BI) infection with 300 CFU of *M. marinum* (*Mm*) (**A, B**). We transiently overexpressed *cxcr3.3* in AB/TL embryos by injection of a *CMV: cxcr3.3* construct at 0 hpf and observed that bacterial burden was lower in larvae overexpressing the gene than in non-injected controls at 4 dpi (**C, D**). To rescue the *cxcr3.3-/-* phenotype we restored the expression of the gene by transiently overexpressing it (*CMV: cxcr3.3*) in one half of the *cxcr3.3* mutants (*cxcr3.3-/-* rescued). The bacterial burden was lower in the rescued group than in non-injected *cxcr3.3* mutants (*cxcr3.3-/-*) and similar to the bacterial burden in WT controls (**E, F**). Results from qPCR show that *cxcr3.2* expression remained unaltered in the *cxcr3.3* mutants and that it was induced upon infection (**G**), while *cxcr3.3* expression was lower in *cxcr3.2-/-* and was moderately induced upon *Mm* infection (**H**). The ligand *cxcl11aa* was induced upon infection independently of any of the *cxcr3* genes. In all cases, systemic infection was done at 28 hpf in the BI with 300 CFU of *Mm*. The bacterial burden data were analyzed using a two-tailed t-test (**A-C**) and a One-way ANOVA (**E**). Results are shown as mean ± SEM (ns $p > 0.05$, * $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$) and combine data of 3 independent replicates of 20-30 larvae each. The qPCR data were analyzed with the $2^{-\Delta\Delta Ct}$ method and a One-way ANOVA. Results are plotted as mean ± SEM (ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

bacteria, we injected *ΔERP M. marinum* into the circulation of WT and mutant larvae and quantified bacterial clusters in the tail area at 2 dpi. **Figure 4A** shows no difference between WT and mutants regarding the total number of bacterial clusters in the tail area. We divided bacterial clusters into three groups according to the number of bacteria they contained: 1-5 bacteria (small cluster), 6-10 bacteria (medium-sized cluster) and >10 (large cluster) as shown in the representative image illustrating the cluster size categories in **Figure 4B**. The frequency distributions of the 3 different cluster sizes in each genotype were compared and no significant difference was found (**Figure 4C**).

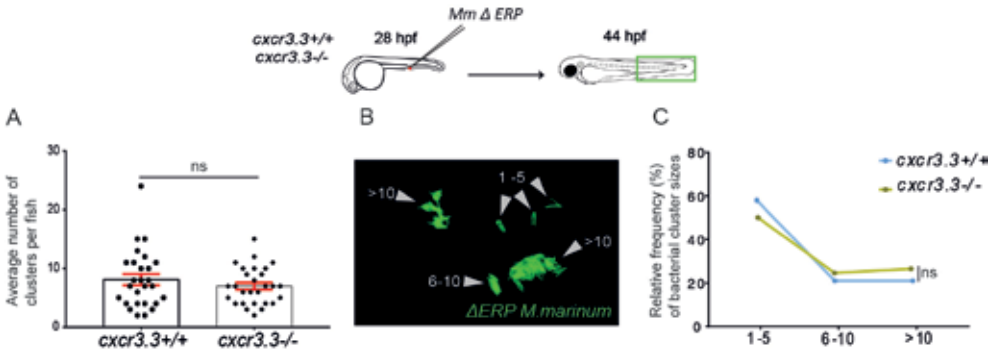

Figure 4. Macrophages lacking Cxcr3.3 efficiently clear intracellular bacteria. Cxcr3.3 deficient larvae and their WT siblings were infected in the BI at 28 hpf with 200 CFU of the *ΔERP M. marinum-wasabi* strain that is unable to survive and replicate inside acidic compartments and can be easily cleared by macrophages. The total number of bacterial clusters in every fish was quantified (**A**). We divided the bacterial clusters into 3 groups based on the number of bacteria they contained (1-5, 1-6 and > 10) to assess bacterial clearance at 44 hpi (**B**). No difference between WT and *cxcr3.3-/-* cluster size distributions (frequency in %) was found (**C**). A Mann-Whitney test was conducted to analyze the overall bacterial burden of the pooled data of 3 independent replicates of 9 fish each. Data are shown as mean ± SEM (**A**). A Kolmogorov-Smirnov test was used to analyze the distribution of bacterial cluster sizes (**C**) (ns $p > 0.05$).

Mycobacterial clearance remained unaffected in the absence of Cxcr3.3, suggesting that the poor control of the infection in *cxcr3.3* mutants is not due to a deficient bacterial clearance. As a positive control, we also ran this assay using DNA-damage regulated autophagy modulator 1 *(dram1)* mutant larvae, and their WT siblings, since *dram1* mutants cannot efficiently clear *Mm* [37]. The total number of clusters was higher in *dram1* mutants and large bacterial clusters were more frequent (**Supplementary figure 3**). Therefore, we conclude that macrophages in *cxcr3.3* mutants, contrary to *dram1* mutants, are not affected in their microbicidal capacity.

Cxcr3.3-deficient macrophages show enhanced recruitment to sites of infection, towards Cxcl11aa, and to sites of injury

Several studies have shown that macrophage recruitment is essential for bacterial clearance and containment during mycobacterial pathogenesis but supports bacterial dissemination and granuloma formation at early stages of the infection [55, 56]. We previously found that *cxcr3.2* mutant larvae showed attenuated recruitment of macrophages to sites of infection and towards Cxcl11aa ligand. This study suggested that macrophage-mediated dissemination of bacteria was reduced due to this recruitment deficiency in *cxcr3.2* mutants since fewer cells would become infected with *M. marinum* [29]. We addressed cell recruitment to examine whether the process was altered in absence of the Cxcr3.3 receptor. We infected 2-day old larvae in the hindbrain ventricle with either *M. marinum* or Cxcl11aa protein and quantified the macrophages that infiltrated into the cavity at 3 hpi. In both cases, we observed enhanced recruitment to the site of injection in *cxcr3.3* mutants (**Figure 5A-D**). In contrast, recruitment was attenuated in *cxcr3.2* mutants (**Figure 5A-D**), in line with our previous results [29]. The response to mechanical damage was also assessed using the tail-amputation model. The tail fins of WT, *cxcr3.2* mutant and *cxcr3.3* mutant larvae were dissected and macrophages within an area of 500 μm from the cut towards the trunk were quantified as recruited cells. Here too, we observed opposing results between the Cxcr3 mutants: more cells were recruited in the *cxcr3.3* mutants and fewer cells were recruited to the site of damage in the *cxcr3.2* deficient larvae (**Figure 5E, F**). We conclude that Cxcr3.3 and Cxcr3.2 deficiencies have opposing phenotypes regarding macrophage recruitment to sites of infection and injury or to a source of Cxcl11aa chemokine. While attenuated macrophage recruitment in *cxcr3.2* mutants favors bacterial contention [29], enhanced recruitment of macrophages to sites of infection in *cxcr3.3* mutants might be facilitating macrophage-mediated dissemination of bacteria, resulting in the increased bacterial burden observed in our infection experiments.

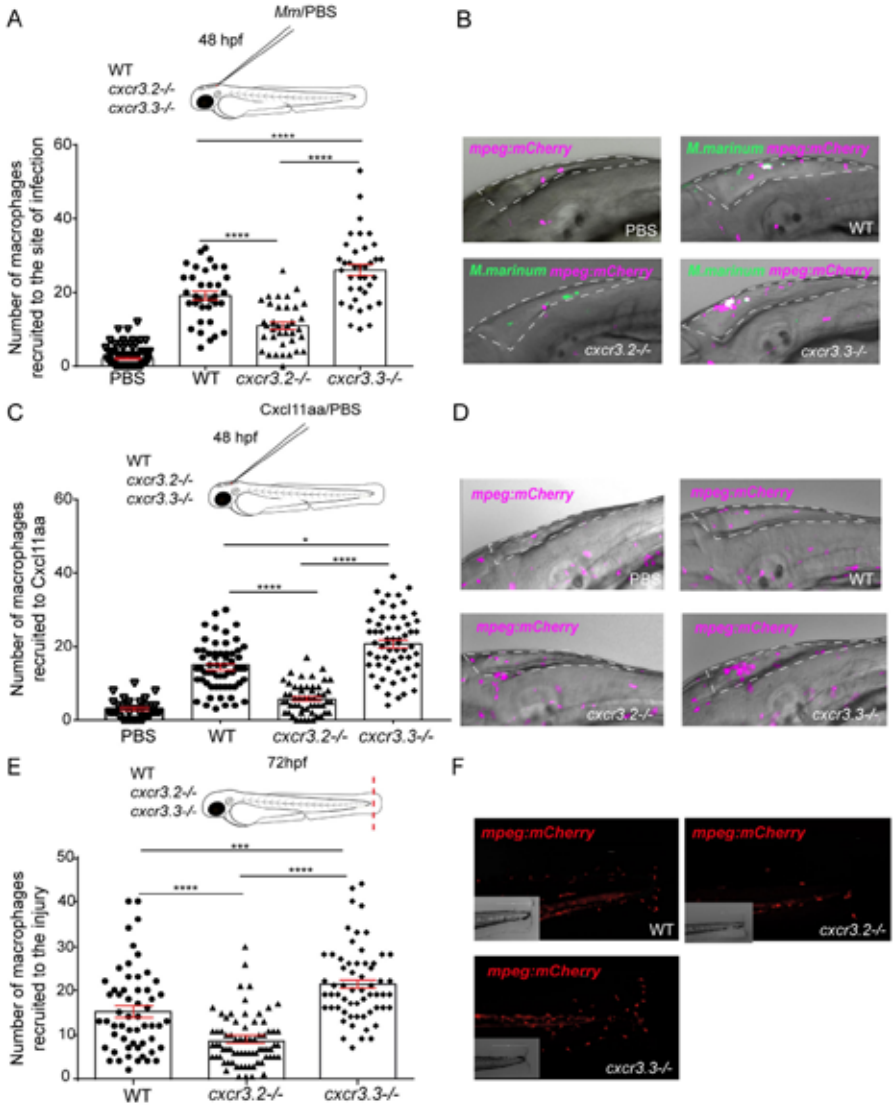

Figure 5. Macrophages lacking Cxcr3.3 show enhanced recruitment to sites of infection, towards Cxcl11aa, and to sites of mechanical damage. Significantly fewer cells were recruited to the hindbrain ventricle *in cxcr3.2-/-* at 3 hpi with *Mm* and more macrophages were recruited to the same site in *cxcr3.3-/-* compared to WT controls (**A, B**). The same trend was observed when 1nL of Cxcl11aa protein (10 ng/mL) was injected in the same experimental setup (**C, D**). To assess macrophage recruitment to sites of injury, we used the tail-amputation model and observed enhanced recruitment of macrophages in *cxcr3.3-/-* larvae and attenuated recruitment of macrophages in *cxcr3.2-/-* relative to WT at 4 hpa (**E, F**). The PBS injected control group combines WT, *cxcr3.2* and *cxcr3.3* mutants and shows no cell recruitment at 3 hpi. In all cases, statistical analyses were done with pooled data of three independent replicates (20-30 larvae per group each). A Kruskal-Wallis test was used

to assess significance (* $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$) and data are shown as mean ± SEM.

Cxcr3.3 depletion has no significant effect on neutrophil recruitment to sites of infection or injury

Although macrophages are the first responders towards mycobacterial infection and the main components of granulomas, neutrophils are also recruited to infectious foci and participate in the early immune response [57, 58]. Besides, both Cxcr3.2 and Cxcr3.3 are also expressed on this cell-type [29]. Therefore, we assessed the effect of the *cxcr3.2* and *cxcr3.3* mutations on neutrophil recruitment to local *Mm* infection and upon injury similar as for macrophages in the previous section (**Figure 6**). When *Mm* was locally injected into the hindbrain, fewer neutrophils were recruited to the cavity in *cxcr3.2* mutants at 3 hpi, while there was no difference between WT and *cxcr3.3* mutants (**Figure 6 A-B**). Using the tail-amputation model to assess cell recruitment, we observed the same pattern: the lack of *cxcr3.2* reduced neutrophil recruitment to injury while recruitment remained unaffected in *cxcr3.3* mutants (**Figure 6C-D**). Our data suggest that Cxcr3.2 is required for neutrophil recruitment, as shown by previous studies [59], and that the effect of the *cxcr3.3* mutation does not significantly impact the migratory properties of this cell type.

Macrophages lacking Cxcr3.3 move faster than WT cells under basal conditions and upon mechanical damage, and have an elongated and branched morphology

We previously reported that macrophage recruitment to sites infection was attenuated in *cxcr3.2* mutant macrophages because cells were less motile [29]. To further examine the role of cell recruitment in *M. marinum* pathogenesis, we assessed if macrophage motility was also affected when Cxcr3.3 was ablated. Cell motility was reviewed concerning total cell displacement and average speed. No significant difference was found in total cell displacement under basal conditions (**Figure 7A, B-1**) but *cxcr3.3* mutant macrophages moved faster than the other two groups (**Figure 7A, B-2**). To induce directional migration of macrophages we used the tail-amputation model. The tracks covered by *cxcr3.2* mutant macrophages were shorter when we induced directed migration (**Figure 7C, D-1**). In contrast, Cxcr3.3-deficient macrophages moved faster than the remaining groups when the tail-amputation model was employed (**Figure 7C, D-2, Supplementary videos 1**). Cell circularity index (CI) was assessed as an indicator of motility and activation status of macrophages. Both *cxcr3* mutant CI distributions differ from the WT. The distribution of the CI values of Cxcr3.3-depleted macrophages shows that more cells are branched and elongated, while the CI value distribution in the *cxcr3.2* mutants suggests that macrophages are rounder (**Figure 7E**).

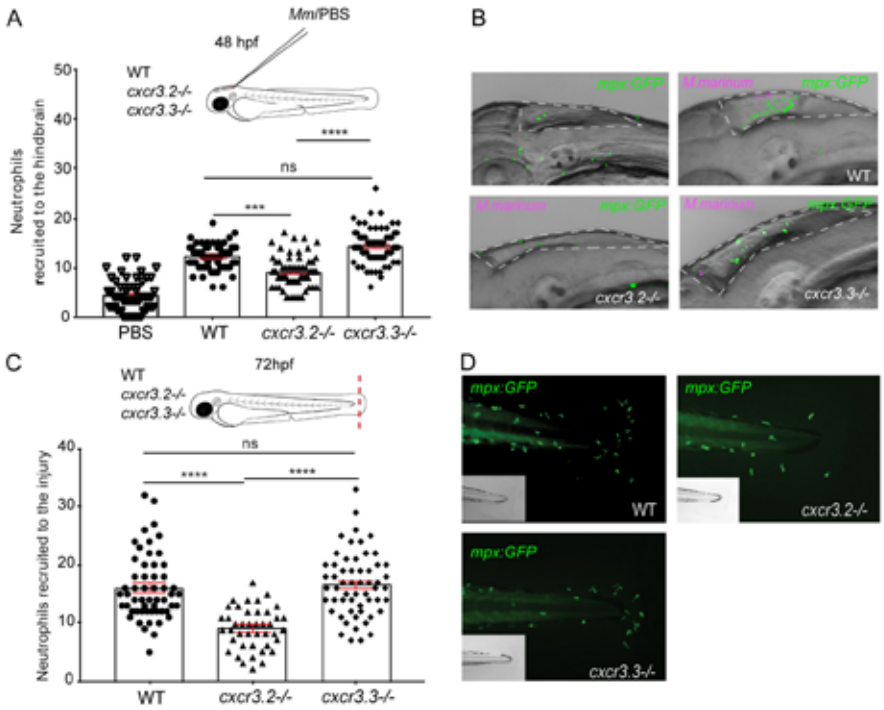

Figure 6. Neutrophil recruitment to sites of infection and injury is not altered in *cxcr3.3* mutants. 100 CFU of *Mm*-mCherry were injected in the hb ventricle of 2-day-old WT, *cxcr3.2* and *cxcr3.3* mutant larvae to assess neutrophil (*mpx*: e*GFP*) recruitment to the infection site at 3 hpi. The number of cells that infiltrated the cavity was lower in *cxcr3.2* mutants but remained unchanged in WT and *cxcr3.3* mutants (**A-B**). The tail fin of WT larvae and *cxcr3.2* and *cxcr3.3* mutants was amputated and neutrophil recruitment was assessed at 4 hours post amputation. There were fewer recruited neutrophils in the *cxcr3.2* mutants while there was no difference between *cxcr3.3* mutants and WT (**C-D**). The PBS injected control group PBS combines WT, *cxcr3.2* and *cxcr3.3* mutants and shows no cell recruitment at 3 hpi. In all cases, statistical analyses were done with pooled data of three independent replicates (20-30 larvae per group each). A Kruskal-Wallis test was used to assess significance (ns $p > 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$) and data are shown as mean ± SEM.

The most frequent CI interval within WT macrophages was 0.4-0.6 (42%), for *cxcr3.2* mutants it was 0.4-0.8 (71%) and for *cxcr3.3* mutants, 0.2-0.4 (39%) (**Figure 7F**). To further confirm that *cxcr3.2* and *cxcr3.3* mutants have different activation profiles we assessed the transcriptional profile of the inflammatory markers *tnfa*, *cxcl11aa* and *il1b* at 4 hours post amputation in the three groups and found that *tnfa* and *cxcl11aa* were upregulated in *cxcr3.3* mutants (**Supplementary figure 4**). Taken together, these data

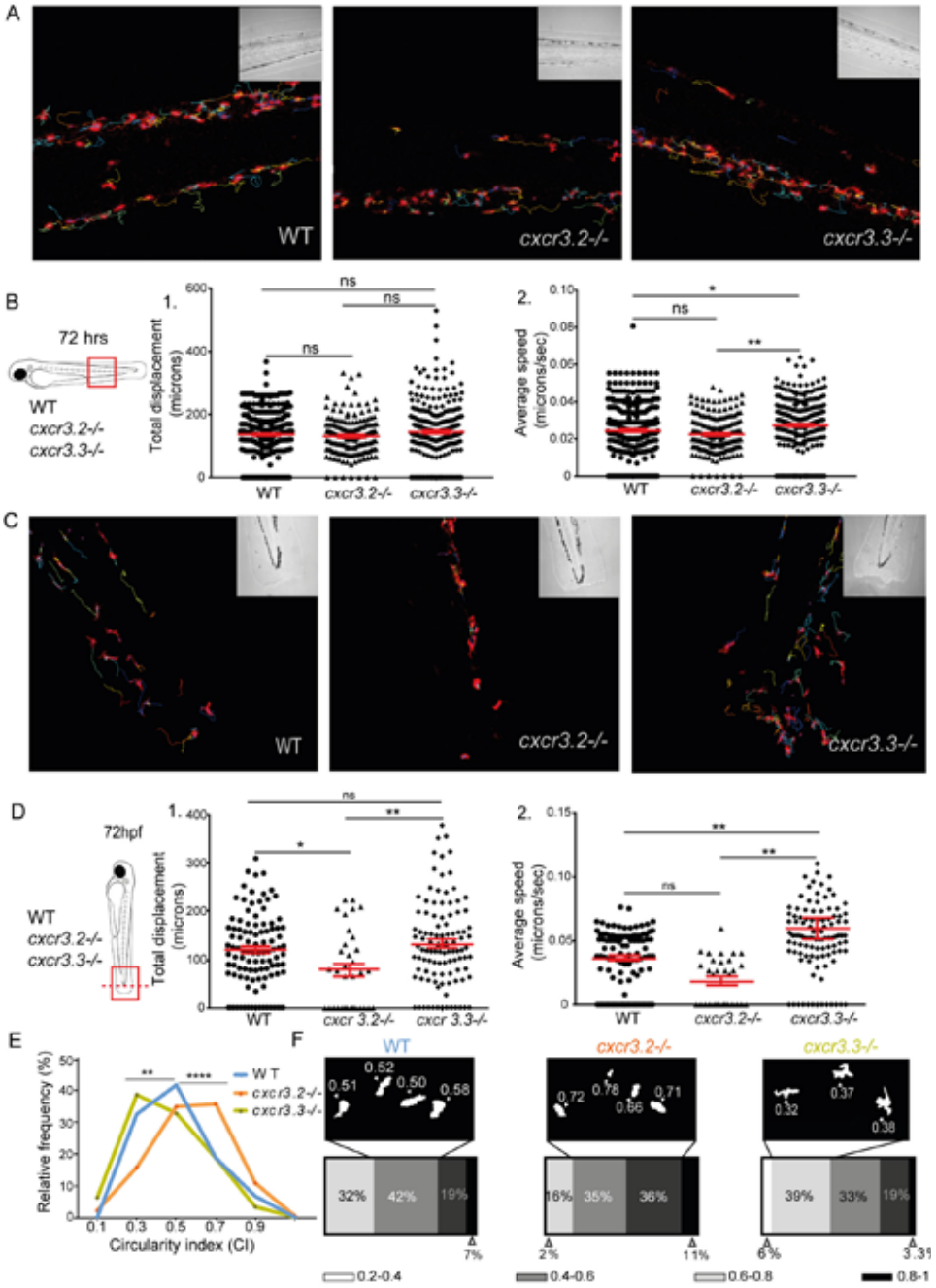

Figure 7. Cxcr3.3 depleted macrophages move faster than WT cells under basal conditions and upon mechanical damage and have a lower circularity index (CI). Panel **A** shows representative images of tracks of macrophages of 3-day-old larvae from the three genotypes under

unchallenged conditions (random patrolling). Macrophages were tracked for 3 h and images were taken every 2 minutes. Graphs in **B** show the total displacement of all cells tracked shortly after amputation in each group throughout 3h (**B-2**) and the average speed of each cell (**B-2**). In this case, macrophages were tracked for 1.5 h and images were acquired every 1 minute. There was no significant difference between the groups in terms of total cell displacement (**B-1**), however *cxcr3.3-/-* macrophages did move faster than the remaining groups as indicated by the dot-plots in (**B-2**). Panel **C** shows representative images of macrophage tracks of the three groups directly after a tail amputation. The tracks of *cxcr3.2-/-* macrophages were shorter than those of the remaining groups (**D-1**) and *cxcr3.3-/-* macrophages moved faster than the other two groups when mechanical damage was inflicted (**D-2**). Data of unchallenged larvae were collected from two independent replicates (5 larvae per group each) and for the tail-amputation model data from 3 independent replicates (4 larvae per group each) were pooled together for analysis. A One-way ANOVA was performed to test for significance (ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$) of the most frequent CI and data are shown as mean ± SEM. The circularity index (CI) distributions of both *cxcr3.2-/-* and *cxc3.3-/-* differ from the WT control but are skewed in opposite directions as low CI values are more frequent in *cxcr3.3* mutants than in WT and high CI values are more frequent in *cxcr3.2* mutants as shown by the curves. (**E**). Panel **F** shows representative images interval in each group and the bar displays the percentage of each CI category within each genotype. A Kolmogorov-Smirnov test was used to evaluate the CI value distributions using the WT data as reference distribution (** $p \leq 0.01$, **** $p \leq 0.0001$).

suggest that macrophage recruitment in *cxcr3.3* mutants results from a faster displacement of these cells to reach sites of infection or other inflammatory stimuli. This increased speed is linked to a higher macrophage activation status (lower CI values) and a pro-inflammatory phenotype of the *cxcr3.3* mutant fish. Therefore, we propose that the progression of *M. marinum* infection is accelerated in *cxcr3.3* mutants by facilitating the spreading of bacteria into newly recruited macrophages and the subsequent seeding of secondary granulomas

Enhanced motility of *cxcr3.3* mutant macrophages facilitates cell-mediated *M. marinum* dissemination

Taking into account that *cxcr3.3* mutant macrophages move faster and are recruited more efficiently to sites of infection, we wanted to know whether enhanced motility of macrophages in *cxcr3.3* mutants could facilitate bacterial dissemination by accelerating granuloma formation and seeding of secondary granulomas. We addressed our question by locally injecting *Mm* into the hindbrain of WT, *cxcr3.2* and *cxcr3.3* mutants at 28 hpf and by assessing the percentage of infected larvae that developed distal granulomas at 4 dpi (**Figure 8A**), as well as the number and size of such granulomas in each group (**Figure 8C, D**). Our data show that *cxcr3.3* mutant larvae more frequently developed distal granulomas (22%) than the other two groups (**Figure 8A**). In addition, the average number of the distal granulomas per fish within this group was higher (**Figure 8C**) and the quantified structures were also larger (**Figure 8D**). Consistent with previous work [29], a small proportion of *cxcr3.2* mutant larvae (5%) developed fewer and smaller distal granulomas compared with the wild type (12.7%). Our data suggest that

cxcr3.3 mutant macrophages favor bacterial dissemination and the seeding of secondary granulomas due to their enhanced recruitment to sites of infection and their higher speed.

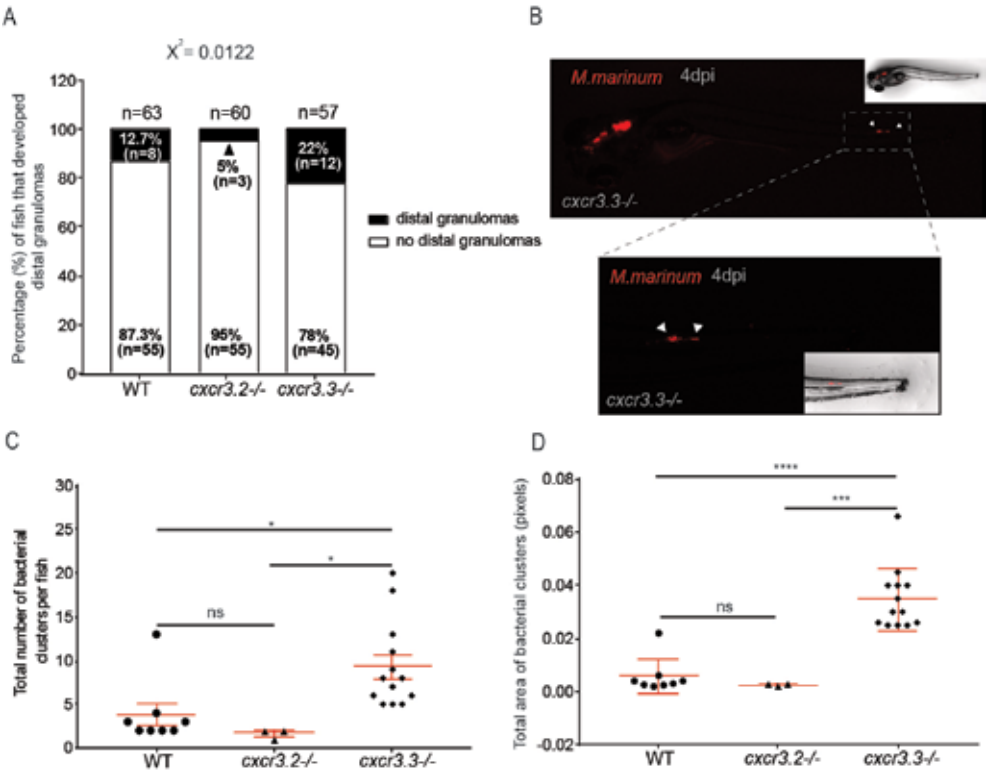

Figure 8. Enhanced motility of *cxcr3.3* mutant macrophages facilitates bacterial dissemination. Four days after local infection with 200 CFU of *Mm* in the hb, *cxcr3.3* mutants developed more distal granulomas (22%) than WT (12.7%) and *cxcr3.2* mutants (5%) while the latter developed fewer than the other two groups (**A**). Embryos from the three genotypes were infected at 28 hpf and imaged under the stereo fluorescence microscope (whole body and zoom to the tail) at 4 dpi. **B** illustrates the imaging process of a representative *cxcr3.3* mutant larvae. Cxcr3.3 depleted larvae developed more distal granulomas per fish (**C**) and these granulomas were also larger in *cxcr3.3* mutants than the other two groups, while *cxcr3.2* mutants showed an opposite trend (**D**). Graphs show pooled data from four independent replicates, each of 12-15 infected larvae per group. The number and size of distal granulomas were determined using the "analyze particle" function in Fiji. A $\chi 2$ test was conducted to assess differences in the proportion of larvae that developed distal granulomas within the 3 groups and a One-way ANOVA to compare the number and size of distal granulomas (ns $p > 0.05$, * $p \leq 0.05$, *** $p \leq 0.001$ and **** $p \leq 0.001$). Data are shown as mean ± SEM.

Chemical inhibition of both Cxcr3 receptors affects only macrophages expressing Cxcr3.2 and phenocopies *cxcr3.2* mutants regarding bacterial burden and macrophage recruitment efficiency

To further inquire into the roles and interactions of Cxcr3.2 and Cxcr3.3 we chemically inhibited both receptors simultaneously and addressed macrophage recruitment using the tail-amputation model and the *M. marinum* infection model. To this end, we used the allosteric CXCR3-specific inhibitor NBI 74330, of which the binding site is highly conserved in the Cxcr3.2 and Cxcr3.3 protein structures [29]. WT, *cxcr3.2* mutant and *cxcr3.3* mutant larvae were bath-exposed for 3 h before amputation and for another 4 h following tail-amputation to NBI 74330 (50 μM) or to the vehicle DMSO (0.05%) as a control. A significant reduction in the number of recruited macrophages occurred in WT and *cxcr3.3* mutants, but there was no decline in cell recruitment in *cxcr3.2* mutants when exposed to the inhibitor (**Figure 9A-D**).

Similarly, WT larvae were bath exposed to NBI 74330 (25 μM) for 3h prior systemic infection with *M. marinum* and kept in NBI 74330 (25 μM) for the following 4 days. Inhibition of both Cxcr3 receptors resulted in a lower bacterial burden than that of larvae treated with DMSO (**Figure 9E, F**) and thereby phenocopied the effects of the *cxcr3.2* mutation [29] or *cxcr3.3* overexpression (this study). These results support our hypothesis that Cxcr3.2, an active GPCR, is essential for macrophage motility and recruitment to different stimuli while Cxcr3.3, an ACKR with predicted scavenger function, does not play a direct role on these processes but indirectly regulates them by competing with active receptors for shared ligands.

Discussion

Our findings illustrate the evolution of regulatory mechanisms in chemokine signaling networks and show how positive or negative dysregulation of the CXCR3 signaling axis results in opposite outcomes on macrophage behavior and innate host defense against mycobacterial infection. The *Mycobacterium tuberculosis* epidemiology highlights the urgent need to develop new clinical strategies to treat the infection given the growing incidence of multidrug-resistant strains and the high prevalence of the infection worldwide [12, 60]. GPCRs, such as chemokine receptors, are the largest protein family targeted by approved pharmacological therapies [61]. Therefore, it is important to further our understanding of the fundamental regulatory mechanisms of GPCR-related pathways. In the present study, we used the zebrafish model to functionally characterize the antagonistic interplay between two CXCR3 paralogs in the context of mycobacterial infection and mechanical damage. Our results suggest that the potential scavenging activity of an atypical CXCR3 paralog, Cxcr3.3, fine-tunes the activity of the functional CXCR3 paralog, Cxcr3.2, serving as a regulatory mechanism for the modulation of the immune

response. These findings highlight the relevance of ACKRs as regulatory components of chemokine signaling networks. At present, 5 ACKRs have been described in vertebrates, namely, ACKR1 (DARK), ACKR2, ACKR3 (CXCR7), ACKR4, and ACKR5 [12, 18]. The identification of ACKRs and the subsequent classification of these receptors within the subfamily is complex given their structural heterogeneity and the limited phylogenetic homology [17, 18, 15]. However, as in all GPCRs, the E/DRY motif and microswitch elements are indicative of the activation status and function of a receptor [16].

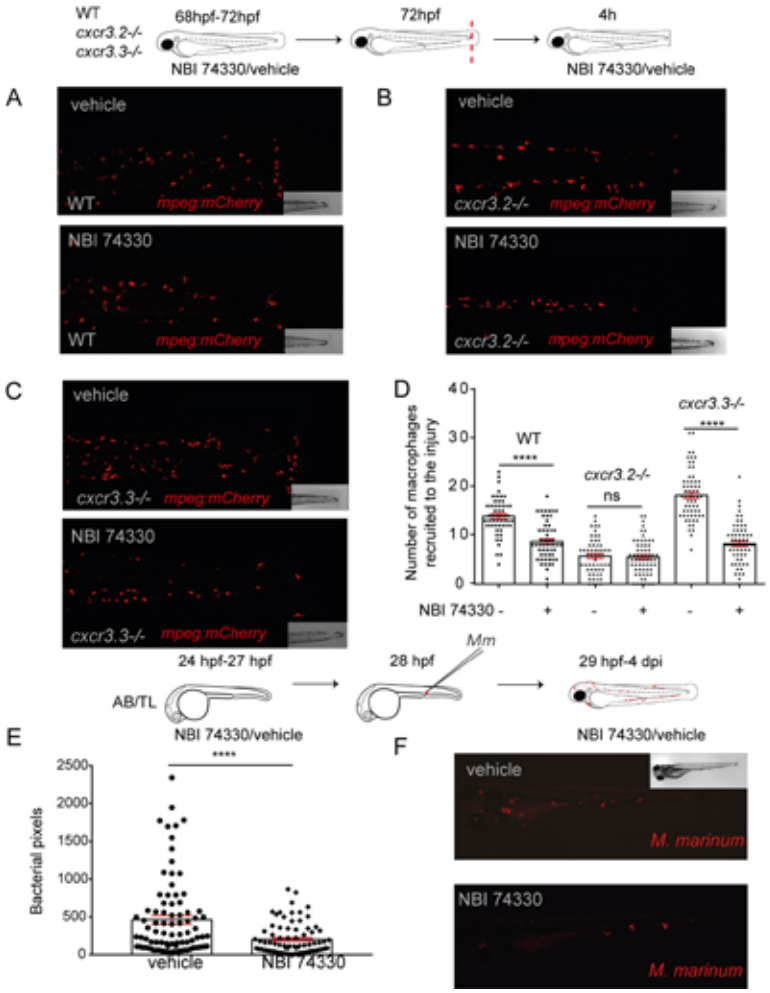

Figure 9. Chemical inhibition of both Cxcr3 receptors affects only macrophages expressing Cxcr3.2 and renders a similar bacterial burden and macrophage recruitment efficiency as *cxcr3.2* mutants. After bath exposure of 3-day old larvae to either the CXCR3-specific inhibitor NBI 74330 (50 μM) or vehicle (DMSO 0.01%), before and after tail-amputation showed that cell recruitment to the site of injury was reduced in macrophages expressing Cxcr3.2, namely WT

and *cxcr3.3-/-* (**A, C**), while no further decline in cell recruitment was observed in *cxcr3.2* mutants (**B, D**). Chemical inhibition of both Cxcr3 receptors with NBI 74330 (25 μM) before and after systemic infection with *Mm* resulted in a lower bacterial burden at 4 dpi than in the vehicle-treated control and resembles the *cxcr3.2* mutant phenotype (**E, F**). The data of three independent replicates were pooled and are presented as mean ± SEM. A Kruskal-Wallis test was conducted to assess significance (ns $p > 0.05$, **** $p \leq 0.0001$) in the macrophage recruitment assay. Only the p values between each condition (vehicle/ NBI 74330) within each group are shown (D). Bacterial burden data were analyzed using a two-tailed t-test and data are shown as mean ± SEM (**** $p \leq 0.0001$).

Microswitches stabilize the active conformation of GPCRs and are highly conserved residues, which are unchanged in Cxcr3.2 but not in Cxcr3.3 [13, 16]. The Asp (D) and the Arg (R) of the E/DRY- motif are key residues to stabilize the inactive conformation of GPCRs by forming a salt bridge between the 3rd IC loop and TM6 that blocks G-protein coupling. This so-called "iconic-lock" breaks upon binding of an agonist and triggers structural rearrangements that expose the G-protein docking site and enables canonical (G-protein-dependent) downstream signaling [16]. Substitutions in the E/D and Y within the E/DRY-motif are commonly associated with the permanent activation of the receptor and gain of function events, while substitutions of the R, as found in Cxcr3.3 (DCY motif), have been shown to result in the permanent "locking" of the receptor and a consequent loss of function [16, 54, 62]. The E/DRY motif also interacts with the intracellular COOH terminus that is critical for GPCR activation and with Gα subunits. It is noteworthy to mention that chemokine receptors can also signal in a G-protein-independent manner through β-arrestin in the context of chemotaxis, and that this pathway is associated with the internalization and subsequent intracellular degradation of ligands [16, 62]. Altogether, this information led us to propose that Cxcr3.3 is an ACKR.

The zebrafish genome encodes a family of seven *cxcl11*-like paralogous genes, which are thought to share common ancestry with *CXCL9-10-11*, the ligands of human CXCR3 [29]. We have previously shown that one of the *cxcl11*-like genes, *cxcl11aa*, is strongly inducible by mycobacterial infection and by mechanical damage [29, 63]. Subsequently, we used an *in vivo* macrophage migration assay in *cxcr3.2* mutants and wild-type siblings to demonstrate that purified Cxcl11aa protein acts as a ligand for the Cxcr3.2 receptor. Based on the structural conservation of the ligand-binding site Cxcr3.3 is predicted to bind the same ligands as Cxcr3.2. This is consistent with several studies reporting that mutations in GPCRs may affect the structure of the receptor preventing the opening of the intercellular cavity required for G-protein coupling and subsequent signaling, while ligand affinity remains unchanged [16]. Furthermore, the fact that the top hits in our ligand prediction analysis are shared by both Cxcr3 paralogs strongly suggests that both receptors can bind the same ligands due to the highly conserved hydrophobic residues in the ligand-binding site. Studies showing that signaling by CXCL11 and CXCL12 chemokines is subject to ACKR regulation [13, 18], set a precedent for our hypothesis

that both receptors can bind the same ligands but only Cxcr3.2 can signal in a canonical manner, while Cxcr3.3 acts as a regulator by scavenging shared ligands. Nevertheless, biochemical data are required to fully confirm our hypothesis.

To deconstruct the proposed antagonism of Cxcr3.3 on Cxcr3.2 activity, we first compared the overall outcomes of *M. marinum* infection in *cxcr3.2* and *cxcr3.3* mutants. In agreement with our hypothesis, we observed that *cxcr3.2* and *cxcr3.3* mutants have opposite infection phenotypes. While our previous results showed that *cxcr3.2* mutants have increased resistance to mycobacterial infection [29], similar to *cxcr3* mutant mice [30], the *cxcr3.3* mutant generated in this study is more susceptible to *M. marinum*. The increased infection burden of *cxcr3.3* mutants could be reverted to wild-type levels by injection of *cxcr3.3* mRNA, confirming the specificity of the mutant phenotype. A reduced infection burden, similar to the *cxcr3.2* mutant phenotype, was induced when *cxcr3.3* was overexpressed in wild-type AB/TL embryos, further supporting the notion that Cxcr3.2 and Cxcr3.3 have contrasting functions. We asked whether the underlying causes of the opposite effects of Cxcr3.2 and Cxcr3.3 on mycobacterial infection were due to essentially antagonistic functions or to a dysregulation of the transcription of the genes for the Cxcr3.2 and Cxcr3.3 receptors or the Cxcl11aa ligand. Gene expression profiles showed that *cxcr3.2* and *cxcl11aa* are induced upon infection with *M. marinum* and that *cxcr3.3* expression depends on *cxcr3.2*. The infection-driven induction of *cxcl11aa* remains unaltered in the *cxcr3.2* and *cxcr3.3* mutants, suggesting that the transcriptional regulation of the axis does not involve the ligand. While *cxcr3.3* expression levels were lower in *cxcr3.2* mutants, no alteration of *cxcr3.2* expression was detected in *cxcr3.3* mutants. Therefore, the increased infection susceptibility of *cxcr3.3* mutants cannot be explained by differences in the level of the functional Cxcr3.2 receptor or the Cxcl11aa ligand. Taking these data together, we propose that the Cxcr3-Cxcl11 signaling axis is regulated at least at two levels. At the transcriptional level, infection drives the expression of *cxcr3.2* (and indirectly *cxcr3.3*) and *cxcl11aa.* At the functional level, Cxcr3.2 signals in response to Cxcl1aa ligand, while Cxcr3.3, given its ACKR-like features, may function to negatively regulate Cxcr3.2 activity.

The increased infection burden of *cxcr3.3* mutants could either be due to a defective bacterial clearance or to altered macrophage migration properties, which can have major effects on the development of mycobacterial infection [29, 64, 8]. We demonstrated that *cxcr3.3* mutants can clear bacteria effectively and proceeded to evaluate if an altered macrophage migration could be facilitating bacterial dissemination. We obtained results supporting the functional antagonism between Cxcr3.2 and Cxcr3.3 when we locally injected *M. marinum* or purified Cxcl11aa protein into the hindbrain cavity. In both cases, we observed enhanced recruitment of macrophages to the site of injection in *cxcr3.3* mutants, while *cxcr3.2* mutants displayed reduced cell recruitment. Interestingly, while neutrophil recruitment was reduced in the *cxcr3.2* mutant, it remained unal-

tered in *cxcr3.3* mutants, suggesting that Cxcr3.3 has no effect on neutrophil migratory properties.
To examine whether altered cell motility was the underlying reason for enhanced recruitment in *cxcr3.3* mutant macrophages, we used a tail-amputation assay to assess migration in terms of total cell displacement and average speed. We showed that *cxcr3.3* mutant macrophages move faster than WT controls. To test our hypothesis, we assessed bacterial dissemination and confirmed that, in the context of *M. marinum* infection, the overall worse outcome in *cxcr3.3* mutant larvae was linked to amplified macrophage-mediated dissemination of bacteria that is facilitated by the higher speed of migrating macrophages and favors the formation of secondary granulomas. Since more macrophages were recruited when Cxcl11aa was injected into the hindbrain cavity and upon tail-amputation, we propose that the enhanced macrophage recruitment in *cxcr3.3* mutants is not a specific *M. marinum*-induced phenotype, but rather a Cxcl11-dependent response that can also result from wound-induced inflammation or other Cxcl11aa-inducing stimuli.

The circularity index (CI) is a measure indicative of the activation status of macrophages, with low CI values (stretched morphology) corresponding to a high activation status [65, 66]. The predominance of macrophages with low CI values in *cxcr3.3* mutants suggests that these cells have a higher activation status and that they are more responsive to stimuli in their environment. Cxcr3.3 depleted larvae showed an overall upregulation of inflammatory markers (*tnfa* and *cxcl11aa*) at 4 hpa. We suggest that the inflammatory phenotype of Cxcr3.3-deficient larvae reflects a dysregulation in the Cxcr3-Cxcl11 signaling axis, supported by the upregulation of *cxcl11aa*, that results in an exacerbated Cxcr3.2 signaling in the absence of the ligand-scavenging function of Cxcr3.3. In support of this model, the simultaneous chemical inhibition of the two Cxcr3 paralogs showed that only macrophages expressing Cxcr3.2 were affected and that the inhibitor treatment phenocopied *cxcr3.2* mutants regarding *M. marinum* burden and wound-induced macrophage recruitment. These data provide further evidence that Cxcr3.2 is directly involved in leukocyte trafficking, while Cxcr3.3 only fine-tunes the process by shaping the chemokine gradient and the availability of shared ligands.

Although we found that enhancement of Cxcr3.2 signaling due to the loss of Cxcr3.3 is detrimental in *M. marinum* infection, it might be beneficial in the context of other infections or in other processes dependent on macrophage recruitment, such as tissue repair and regeneration. Furthermore, it should be noted that the function of a chemokine receptor is primarily dependent on the type of cell expressing it, as the sub-set of receptors expressed by the cell and the intracellular integration of the signals have been shown to be determinant for functional specificity [28]. While our study revealed that macrophage migration is modulated by an antagonistic interplay between the Cxcr3.2 and Cxcr3.3 receptors, it remains to be determined how interactions between Cxcr3

paralogs may affect the function of other innate and adaptive immune cells. Although there is only one copy of CXCR3 in humans, there are 3 splice variants of the gene (CXCR3-A, CXCR3-B, and CXCR3-alt), and a regulatory mechanism for fine-tuning of CXCR3 function also exists. The splice variants CXCR3-A and CXCR3-B differ in their N and C termini and carry out antagonistic functions. CXCR3-A mediates chemotaxis and proliferation, while CXCR3-B inhibits cell migration and proliferation, and induces apoptosis [67, 68]. Both splice forms can bind to CXCL9-11 chemokines but mediate opposite functions. While there are no splice variants of *cxcr3.2* and *cxcr3.3* in zebrafish [69], the regulatory antagonism between the two paralogs resembles the interaction between the two human CXCR3 splice variants, which might suggest a form of convergent evolution. However, this functional diversification of CXCR3 variants is not conserved in the murine model, where CXCR3 is a single copy gene and no splice variants have been identified so far [30, 67].

In conclusion, our work illustrates the antagonistic interaction between the two CXCR3 paralogs Cxcr3.2 and Cxcr3.3 in zebrafish. The structural analysis of Cxcr3.3 supports that this receptor is unable to signal in the conventional G-protein-dependent way, but that it can still bind ligands and shape chemokine gradients, thereby regulating active receptors with shared ligands. Our experimental data show that the absence of the scavenging function of Cxcr3.3 is detrimental in the context of mycobacterial infection due to an exacerbated Cxcr3.2 signaling and a consequently enhanced macrophage motility that facilitates bacterial dissemination. However, we propose that enhanced macrophage motility could be benign in other contexts, such as tissue repair. Our findings suggest an extensive crosstalk between several chemokine signaling axes such as CXCR3-CXCL11 andCXCR4-ACKR3 (CXCR7), since ACKR3 also binds CXCL11 besides CXCL12 [26, 18]. Furthermore, ACKR1 a silent receptor that does not scavenge chemokines but redistributes them to mediate leukocyte extravasation, shares the CXCL11 and CXCL4 ligands with CXCR3 [70, 71]. The complexity of signaling axis integration, further emphasizes the relevance of unraveling the fundamental mechanistic principles underlying intricate chemokine networks. Our findings contribute to understanding one such mechanistic interaction and suggest that a more comprehensive ACKR classification needs to be developed to aid the understanding of complex chemokine signaling regulation.

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Supplementary materials

Supplementary table 1. Top hits for predicted ligands of Cxcr3.2 and Cxcr3.3.

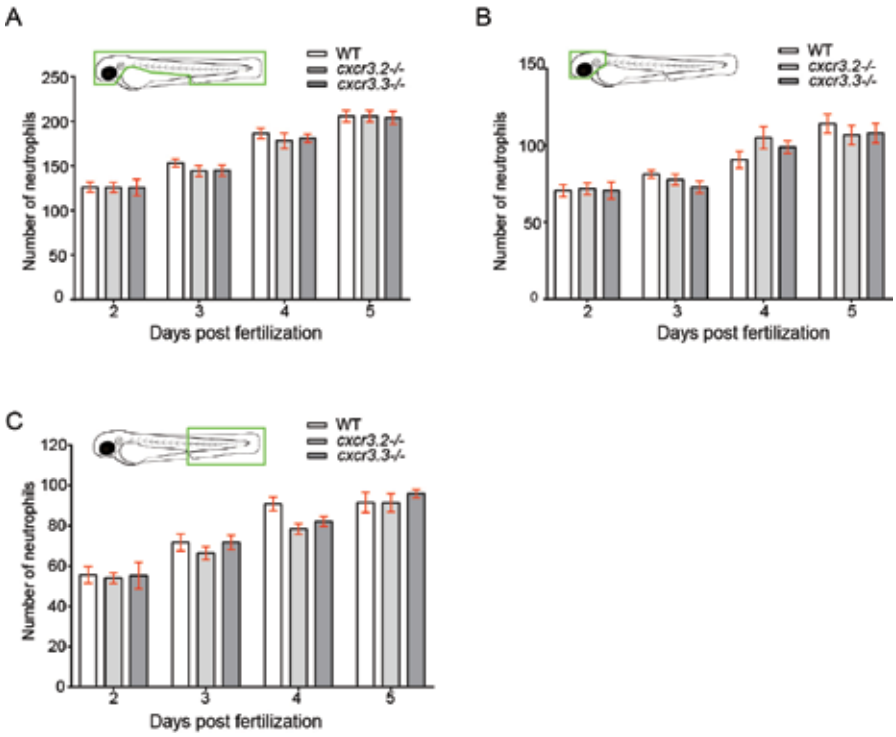
	Ligand name	C-score
1	0NN	0.18
2	DGW	0.12
3	2CV	0.5
4	Y01	0.05
	Ligand name	**C-score**
1	DGW	0.12
2	0NN	0.10
3	Y01	0.05
4	2CV	0.04

**c-score-confidence index*

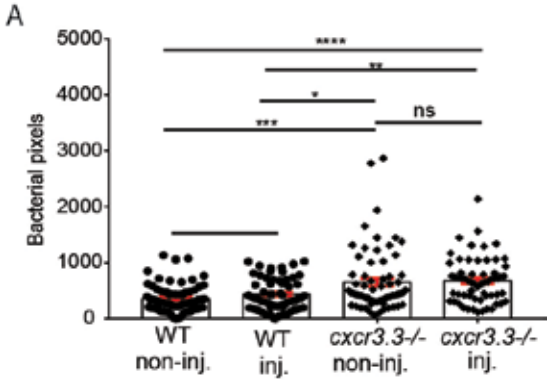
Supplementary table 2. Accession numbers of sequences used for the phylogenetic tree.

	Species	Accession number
1.	Cavefish (CF) Ackr4	ENST00000249887.2
2.	Cavefish (CF) Ackr4b	ENSAMXG00000025769
3.	Cavefish (CF)Cxcr3.2	ENSAMXG00000035350
4.	Cavefish (CF)Cxcr3.3	ENSAMXG00000018866
5.	Herring (CH) CXCR3	XP_012694805.1
6.	Cod (COD)Ackr3b	ENSGMOG00000019953
7.	Cod (COD)Cxcr3.3	ENSGMOG00000016951
8.	Coelacanth (COE) Ackr2	ENSLACG00000000319
9.	Coelacanth (COE) Ackr3	ENSLACG00000016030
10.	Coelacanth (COE) Ackr4	ENSLACG00000007877
11.	Coelacanth (COE) Cxcr3.1	XP_005999214.1
12.	Coelacanth (COE) Cxcr3.2	XP_014343707.1
13.	Elephant shark (ES) Cxcr3	XP_007909361.1

14. Frog (FR) Ackr3	ENSXETG00000003296
15. Frog (FR) Cxcr3	ENSXETG00000024989
16. Fugu (FU) Ackr4a	XP_003978861.2
17. Fugu (FU) Ackr4b	XP_011606262.1
18. Fugu (FU) Cxcr3.1	XP_003966387.1
19. Fugu (FU) Cxcr3.3	XP_003966388.2
20. Human (HU) Ackr1	ENSG00000186810
21. Human (HU) Ackr2	ENSG00000144648
22. Human (HU) Ackr3	ENSG00000144476
23. Human (HU) Ackr4	ENSG00000129048
24. Human (HU) Cxcr3	ENSG00000186810
25. Lamprey (LAM) Ackr3	XP_007909361.1
26. Mouse (MO) Ackr2	ENSMUSG00000044534
27. Mouse (MO) Ackr3	ENSMUSG00000044337
28. Mouse (MO) Ackr4	ENSMUSG00000079355
29. Mouse (MO) Cxcr3	ENSMUSG00000050232
30. Asian arowana (SF) Cxcr3	XP_018587703.1
31. Asian arowana (SF) Cxcr3a	KPP61297.1
32. Spotted gar (SG) Ackr2	ENSLOCG00000009409
33. Spotted gar (SG) Ackr4a	ENSLOCG00000018134
34. Spotted gar (SG) Ackr3	ENSLOCG00000004865
35. Spotted gar (SG) Cxcr3.1	XP_015224255.1
36. Spotted gar (SG) Cxcr3.3	AWO96327.1
37. Zebrafish (ZF) Ackr3a	ENSDART00000090414
38. Zebrafish (ZF) Ackr3.2	ENSDARG00000058179
39. Zebrafish (ZF) Ackr4a	ENSDART00000145446
40. Zebrafish (ZF) Ackr4b	ENSDART00000058703
41. Zebrafish (ZF) Cxcr3.1	ENSDARG00000078177
42. Zebrafish (ZF) Cxcr3.2	ENSDARG00000041041
43. Zebrafish (ZF) Cxcr3.3	ENSDART00000146611

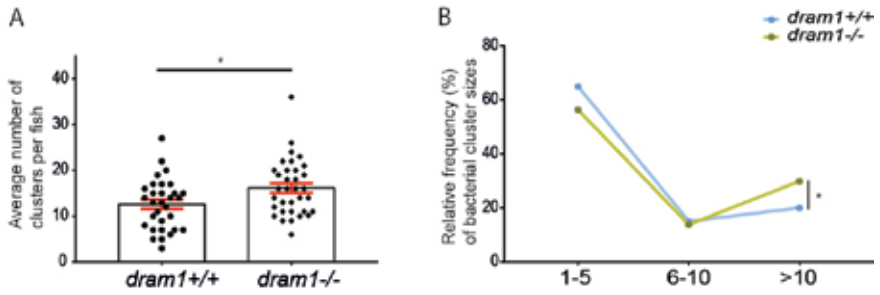


Supplementary figure 1. Neutrophil development is unaltered in *cxcr3.3* mutants. No evident morphological aberrations were observed in *cxcr3.3-/-* larvae within the first 5 dpf and the numbers of neutrophils in whole body (**A**), head (**B**), and tail (**C**) were unaltered in *cxcr3.3* and *cxcr3.2* mutants. The cell numbers corresponding to each day are the average of 35 larvae of each of the 3 groups (genotypes). Data were analyzed using a two-way ANOVA and are shown as mean ± SEM (ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

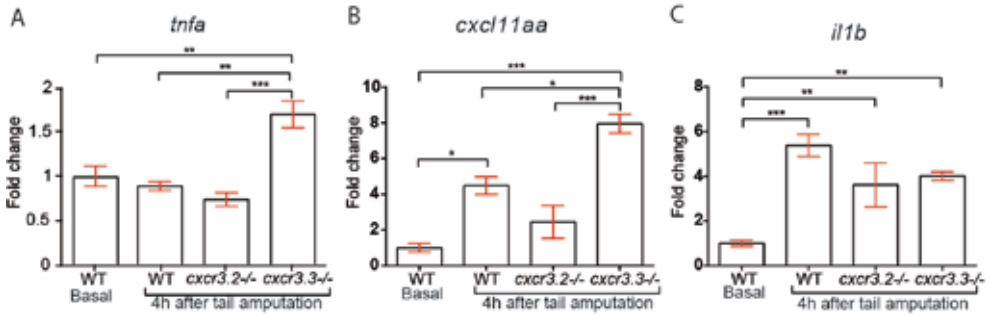


Supplementary figure 2. Injection of an empty CMV vector had no effect on bacterial burden after systemic *Mm* infection. WT and *cxcr3.3* mutant larvae were injected with an empty *CMV* vector at 0 hpf and bacterial burden was assessed at 4dpi. The injection of the empty vector had no effect on the outcome of *Mm* infection on either group (**A**). We conducted a One-way ANOVA

to test for significance. Results are plotted as mean ± SEM (ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).



Supplementary figure 3. *dram1* mutant larvae cannot clear mycobacterial infection efficiently. *dram1* mutant embryos and their WT siblings were systemically infected at 28 hpf with 200 CFU of the Δ*ERP wasabi M. marinum* strain. The total number of bacterial clusters in every fish was quantified (**A**) and bacterial clusters were divided into 3 groups based on the number of bacteria they contained (1-5, 1-6 and > 10) to assess bacterial clearance at 44 hpi (**B**). Dram1-depleted larvae had more bacterial cluster than their WT siblings and developed larger bacterial clusters.



Supplementary figure 4. *cxcr3.3* mutants have a predominantly pro-inflammatory phenotype. To assess the activation status of WT, *cxcr3.2* and *cxcr3.3* mutant larvae, we assess the transcriptional profile of the three M1 markers, *tnfa* (**A**), *cxcl11aa* (**B**) and *Il1b* (**C**) at 4hpa. In *cxcr3.3* mutants the former two (**A-B**) were upregulated while there was no difference in il1b (**C**) among the three groups.

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Online supplementary videos 1. Representative time-lapses of WT, *cxcr3.2* mutants, and *cxcr3.3* mutant larvae after tail amputation. Time-lapses show macrophages of WT, *cxcr3.2* mutants, and *cxcr3.3* mutant larvae migrating towards the injury. Images from a z-stack of the injured area were acquired every 60 sec for 1.5 h and combined into max projection time-lapse.

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3 Disruption of Cxcr3 chemotactic signaling alters lysosomal function and renders macrophages more microbicidal

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Abstract

Chemotaxis and lysosomal function are closely intertwined processes essential for the inflammatory response and clearance of intracellular bacteria. We used the zebrafish model to examine the link between chemotactic signaling and lysosome physiology in macrophages during mycobacterial infection and wound-induced inflammation *in vivo*. Macrophages from zebrafish larvae mutated in a Cxcr3 family chemokine receptor display upregulated expression of vesicle trafficking and lysosomal genes and possess enlarged, highly acidic lysosomes that enhance intracellular bacterial clearance. This increased microbicidal capacity could be phenocopied by blocking the lysosomal Transcription Factor EC, while its overexpression counteracted the protective effect of chemokine receptor mutation. Tracking macrophage migration in zebrafish revealed that lysosomes of chemokine receptor mutants accumulate in the front half of the cell, preventing macrophages to polarize during chemotaxis and reach sites of inflammation. Our work shows that chemotactic signaling affects lysosomal properties and localization during chemotaxis, key aspects of the inflammatory response.

Introduction

Leukocytes differentially express chemokine receptors to sense chemotactic cues that direct them to inflammatory sites [1, 2]. Following chemotactic stimulation, leukocytes acquire a polarized phenotype characterized by clearly identifiable lamellipodia (leading edge) and a uropod (rear edge) that involves both the contractile machinery of the cell and the intracellular vesicle trafficking system [3]. Lysosomes are closely related to cell motility due to their role in lipid catabolism and vesicle trafficking during chemotaxis. The Ca^{2+} release triggered by chemokine receptors induces the fusion of lysosomes with the plasma membrane at the uropod to sustain cell shape remodeling through the delivery of endomembranes and to detach the uropod during chemotaxis [4, 3, 5, 6, 7]. Synaptotagmins (calcium-sensing vesicle-fusion proteins) and Rab GTPases are critical regulators of vesicular trafficking and lysosomal exocytosis and link the chemokine signaling-dependent Ca^{2+} flux to lysosomal function [4, 8, 9]. Processes associating cell motility and lysosomal function are only partially understood and the effect of chemokine signaling on lysosomal function during inflammatory processes *in vivo* remains largely unknown.

Lysosomes are the primary degradative organelles and critical regulators of cell metabolism, and survival [10, 11]. The mammalian/mechanistic target of rapamycin complex 1 (mTORC1), a kinase complex anchored to the lysosomal membrane, is one of the main regulators of lysosomal function [12, 13]. The serine/threonine kinase mTOR phosphorylates the master gene of lysosomal biogenesis TFEB (transcription factor TB) to prevent its translocation to the nucleus [14, 15, 16]. TFEB is a member of the basic helix-loop-helix leucine zipper family of transcription factors that bind to the CLEAR (Coordinated Lysosomal Expression and Regulation) elements (GTCACGTGAC) in the promoter regions of autophagic and lysosomal genes [14, 15]. It belongs to the microphthalmia-associated transcription factor and TFE (MiTF/TFE) that also includes TFEC (transcription factor EC), TFE3 (transcription factor E3) and MITF (Melanocyte inducing transcription factor), which are also under mTORC1 regulation [14, 16, 17]. TFE3 homodimers or TFE3-TFEB heterodimers cooperatively orchestrate lysosomal biogenesis and exocytosis by binding to an overlapping set of CLEAR elements [18, 19]. The role of TFEC in development is well known but its involvement in lysosomal function remains poorly understood [20, 21]. Early reports suggest that TFEC acts as a repressor of lysosomal biogenesis [22, 23]. It was later suggested that different isoforms of TFEC can enhance lysosomal biogenesis in a cell-specific manner, and therefore TFEC is now ascribed mostly a dual role [24, 25]. Lysosomes carry out multiple cell-specific tasks [26, 27]. In macrophages and other phagocytic cells, lysosomes are involved in pro-inflammatory, chemoattractant, and antimicrobial responses [5, 26, 28, 29]. Macrophage recognition of pathogen- and damage'associated molecular patterns (PAMPs and DAMPs) activates and primes lysosomes for pathogen degradation and chemotaxis in a mTORC1-independent manner [5, 30, 31]. Macrophages activated by TLR (Toll-like receptor) sensing of live bacteria or LPS (lipopolysaccharide) show accumulation of TFE3 in the nucleus and the induction of immune genes directly implicated in the inflammatory response [28, 32]. Likewise, the activation of TFEB leads to increased phagosomal acidification and a significant increase in the total number of lysosomes [28, 31, 27]. By contrast, depletion of TFEB or TFE3 results in dampening of cytokine and chemokine secretion [28, 29, 27]. Thus, the function of the lysosomal transcriptional regulators is tightly linked to the production of chemotactic signals directing macrophage migration.

Pathogen sensing through TLRs triggers the release of intracellular calcium from the lysosome through the MCOLIN 1 (mucolipin 1) ion-channel and activates calcineurin, which dephosphorylates TFEB and facilitates its translocation to the nucleus independently of mTORC1 [5, 33, 34, 32]. Induction of microbicidal genes also occurs independently of mTORC1 upon overexpression of the master metabolic modulator 5'AMPK (activated protein kinase) and depletion of its negative regulator FLCN (folliculin), which acts upstream of TFEB/TFE3 [31]. An additional mTORC1-independent mechanism involves the Rag-Ragulator complex in microglia [30, 35]. The Rag-Ragu-

lator complex refers to heterodimeric complexes of RagA or RagB GTPases coupled to either RagC or RagD [36, 37, 38]. These complexes anchor TFEB/TFE3 to the lysosomal membrane and control the intracellular distribution of the transcription factors [37, 39, 40]. In a study using zebrafish, RNA sequencing analyses showed upregulation of multiple lysosomal genes in microglia of RagA (*rraga*) mutant zebrafish larvae [30].

In the present study, we investigated the link between chemotactic signaling and lysosomal function *in vivo* using a *cxcr3.2* mutant zebrafish line which is deficient in a macrophage-attractant chemokine receptor homologous to human CXCR3 [41]. We previously showed that zebrafish larvae lacking this chemokine receptor have increased resistance towards mycobacterial infection and their macrophages show reduced motility and a rounded shape [41, 42]. Here we report that RNAseq data of these macrophages revealed a dysregulation of lysosomal and Golgi- related genes. In agreement, we found that the disruption of chemokine signaling in these cells was linked to increased lysosomal contents. Disruption of the Cxcr3 axis resulted in enhanced clearance of ingested material, specifically a mycobacterial pathogen. Supporting the connection between Cxcr3 chemotactic signaling and lysosomal function, we found that blocking Tfec phenocopied the infection resistance of chemokine receptor mutants, while their enhanced microbicidal capacity was counteracted by increasing *tfec* levels. Finally, we assessed if the aberrant macrophage motility in the chemokine receptor mutants was linked to altered subcellular dynamics of lysosomes during cell migration. Indeed, we observed that cell polarization in mutant macrophages was incomplete, with lysosomes largely failing to shuttle between the leading and trailing edges of the cell. Taken together, these results link macrophage chemotaxis to intracellular vesicular trafficking, showing that disruption of the Cxcr3 axis leads to the induction of lysosomal gene expression and enhanced microbicidal capacity, which primes macrophages for defense against intracellular bacteria.

Materials and Methods

Zebrafish lines and husbandry

Zebrafish were handled 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 committee of Leiden University (License number: 10612). The wt fishline used in this study is AB/TL. The homozygous mutant (*cxcr3.2-/-*) and homozygous wildtype (wt) siblings (*cxcr3.2+/+*) of the *cxcr3.2*^{hu6044} allele were crossed into the Tg (*mpeg1:mCherry-F*)^{ump2} background to visualize macrophages. Zebrafish larvae and eggs were stored 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 infections

and imaging. Larvae were kept in E2 medium (15 mM NaCl; 0.5 mM KCl, 1.0 mM $MgSO_4$, 150 μM KH_2PO_4, 50 μM Na_2HPO_4, 1mM $CaCl_2$; 0.7 mM $NaHCO_3$) for a minimum of 6h prior and during experimental procedures involving pH-rodo and LysoTracker. For confocal imaging, larvae were kept in egg water containing 0.003% PTU (1-phenyl-2-thiourea, Sigma Aldrich) to prevent pigmentation.

FACS, RNA extraction and cDNA synthesis

For RNA sequencing experiments, three biological samples of 150-200 6dpf Tg *(mpeg1:mCherry-F cxcr3.2-/- and cxcr3.2+/+)* larvae were dissociated for FACS following the procedure described in [43]. For qPCR analysis on sorted cells, three biological samples of 100-200 Tg *(mpeg1:mCherry-F cxcr3.2-/- and cxcr3.2+/+)* 5dpf larvae were used. For both procedures, RNA was extracted using the miRNeasy mini kit (Qiagen) according to the manufacturers' instructions. For RNA sequencing, the synthesis of cDNA was done using the SMARTer® Universal Low Input RNA Kit for Sequencing (Clontech) following the manufacturer's guidelines.— For qPCR analysis, cDNA was generated using the iScript™ cDNA Synthesis Kit (Bio-Rad).

RNA-sequencing analysis

Illumina RNA sequencing, mapping and counting of reads was performed as described previously [43]. RNA sequencing data analysis was done with the DESeq2 bioinformatics package (https://bioconductor.org/packages/release/bioc/html/DESeq2 .html) [44]. Before data processing, lowly expressed genes (<50 total reads) were filtered. Genes with a p.adj < 0.05 and $|\log2(\text{fold change})| > 0.5$ cut off were selected for gene ontology analyses (**Supplementary Dataset 1**). Correspondence between human and zebrafish orthologs was derived through g:profiler (http://biit.cs.ut.ee/gprofiler) and manually curated [47] (**Supplementary Table 1**). The significantly affected KEGG pathways were determined by submitting the predicted human orthologs of the significantly regulated zebrafish genes to DAVID bioinformatics tools (david. ncifcrf.gov) [45, 46] (**Supplementary Dataset 2**). The significantly affected Gene Ontology (GO) terms were determined by submitting the predicted human orthologs of the significantly regulated zebrafish genes to PANTHER (geneontology.org). Gene enrichment analysis criteria were Fisher Exact test or False Discovery Rare (FDR) < 0.05 (for DAVID or PANTHER respectively), number of affected genes ≥ 10, fold enrichment ≥ 1.5. Raw data are deposited in the Gene Expression Omnibus database under accession number GSE149942. The complete data analysis (**Supplementary Datasets1-3**) are available following the link: **https://doi.org/10.5281/zenodo.3833848**

Quantitative PCR analysis

For qPCR analyses on *cxcr3.2* expression, three batches of 10 ABT/TL larvae injected with DN-*tfec*, *CMV:tfec* or PBS each, were collected in QIAzol lysis reagent (Qiagen). Similarly, 3 batches of infected and non-infected AB/TL larvae were collected to assess

tfec induction upon infection. 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) for whole larvae, and *eif5* for sorted macrophages and analyzed the data with the 2 –ΔΔCt method**.** Primer sequences can be found in **Supplementary Table 1.** A One-way ANOVA was used to test for significance of the sorted macrophages data and results are plotted as mean ± SEM (ns p> 0.05, * p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001). For *cxcr3.2* expression and *tfec* induction on whole larvae, we used a two-tailed T-test and plotted the results as mean ± SEM (ns p> 0.05, * p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001).

Assessment of microbicidal capacity

To determine the microbicidal capacity of zebrafish larval macrophages, embryos were infected with 200 CFU of the attenuated strain, *ΔERP-M. marinum-mWasabi*. Larvae were infected in the blood island (BI) with 1nL of a *ΔERP-M. marinum-mWasabi* single-use glycerol stock and microinjected at 28hpf. Infected larvae were fixed with 4% paraformaldehyde (PFA) at 44 hpi, mounted in 1.5% low-melting-point agarose (SphaeroQ, Burgos, Spain) and bacterial clusters were quantified under a Zeiss Observer 6.5.32 laser scanning confocal microscope (Carl Zeiss, Sliedrecht, The Netherlands) using a CApochromat 63x/1.20 W Corr UV-VIR-IR objective (Carl Zeiss). We used a Mann-Whitney test to analyze the overall bacterial burden of the pooled data of 2 independent replicates of 12-15 fish each, where data are shown as mean ± SEM. A Kolmogorov-Smirnov test was used to analyze the distribution of bacterial cluster sizes (ns p>0.05, * p≤0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001).

Acidification assessment using pH-rodo

cxcr3.2 mutant and wt larvae were injected with 1 nL of *E. coli* pH-rodo bioparticles conjugate for phagocytosis (Invitrogen) at 28-37 hpf into the BI and imaged over the circulation valley at 30-45 minutes post-injection (mpi). In all cases, the same area was imaged by mounting anesthetized larvae in 1.5% low-melting-point agarose and imaged with Plan-Neofluar 40x/0.9 Imm corr objective on a Zeiss Observer 6.5.32 laser-scanning confocal microscope (Carl Zeiss, Sliedrecht, The Netherlands). Fluorescence intensity was assessed using FIJI/ Image J quantification tools and data were analyzed using a two-tailed T-test. Results are shown as mean ± SEM (ns p> 0.05, * p≤0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001). Results are expressed as % relative to the wt control (100%).

LysoTracker staining of acidic compartments

2-day-old *cxcr3.2* mutant and wt larvae were incubated for 1-2 h with 10μM LysoTrack-

er green DND-26 (Invitrogen) in E2 medium. Larvae were anesthetized following the staining and rinsed 3 times for 5 min each with E2 medium and tricaine. Images of live macrophages were acquired with a Plan-Neofluar 40x/0.9 Imm corr objective on a Zeiss Observer 6.5.32 laser-scanning confocal microscope (Carl Zeiss, Sliedrecht, The Netherlands). To quantify LysoTracker staining within macrophages, the mean intensity of LysoTracker overlapping with *mpeg1:mCherry* signal was measured using FIJI/Image J quantification tools. Data were analyzed using a two-tailed t-test. Results are shownas mean ± SEM (ns p> 0.05, * p≤0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001). Results are expressed as % relative to the wt control (100%).

Systemic infection with *Mycobacterium marinum* and determination of bacterial burden

M. marinum M-strain expressing the fluorescent marker mCherry was grown and prepared freshly for injection as described in [48]. Embryos were systemically infected with 300CFU of *M. marinum*-mCherry by microinjection in the BI at 28hpf. Infected larvae were imaged under a Leica M165C stereo-florescence microscope at 4 days post-infection, and the bacterial burden was determined using a dedicated pixel counting program [49]. Data were analyzed using a two-tailed t-test. Results are shown as mean ± SEM (ns p> 0.05, * p≤0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001) and combined data of 3 independent replicates of 20-30 larvae each.

Transient *tfec* overexpression and Tfec function blockade

An expression construct pcDNA™3.1/V5-His TOPO-*CMV:tfec* was injected into the yolk at 0 hpf to overexpress the gene in wt and *cxcr3.2* mutant larvae. Overexpression levels were verified by qPCR analysis. Tfec function was blocked by injecting a DN-*tfec* construct in wt larvae at 0 hpf. Tfec function blockade was verified through qPCR on *kitlgb*, a downstream target of Tfec [20].

Lysosome localization within migrating macrophages

Time-lapse images of LysoTracker stained macrophages of 3-day-old *cxcr3.2* mutant and wt larvae (5 larvae per genotype) were acquired 1 after tail-amputation every 30 sec for 1h. Larvae were mounted in 1.5 % low-melting-point agarose and microscopy was done using a Leica TCS SP8 MP confocal microscope (Leica Microsystems). Data were analyzed using a Fiji/ImageJ homemade plugin "Lysosomal distribution" (**https://sites.imagej.net/Willemsejj/**). The plugin divides the total area of single macrophages in half and quantifies the proportion of LysoTracker staining in each part of the cell in every time-frame (**Supplementary File 1**). The data were organized by cell and by fish and analyzed with a two-tailed t-test a Mann-Whitney test, respectively. Data are shown as mean ± SEM (ns p > 0.05, * p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001).

Results

Intracellular vesicle trafficking and lysosomal genes are upregulated when the Cxcr3.2 chemotactic signaling is disrupted

The zebrafish Cxcr3.2 chemokine receptor is a functional homolog of human CXCR3. Macrophages lacking this receptor have impaired motility and a rounded shape compared to their wildtype (wt) counterparts [41, 42]. To identify the genes and biological pathways affected by the disruption of Cxcr3 signaling, we isolated macrophages from *cxcr3.2* mutant and wt zebrafish larvae and subjected these to RNA deep sequencing (RNAseq). Principal component analysis (PCA) confirmed overall distinction between the *cxcr3.2* mutant and wt transcriptomic profiles (**Figure 1A**). Differential expression analysis revealed that *cxcr3.2* mutation leads to the downregulation of 490 genes and upregulation of 407 genes (**Supplementary Dataset 1**) and genes related to different subcellular compartments (**Figure 1B, Supplementary Dataset 2**). Classification of these genes by compartment showed that peroxisomal, lysosomal and Golgi-related genes were most frequently up-regulated (**Figure 1C, Supplementary Dataset 2**), although only lysosomal and Golgi related terms were significantly differentially represented in GO or KEGG enrichment analysis, *i.e.* KEGG 'Lysosome', GO Cellular components 'Golgi-associated vesicle', 'Golgi apparatus', 'ER-Golgi intermediate compartment', 'Lysosome', 'Vacuole' and GO Biological process 'Golgi vesicle transport' (**Supplementary Dataset 3**). Differentially expressed genes related to lysosomal and Golgi function could also be classified under different processes, including Golgi stacking, post-Golgi coating, Endoplasmic reticulum (ER) to Golgi trafficking, Golgi post-translational modifications (Golgi-PTM), Endosome-lysosome trafficking, Trans Golgi network (TGN) function, lysosomal biogenesis and maturation, and proton transport (**Figure 1D**). To confirm the upregulation of genes involved in lysosomal function, we ran a qPCR on lysosomal markers *ctsl.1* (lysosomal cysteine protease), *atp6v1c1b* (acidifies intracellular compartments) and *slc36a1* (lysosomal amino acid transporter) and the lysosomal regulators *tfeb*, *tfe3,* and *tfec*. All lysosomal markers showed upregulation comparable to those observed in the RNAseq profile (**Figure 1E,F**). However, the expression of the lysosomal regulatory genes was unaffected, indicating that the effects on lysosomal gene expression cannot be attributed to changes in the transcription of *tfeb*, *tfe3b* or *tfec*. Altogether, our data suggest that disruption of the zebrafish Cxcr3 axis induces a transcriptional increase in genes related to lysosomal function and intracellular vesicle trafficking, independently of expression changes in the lysosomal biogenesis regulators *tfeb*, *tfe3b*, and *tfec*.

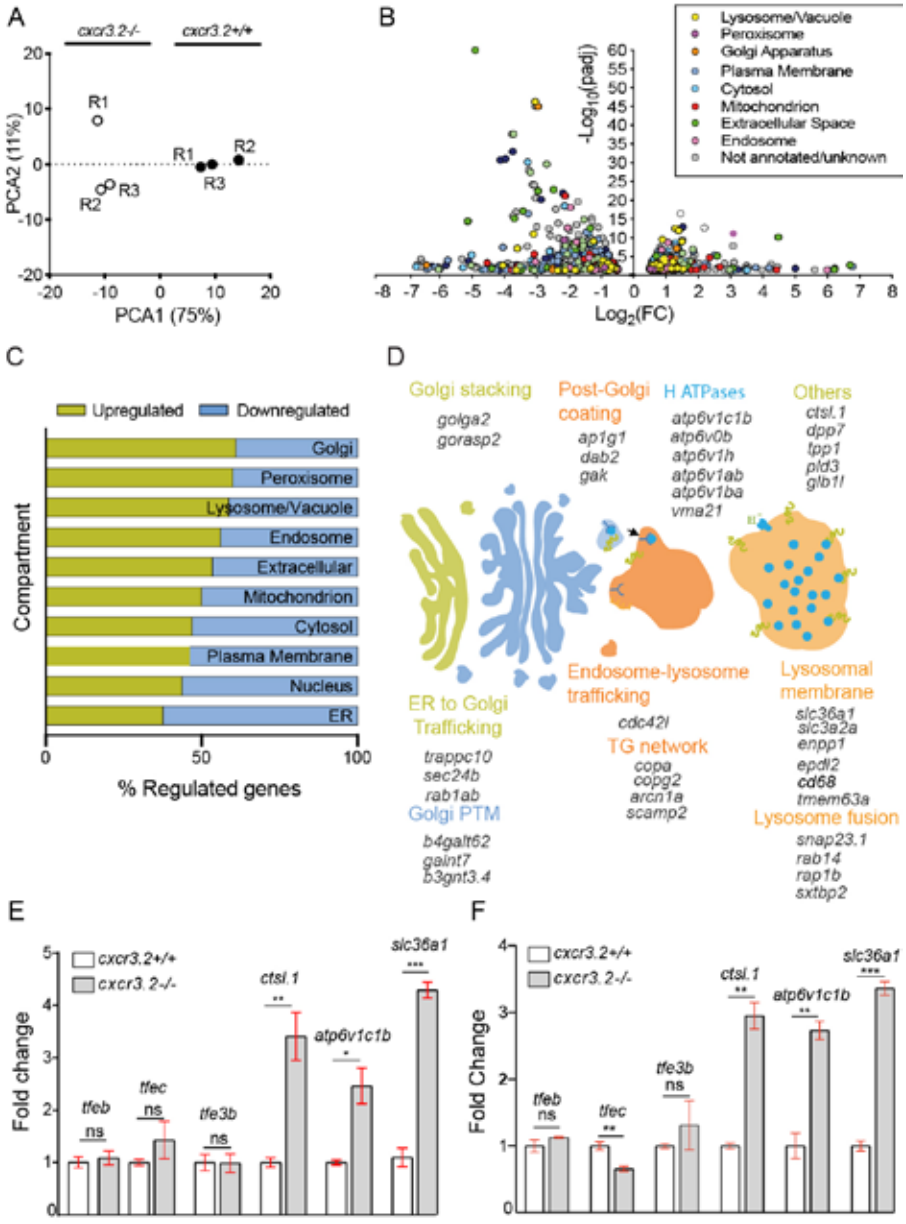

Figure 1. Disruption of Cxcr3.2 signaling transcriptionally induces genes related to lysosomal function and intracellular vesicle trafficking. Principal component analysis (PCA) of *cxcr3.2* mutant and wt transcriptomes. PCA analysis was performed in R on variance-stabilizing transformed (vst) data, using the Deseq2 'plotPCA' command (**A**). Volcano plot of *cxcr3.2* mutant versus wild type differentially expressed genes. Genes are classified and color-coded by cellular compartment annotation. Compartment annotations were obtained from genontology.org according to GO: Cellular component and from KEGG pathways (**B**). Distribution of upregulated (yellow) and downregulated

(blue) genes, classified by compartment as above Lysosomal, Golgi and peroxisome-related genes are more commonly upregulated in *cxcr3.2* mutant macrophages (**C**). Graphical representation of induced genes exerting key functions in Golgi and Lysosomal pathways (**D**). Expression fold change of representative lysosomal markers and transcriptional regulators of lysosomal functions of *cxcr3.2* mutant wt FACS-sorted macrophages, as determined by qPCR (**E**) or RNAseq analysis (**F**). qPCR analysis confirmed that overall lysosomal function is increased in *cxcr3.2* mutants as indicated by the upregulation of lysosomal function markers ctsl.1, atp6v1c1b, and slc36a1, whereas the expression of the lysosomal biogenesis regulators *tfeb*, *tfe3* and *tfec* remained unaltered.These data were analyzed using a two-tailed t-test and results are shown as mean ± SEM (ns $p>0.05$, * $p\leq0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). *Figure 1B-D wASmodified from Torraca, 2016.

Disruption of chemotactic signaling increases the lysosomal content and microbicidal capacity of macrophages

To assess whether altered expression of vesicle trafficking and lysosomal genes impacts the lysosomal function we investigated the microbicidal capacity of macrophages in *cxcr3.2* mutant and wt embryos. We had previously shown that *cxcr3.2* mutation increases overall resistance of zebrafish embryos to *M. marinum*, a mycobacterial pathogen that is widely used to model tuberculosis infection [41, 50, 51]. However, our previous work did not address the competency of individual macrophages in eliminating the mycobacterial infection. Therefore, we infected *cxcr3.2* mutant and wt embryos with the *ΔERP* mutant *M. marinum* strain. This strain lacks the *ERP* (exported repetitive protein) virulence factor that confers resistance to acidity and allows mycobacteria to replicate inside phagolysosomes. As demonstrated in previous studies, clearance of *ΔERP* mutant *M. marinum* by macrophages serves as an indicator of microbicidal efficacy because one can track the clearance of a stationary bacterial population [52]. Our data show that *cxcr3.2* mutant embryos were more efficient at clearing the infection than the wt controls because they developed fewer and smaller bacterial clusters (**Figure 2A-B**). To assess if enhanced clearance of bacteria in *cxcr3.2* mutants was related to a higher phagolysosome and lysosome acidity, we injected pH-rodo *E.coli* bioparticles into the circulation valley of 3 dpf wt and *cxcr3.2* mutant larvae. The pH-rodo *E.coli* bioparticles fluoresce at low pH values and the fluorescence intensity increases with acidity. In line with the RNA sequencing data and the augmented microbicidal efficacy, phagosomes from *cxcr3.2* mutant macrophages were more acidic at 30-40 minutes post-injection (mpi) than macrophages in wt larvae (**Figure 2C-E**). We then assessed whether the upregulation of lysosomal genes had an effect on the size and number of lysosomal vesicles within macrophages. We bath-exposed 2dpf wt and *cxcr3.2* mutant embryos to the intravital LysoTracker dye and proceeded to quantify the area of the staining within single macrophages. Lysosomal contents were more abundant in *cxcr3.2* mutants than in wt controls (**Figure 2F-H**). These *in vivo* experiments support that the upregulation of lysosomal genes in *cxcr3.2* mutants affects both the properties and the number of lysosomal vesicles and acidic compartments, thereby rendering macrophages with a disrupted Cxcr3 chemokine signaling axis more microbicidal than their wt counterparts.

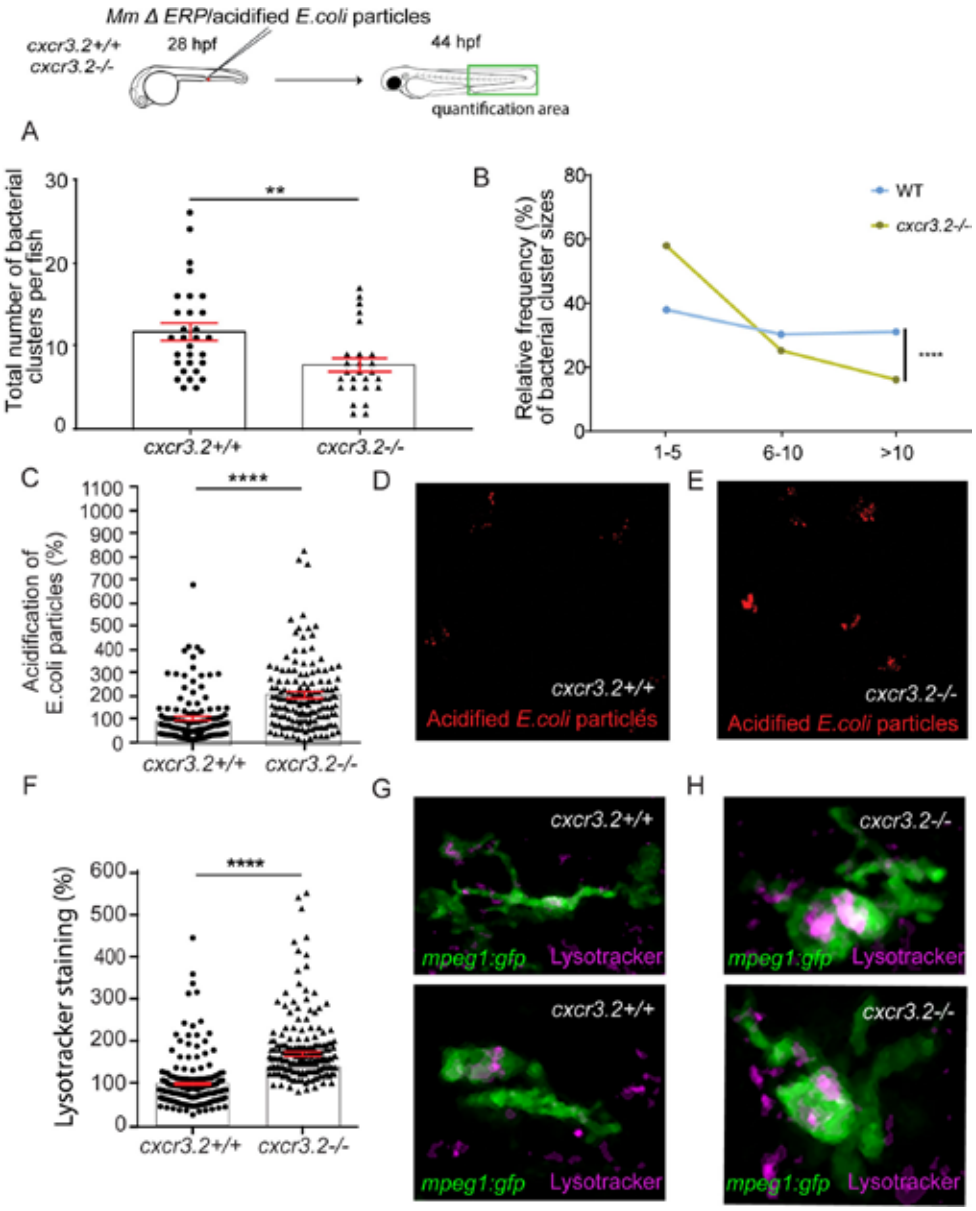

Figure 2. The upregulation of lysosomal genes in *cxcr3.2* mutants is associated with increased microbicidal activity of macrophages. We systemically infected *cxcr3.2* mutant zebrafish embryos and their wt siblings with the attenuated *M. marinum ΔERP wasabi* strain. Quantification of bacterial clusters at 44 hpi in the indicated area showed that infected *cxcr3.2* mutants develop fewer bacterial clusters than their wt siblings (**A**). Furthermore, *cxcr3.2* mutants have a higher frequency of smaller bacterial clusters (1-5 bacteria) and a lower frequency of larger cluster (>10 bacteria) than wt controls (**B**). The normalized intensity (%) of pH-rodo *E.Coli* bioparticle clusters in *cxcr3.2* mutants is

higher than in wt larvae based on fluorescence quantification and representative images (**C-E**). Normalized data of LysoTracker staining show that *cxcr3.2* mutant macrophages have higher lysosomal contents (%) than wt controls based on fluorescence quantification and representative images (2 per genotype) (**F-H**). A Mann-Whitney test was conducted to analyze the total number of bacterial clusters per fish of the pooled data of 2 independent replicates of 12-15 fish each (**A, C**, and **F**) and a Kolmogorov-Smirnov test was used to analyze the distribution of bacterial cluster sizes (**B**). All data are shown as mean ± SEM (**p ≤ 0.01, **** p ≤ 0.0001). Scale bars: 5µm * Figure 2A-F were modified from Torraca, 2016.

Blocking Tfec function phenocopies the increased resistance of *cxcr3.2* mutants to mycobacterial infection

Having linked the *cxcr3.2* mutant phenotype to increased lysosomal contents and enhanced bacterial clearing, we asked whether this phenotype could be evoked by manipulating one of the lysosomal regulators. We chose Transcription factor EC (Tfec) for this purpose because well-characterized molecular tools are available to modulate its function [20]. Tfec isoforms can act either as lysosomal biogenesis repressors or transactivators depending on the cell type that expresses them [24, 25]. Tfec can form heterodimers with Tfe3 which together with the lysosomal biogenesis master gene, Tfeb, coordinates lysosomal biogenesis and function [53, 54]. To assess the predominant role of Tfec on lysosomal function and the innate immune response, we injected a dominant-negative construct (DN-*tfec*) into the yolk of wt embryos at 0 hpf and subsequently infected them with *M. marinum*. Blocking Tfec function (**Supplementary Figure 1B**) resulted in a lower bacterial burden at 4 dpi (**Figure 3A-B**). By contrast, when *tfec* was overexpressed by injecting a *CMV:tfec* construct (**Supplementary Figure 1A**) instead of the dominant-negative construct, larvae had a higher bacterial burden than PBS-injected controls (**Figure 3C-D**). We asked whether *tfec* expression changes upon *M. marinum* infection, but qPCR analyses demonstrated that *M. marinum* infection does not alter *tfec* transcription (**Supplementary Figure 2**). Furthermore, we verified that *tfec* overexpression or Tfec function blockade did not affect expression levels of *cxcr3.2* itself (**Supplementary Figure 3**). Our data show that in the context of innate immunity, *tfec* increases the susceptibility of zebrafish larvae to mycobacterial infection presumably by limiting the clearance of bacteria and that blocking Tfec function phenocopies the increased resistance to *M. marinum* of *cxcr3.2* mutant larvae.

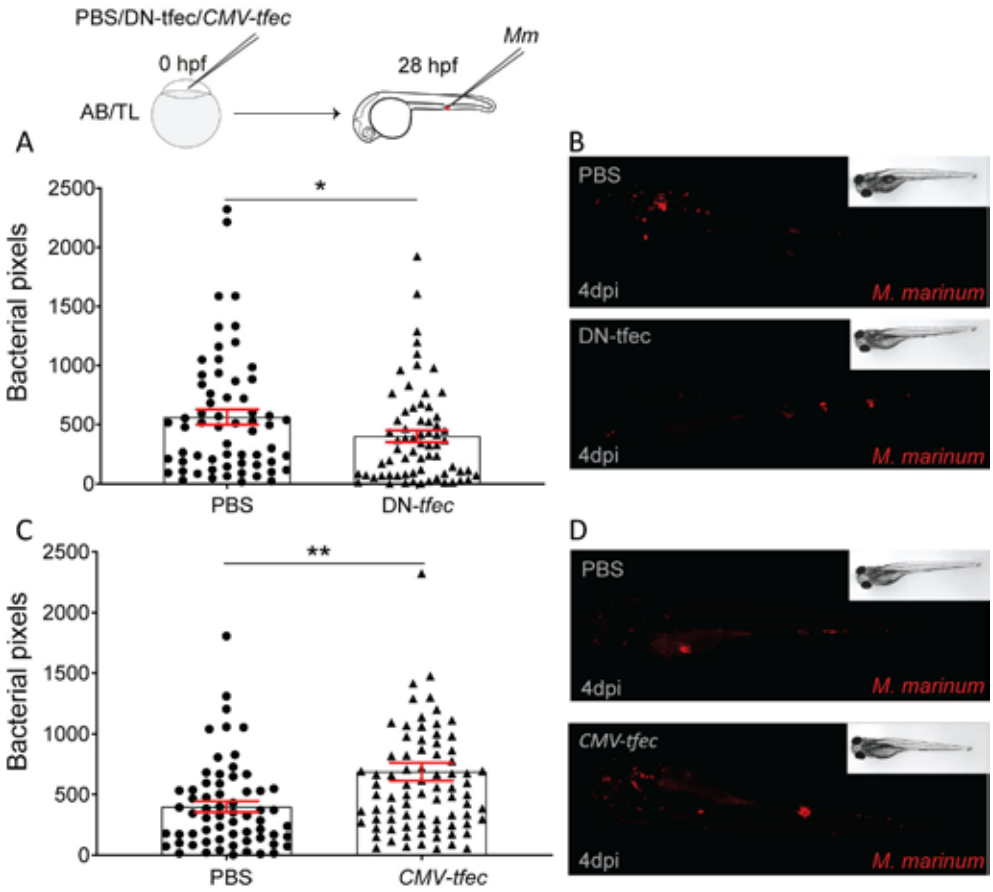

Figure 3. Blocking Tfec function phenocopies the increased resistance of *cxcr3.2* mutants towards *M. marinum* infection. We blocked Tfec function by injecting a dominant-negative construct (DN-*tfec*) (**A-B**) or transiently overexpressed the gene by injecting a *CMV:tfec* construct (**C-D**) in AB/TL eggs at 0 hpf. We subsequently infected the larvae with *M. marinum mCherry* and assessed infection at 4dpi by fluorescent pixel quantification from stereo fluorescence images (representative examples shown). Larvae injected with DN-*tfec* have a lower bacterial burden than PBS injected controls at 4dpi (**A-B**). By contrast, *CMV:tfec* injected larvae have a higher bacterial burden than controls (**C-D**). The bacterial burden data were analyzed using a two-tailed t-test. Results are shown as mean ± SEM (* $p \leq 0.05$, ** $p \leq 0.01$).

Tfec counteracts the enhanced microbicidal capacity of *cxcr3.2* mutants

To confirm if *tfec* alters mycobacterial clearance efficacy by directly affecting the microbicidal capacity of macrophages we used the Δ*ERP* mutant *M. marinum* strain that is sensitive to lysosomal acidification. We blocked Tfec function with the DN-*tfec* construct and observed that larvae developed fewer and smaller bacterial clusters than PBS-inject-

ed controls (**Figure 4A-B**). Next, we transiently overexpressed *tfec* in *cxcr3.2* mutants and confirmed that *tfec* overexpression counteracts the enhanced microbicidal capacity of the *cxcr3.2* mutants towards the *ΔERP* mutant *M. marinum* strain. Tfec overexpression in the *cxcr3.2* mutant background yields an overall bacterial burden comparable to wt controls and larger bacterial clusters, while non-injected mutants preserve their enhanced microbicidal capacity showing a low bacterial burden and fewer large bacterial clusters (**Figure 4C-D**). We conclude that Tfec function counteracts enhanced microbicidal properties of macrophages that arise from disrupting Cxcr3 chemotactic signaling.

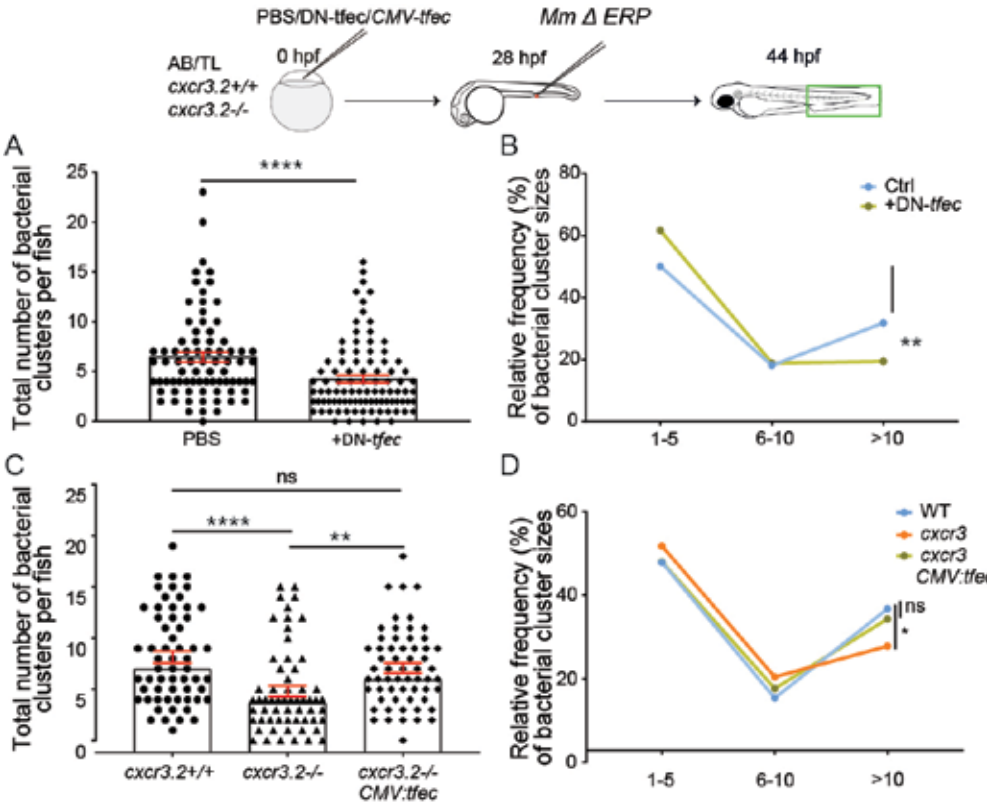

Figure 4. *tfec* overexpression counteracts enhanced microbicidal capacity of *cxcr3.2* mutants. Tfec function was blocked by injecting the DN-*tfec* construct into AB/TL eggs at 0 hpf. Larvae were infected with *the M. marinum ΔERP wasabi* strain. Larvae injected with DN-*tfec* developed fewer and smaller bacterial clusters than PBS injected larvae (**A-B**) and phenocopied *cxcr3.2* mutants in their capacity to clear bacteria (**Figure 2**). Similarly, Tfec was overexpressed by injecting a *CMV:tfec* construct at 0hpf in *cxcr3.2* mutants, prior to infection *with M. marinum ΔERP wasabi*. *CMV:tfec* expressing *cxcr3.2* mutants lose their enhanced microbicidal capacity and have more and larger bacterial clusters than PBS injected mutants, reaching similar levels as the wt controls (**C-D**). The total number of bacterial clusters per fish were analyzed using a Mann-Whitney test and combines the data of 3 independent replicates of 20-30 larvae (**A** and **C**). A Kolmogorov-Smirnov test was used to analyze the distribution

of bacterial cluster sizes between 1-5, 6-10 and >10 bacteria (B and D). All data are shown as mean ± SEM (ns $p>0.05$, * $p\leq0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ **** $p \leq 0.0001$).

The disruption of chemotactic signaling in *cxcr3.2* mutant macrophages alters lysosome trafficking and prevents cell polarization during chemotaxis

Chemokine signaling ultimately triggers the release of intracellular calcium to orchestrate highly dynamic cell membrane rearrangements that result in a polarized phenotype with a clear distinction between the leading edge (lamellipodia) and the trailing edge [4, 3]. Lysosome exocytosis plays a major role in leukocyte chemotaxis as it delivers layers of lipid membrane to sustain plasma membrane turnover and extension towards chemotactic cues, while also mediating the detachment of the uropod [4, 5, 7]. Therefore, as cells move, lysosomal contents shuttle between the lamellipodia and the uropod but transiently concentrate in the latter [9, 8]. Chemokine receptors are required for the formation of the uropod in the rear end of migrating cells [3]. Since *cxcr3.2* mutant macrophages are less motile than wt we assess the localization of lysosomal contents during chemotaxis as an indicator of cell polarization. We stained 3-day-old Tg (*mpeg1-mCherry*) *cxcr3.2* mutant and wt and larvae with the intravital dye LysoTracker and divided the total macrophage area into halves to calculate the anterior-posterior ratio of LysoTracker staining within the cell (**Supplementary File 1**). Macrophages have recognizable leading and trailing edges and lysosomal contents move continuously from the rear to the front (1.15:1) as cells follow a chemotactic signal in wt larvae (**Figure 5A and C**). By contrast, the leading edge and the uropod of *cxcr3.2* mutant macrophages are not well defined and lysosomal contents accumulate in the anterior half of the cell (1.74:1) close to the center in mutant larvae (**Figure 5B and C**). We observed the same trend when single cells were analyzed. The average anteroposterior LysoTracker staining in wt macrophages is 1.13:1 compared with 1.99:1 in *cxcr3.2* mutants (**Figure 5D**). These data show that macrophages lacking Cxcr3.2 are not properly polarized and that lysosomal vesicle trafficking is disrupted in the absence of the chemokine receptor as lysosomes rarely reach the uropod in the absence of Cxcr3 (**Supplementary Figure 4**). This vesicle trafficking defect leads to the accumulation of lysosomal contents and is tightly linked to aberrant macrophage chemotaxis.

Discussion

Recent studies revealed that leukocyte chemotaxis is inextricably intertwined with the subcellular localization and exocytosis of lysosomes [4, 5, 55, 8, 56]. However, our understanding of the complex network of processes linking chemotaxis and lysosomal function in different contexts is only beginning to be understood. Here, we used the zebrafish model to study the conserved Cxcr3 signaling axis, which mediates proinflammatory responses of multiple leukocytes, implicated in several human inflammatory

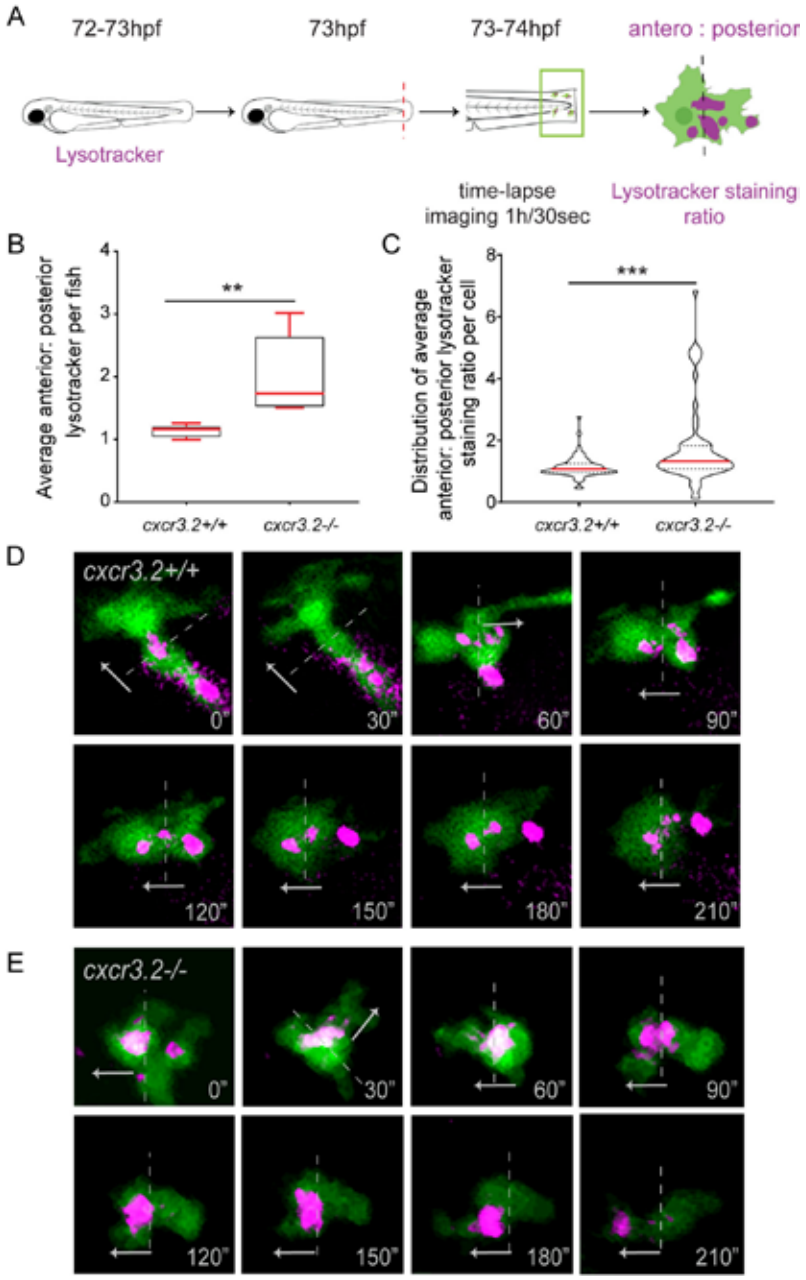

Figure 5. The disruption of Cxcr3.2 signaling in macrophages alters lysosome trafficking and prevents cell polarization during chemotaxis. We assessed the localization of lysosomes during chemotaxis by quantifying the ratio of lysosomal contents in the anterior and posterior halves of migrating macrophages. Tg (*mpeg1-mCherry*) *cxcr3.2* mutant and wt larvae were incubated in LysoTracker and time-lapse images of migrating macrophages after tail-amputation were acquired (**A**). The data shown in **B** and **C** derive from 5 wt and 5 *cxcr3.2* mutant larvae and a total of 63 wt macrophages and 57 mutant macrophages (7≤ cells/fish). The data show the average anterior: posterior LysoTracker

staining ratio per fish (**B**) and the distribution of average staining among cells (**C**). The quantifications and stills at 30sec intervals from representative migration tracks (**D and E**) show that lysosomal contents in wt larvae display a small dispersion in the data (**B and C**) and an even distribution of lysosomes (**D**), while lysosomal contents preferentially accumulate in the anterior half in *cxcr3.2* mutant macrophages (**E**) and show a high variation (**B and C**). The dashed lines indicate the borders between anterior and posterior halves and the arrows indicate the direction in which macrophages move. Data of anterior: posterior LysoTracker staining per fish were analyzed with a Mann-Whitney test and the data per cell were analyzed using a two-tailed t-test. Results are shown as mean ± SEM (**$p \leq 0.01$, ***$p \leq 0.001$). Scale bars: 5μm.

disorders. We show how the disruption of the Cxcr3 chemokine signaling axis in our model leads to transcriptional upregulation of lysosomal genes, increases lysosomal contentsand renders macrophages more microbicidal towards mycobacterial infection despite their altered lysosome trafficking and aberrant motility. These results provide *in vivo* evidence linking lysosomal function to chemotactic signaling that leads us to conclude that disrupting the Cxcr3 chemotactic signaling primes macrophages for better clearance of intracellular infection.

We found a marked dysregulation of lysosomal genes in sorted macrophages of larvae lacking Cxcr3.2, the zebrafish homolog of human CXCR3. In contrast, the expression of lysosomal regulators of the MiTF/TFE protein family remained unaltered, which is in line with previous work showing that members of this protein family are regulated mostly at the posttranscriptional level [23, 25]. The induction of lysosomal genes in *cxcr3.2* mutant macrophages was associated with increased lysosomal contents, higher phagolysosomal acidity, and enhanced clearance of mycobacteria. Previous work by Shen and coworkers used the zebrafish model to assess the lysosomal clearance of apoptotic neuronal debris in RagA (*rraga*) mutant larvae [30]. They reported enlarged lysosomes like in *cxcr3.2* depleted larvae, but a low acidity and poor clearance of apoptotic debris as opposed to our observations in larvae with disrupted chemokine signaling. The RagA GTPase is part of the Rag-Ragulator that anchors TBEB/TFE3 to the lysosomal membrane and interacts with v-ATPases on the lysosomal membrane to acidify the lysosomal lumen [37, 39]. Therefore, the absence of *raga* prevents the anchoring of Tfeb/Tfe3 to the lysosomal membrane and the interaction with v-ATPases, while it promotes the translocation of the transcription factors to the nucleus, arguably leading to a sustained *tfeb*-driven induction of lysosomal genes and a low intraphagosomal acidity [30, 40].

Our results show that the *atp6v1c1b*, and *slc361* genes, that code for the subunit C of the lysosomal v-ATPase (a direct downstream target of Tfeb [14]) and a transmembrane amino acid carrier, respectively, were strongly induced in *cxcr3.2* mutant macrophages together with other lysosomal genes that could be responsible for the highly acidic phagolysosomes in macrophages upon disruption of the Cxcr3 axis. The upregulation of *ctsl.1*, which encodes the endopeptidase Cathepsin L.1 that is involved in catabolic processes and the immune response, could also be linked to the enhanced clearance of bacteria in *cxcr3.2* mutant macrophages.

We observed that blocking the function of Tfec in wt larvae had the same host-protective effect as the *cxcr3.2* mutation upon mycobacterial infection and that *tfec* overexpression resulted in poor control of bacterial dissemination (**Figure 3**). Furthermore, *tfec* overexpression in *cxcr3.2* mutants reverted the protective effect of the mutation and the enhanced clearance of intracellular bacteria (**Figure 4**). Unlike the remaining members of the MiTF/TFE protein family, Tfec is not ubiquitously expressed and, since a Tfec isoform lacks an acidic domain associated with the transactivating function of the other three transcription factors, it can strongly inhibit the Tfe3-mediated gene transactivation [22, 23, 25]. Based on our observations, we posit that *tfec* antagonizes the *tfe3*-driven transactivation of lysosomal and pro-inflammatory genes and that, therefore, blocking Tfec function leads to enhanced lysosomal function and pathogen resistance. Altogether, these results support that the highly microbicidal phenotype of macrophages with a disrupted Cxcr3 axis is associated with deregulations in lysosomal function.

Lysosomes are major players in a concatenated series of molecular pathways required for chemotaxis in macrophages [4, 57]. As previously shown by our group, *cxcr3.2* mutant macrophages have reduced motility and a rounded shape [41, 42]. Here we showed that Cxcr3.2-depleted macrophages do not show a clear polarized phenotype and that lysosomes localize mainly in the leading edge of the cell and rarely reach the uropod. The disruption of two other chemokine signaling axes, CXCR4/CXCL12 and CCR2/CCL2, was found to result in reduced T-cell migration when synaptotagmin SYT7 and the related protein SYTL5 were downregulated [4]. Synaptotagmins are proteins that sense chemoattractant-induced Ca^{2+} to control lysosomal exocytosis and to release the uropod during chemotaxis. Taking these observations as a precedent, the disruption of the Cxcr3 axis in our study might affect the intracellular levels and distribution of intracellular chemokine receptor-induced Ca^{2+}, leading to ER stress and the observed accumulation of lysosomal contents due to calcineurin-independent Tfeb translocation to the nucleus [53, 58]. Moreover, vesicle trafficking and lysosome exocytosis might be compromised at low intracellular Ca^{2+} concentrations further contributing to the accumulation of lysosomes in *cxcr3.2* mutant macrophages and the resulting aberrant motility of these cells.

The identity of a cell is the major determinant of the functional specificity of a chemokine receptor-ligand interaction [59]. Cells are exposed to multiple extracellular signals that are processed simultaneously. In the context of inflammation, macrophages are exposed to both chemotactic signaling and pathogen- and damage-driven signaling, like TLR-sensing, which also triggers the release of intracellular Ca^{2+} from the lysosome, ER and Golgi apparatus [28, 30, 29]. TLR-signaling activates calcineurin which in turn dephosphorylates TFE3 that induces pro-inflammatory cytokines and potentially primes lysosomes for pathogen clearance [28]. Therefore, the reduced chemotaxis but increased pathogen resistance of *cxcr3.2* mutant macrophages might reflect that extracellular cues

were integrated in such a way that the intracellular pathway aimed at killing pathogens overpowered the one guiding the cell towards inflammatory stimuli, thereby priming macrophages for a better defense against pathogens.

Our results support that disturbances in the Cxcr3-dependent chemokine signaling network affect intracellular vesicle trafficking and lysosome exocytosis of macrophages, thereby preventing them from acquiring a polarized phenotype and to migrate towards inflammatory foci whilst rendering them more microbicidal. Our work contributes to further our understanding of chemotaxis as a complex process that incorporates various physiological processes and integrates different extracellular cues. It emphasizes the importance of vesicle trafficking during chemotaxis and the relevance of transcriptional and posttranscriptional regulation of lysosome function in immunity. The power of the zebrafish model for intravital imaging enabled us to show that there is a direct link between chemokine signaling and lysosomal function that affects both the microbicidal properties and the motility of macrophages.

Acknowledgments
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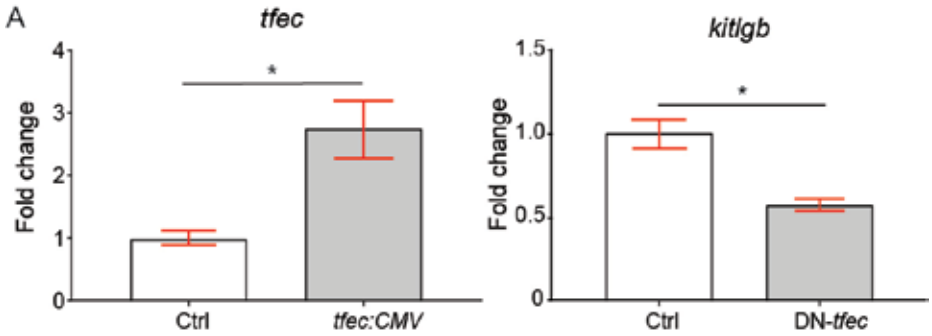
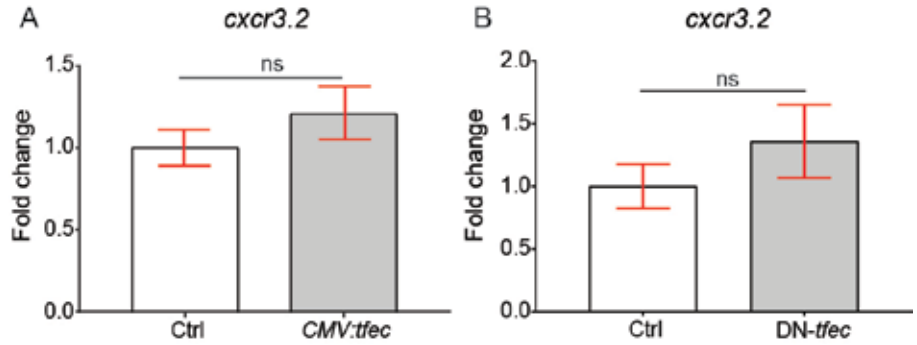
Supplementary materials

Supplementary Table 1. Primer used for the qPCR analysis.

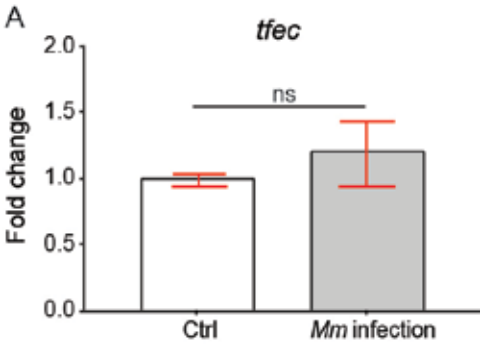
Primer	Forward	Reverse
eif5	CAAGTTTGTGCTGTGTCCCG	AGCCTTGCAGGAGTTTCCAA
ppiab	ACACTGAAACACGGAGGCAAAG	CATCCACAACCTTCCCGAACAC
tfeb	GCATTACATGCAGCATCGCATGCC	CGTGTACACATCCAAATGACTGCTGG
tfec	AACAGTACCTCGCTTTGGGC	CAGTGTTCCCAGCTCCTTGA
tfe3b	CTAGGCTCCAACAAAGAGGAGATG	CAAAATGGTTCCCTTGTTCCAGCGC
ctsl.1	CTGGAGGGACAAGGGCTATG3	CTATGGCAACAGATATGGGGCC
atp6v1c1b	GAGTGTGATGCTGTTTGACGGG	CCCACTGGAACCGGGTAATG
slc36a1	GGAGAACGTGTCGTGGCTAA	CTTCAGCGTGGCTATGACTTCCAT



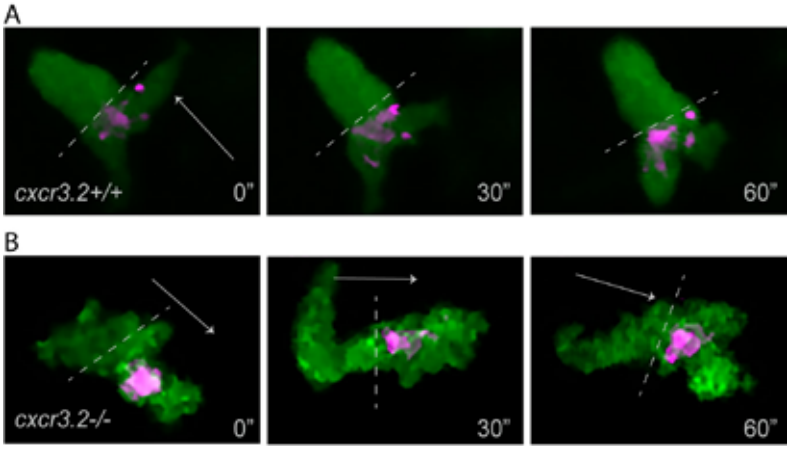
Supplementary Figure 1. qPCR validation of *tfec* overexpression and blockade. qPCR analyses on *CMV:tfec* and DN-*tfec* injected larvae were conducted at 2dpi. Data show that *tfec* was upregulated (n fold=2.65) in *CMV-tfec* injected larvae (**A**). Blocking Tfec function by DN-*tfec* injection results in decreased transcription of the downstream target *kitlgb* . Data were analyzed using a two-tailed t-test and data are shown as mean ± SEM (ns p>0.05, * p≤0.05, **p ≤ 0.01, *** p ≤ 0.001).



Supplementary Figure 2. Blocking Tfec function or overexpressing *tfec* does not affect the transcription of *cxcr3.2*. Expression of *cxcr3.2* is not transcriptionally affected by Tfec overexpression (**A**) or *tfec* function blockade (**B**). AB/TL larvae were injected with either *DN-tfec*, *CMV:tfec*, or PBS in the yolk at 0 hpf. *cxcr3.2* expression was analyzed through quantitative PCR. Data were analyzed using a two-tailed t-test. Results are shown as mean ± SEM (ns P>0.05).



Supplementary Figure 3. *tfec* is not induced upon *M. marinum* infection. AB/TL larvae were systemically infected with *M. marinum* and samples were collected at 2dpi. qPCR analysis of *tfec* indicated no difference between infected larvae and controls. The data were analyzed using a two-tailed t-test and data are shown as mean ± SEM (ns $p>0.05$, * $p\leq0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).



Supplementary Figure 4. Lysosomal contents are rarely found in the uropod of *cxcr3.2* mutant macrophages. During chemotaxis, a clear leading edge and uropod can be recognized in wt macrophages and lysosomes localize in the trailing edge to detach the uropod during cell migration (**A**). Lysosomal contents in *cxcr3.2* mutants generally remain confined to the leading edge and the middle of migrating macrophages and are rarely found in the uropod (**B**). Images are stills at 30sec intervals from representative migration tracks. The dashed lines indicate the borders between anterior and posterior halves and the arrows indicate the direction in which macrophages move. Scale bars: 5μm.

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Supplementary Dataset 1: Differentially expressed genes in *cxcr3.2* mutant vs wt FACS-sorted macrophages and orthologous correspondences to human genes.

Supplementary Dataset 2: Classification of differentially expressed genes by cell compartment.

Supplementary Dataset 3: KEGG and Gene Ontology enrichment analysis for the differentially expressed genes of *cxcr3.2* mutant vs wt FACS-sorted.

https://sites.imagej.net/Willemsejj/

Supplementary File 1: Java script for "Lysosomal distribution" Fiji/ ImageJ plugin.

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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.

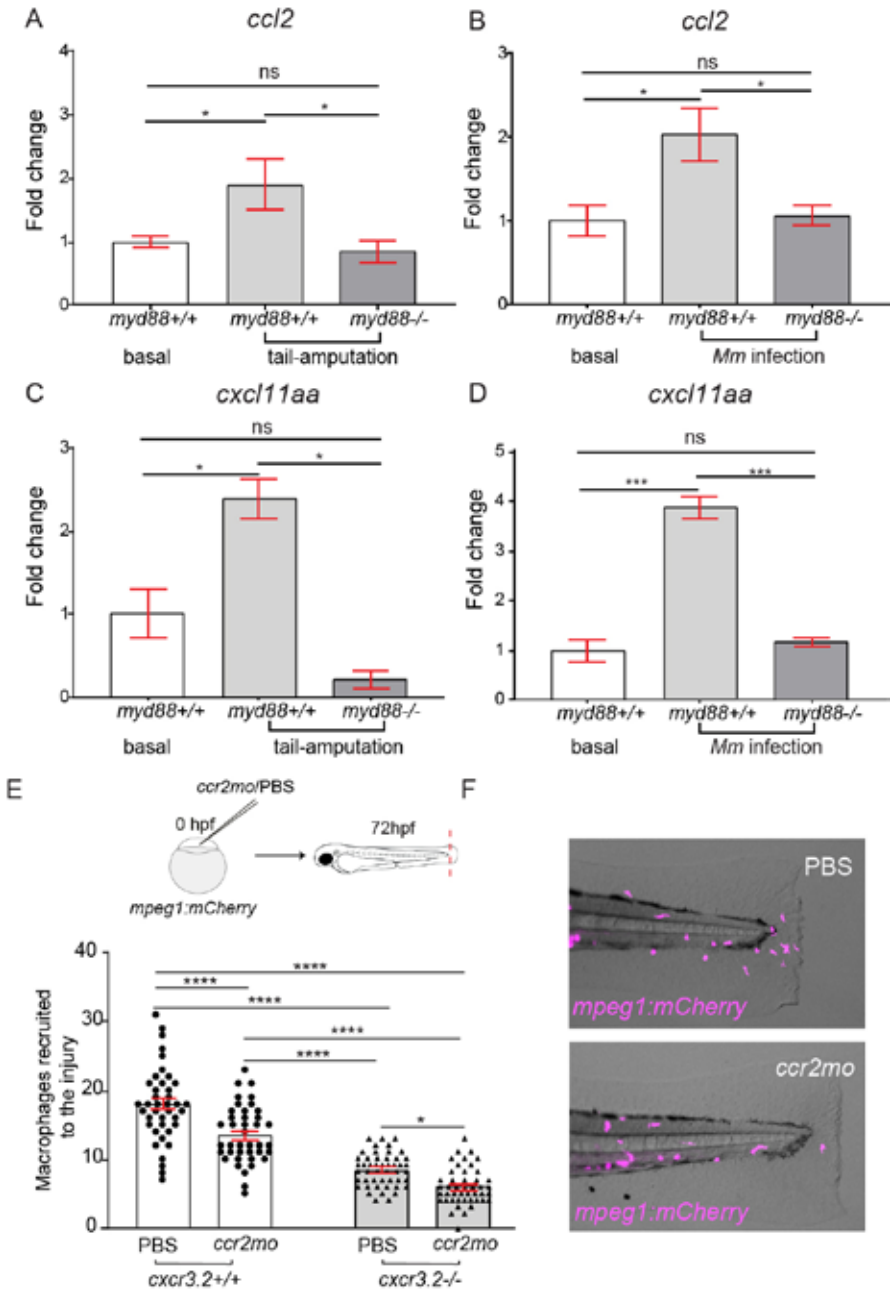

Figure 1. The Ccl2/Ccr2 and Cxcl11aa/Cxcr3.2 chemokine axes contribute to inflammation in zebrafish. Quantitative PCR data on whole larvae show that both *ccl2* **(A-B)** and *cxcl11aa* **(C-**

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.

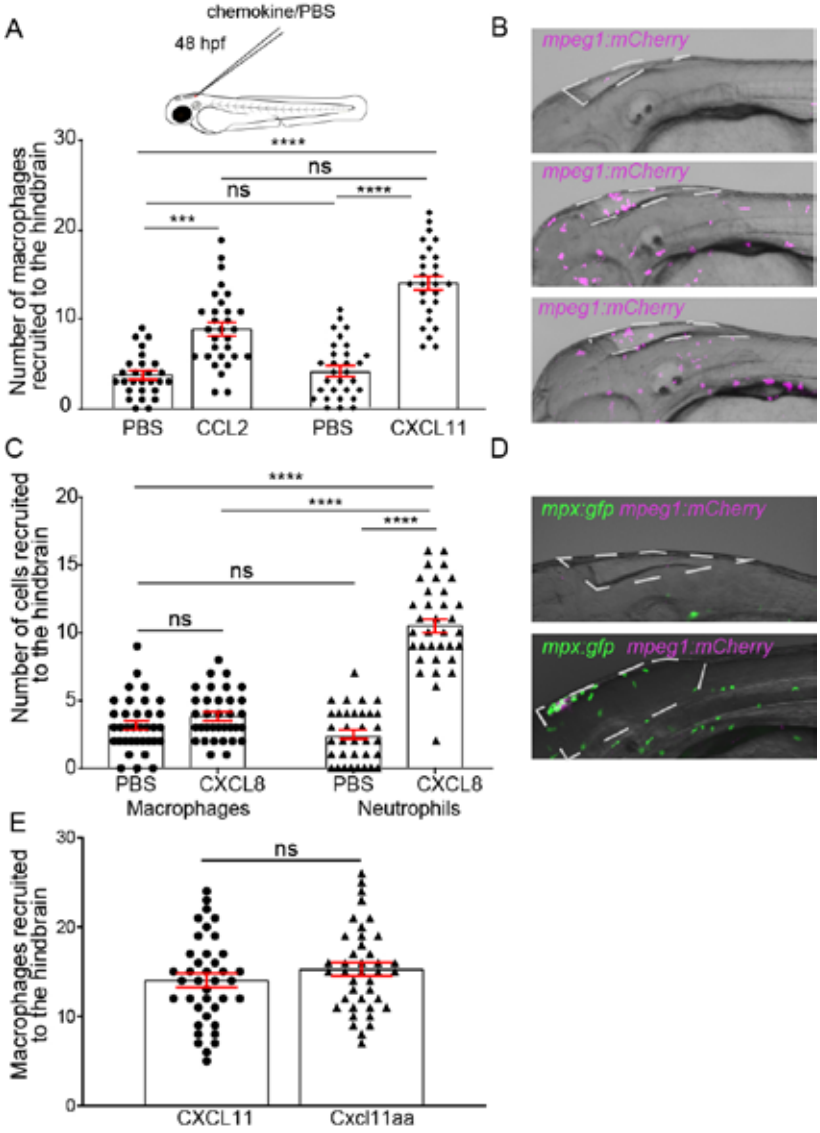

Figure 2. Locally injected human CCL2 and CXCL11 proteins attract macrophages to the hindbrain ventricle of zebrafish larvae. Macrophages (*mpeg1:mCherry*) are recruited to the hind-

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 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

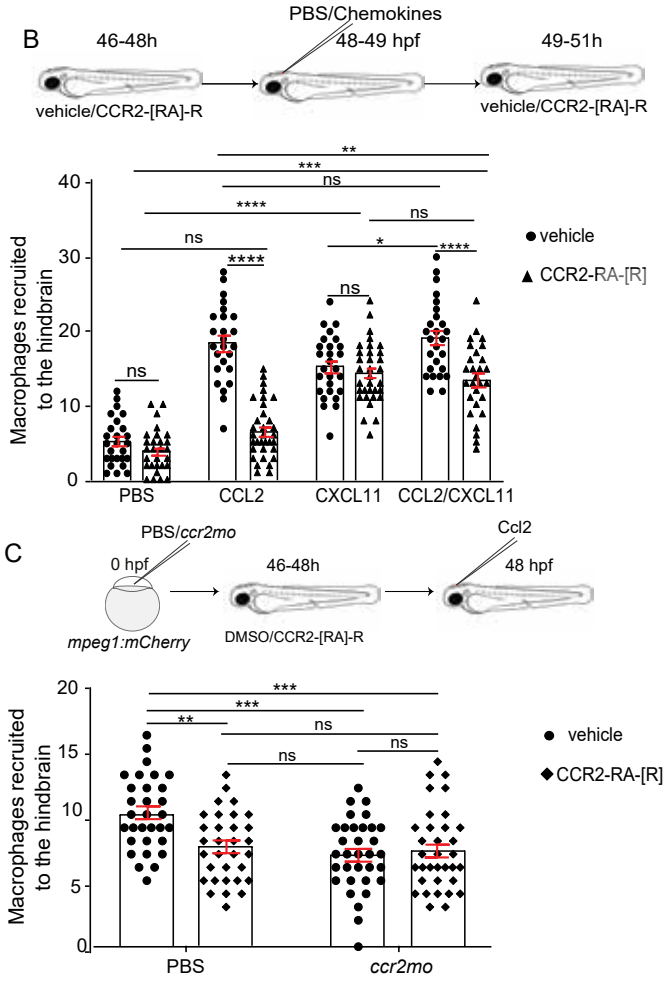

Figure 3. The human CCR2 inhibitor CCR2-RA-[R] inhibits macrophage recruitment in zebrafish. Comparison between whole protein sequences (top) and key residues involved in the intracellular binding of CCR2-RA-[R] in human CCR2 (bottom). Critical residues for intracellular ligand-binding in human CCR2 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-

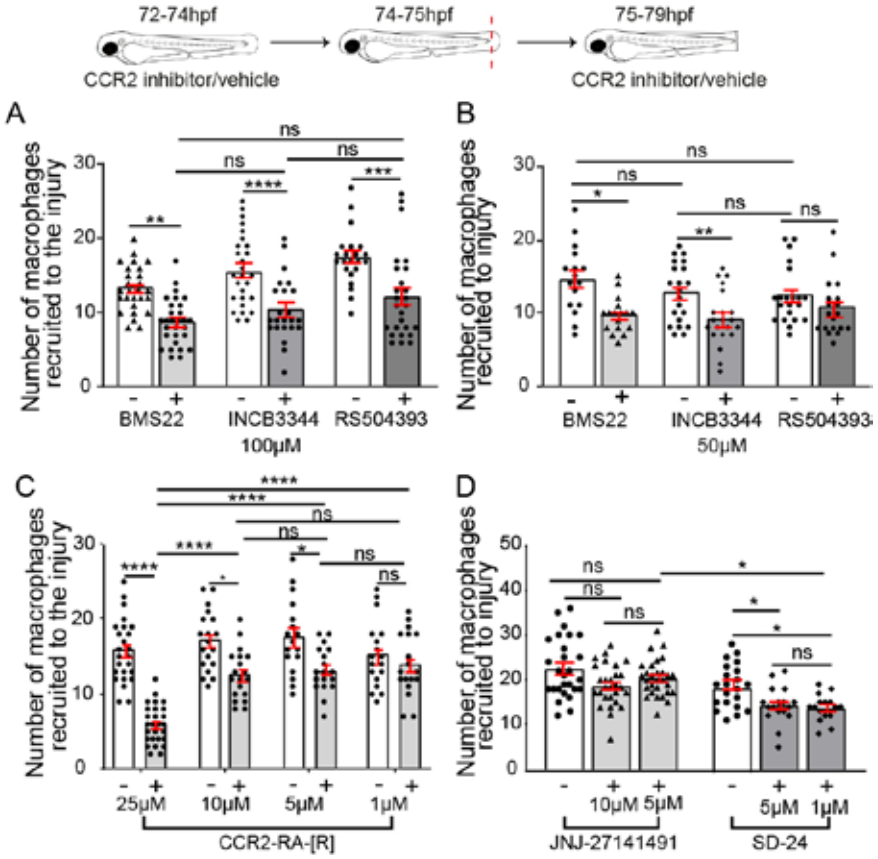

Figure 4. Zebrafish is a powerful screening platform for CCR2 inhibitors. Orthosteric CCR2 inhibitors BMS22, INCB3344, and RS504393 reduce macrophage recruitment to injury at a concentration of 100 µM (**A**). BMS22 and INCB3344 also reduce macrophage recruitment at 50 µM but RS504393 no longer exerts 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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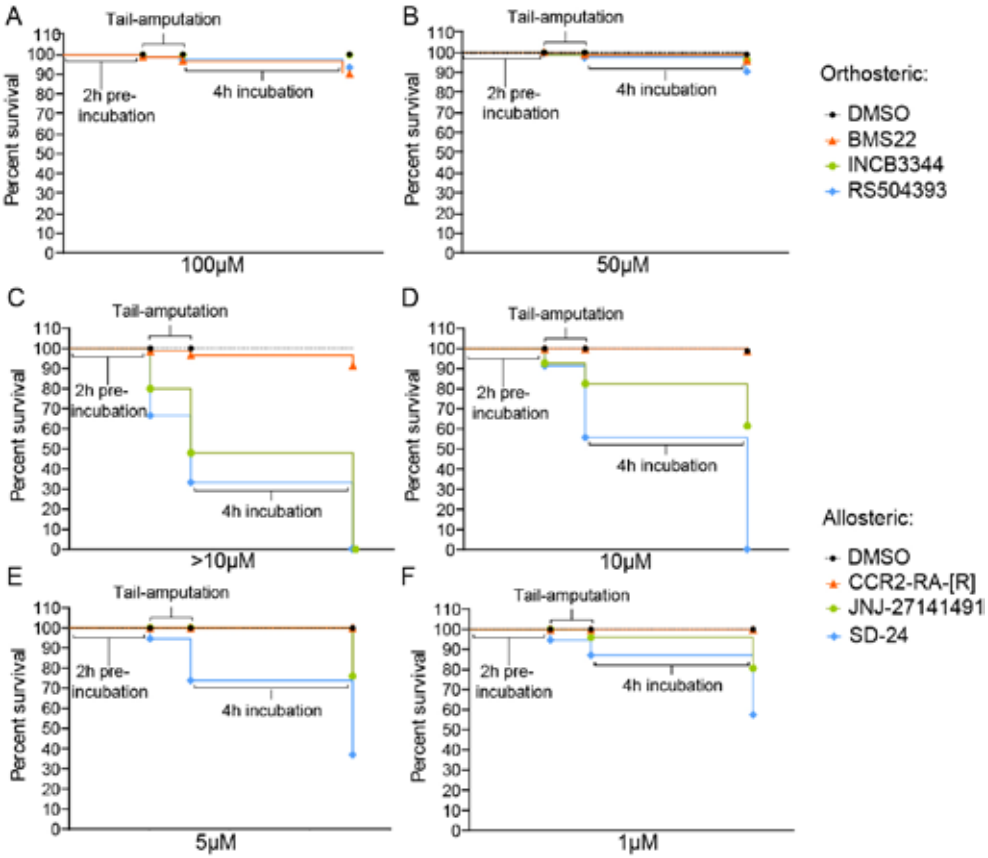
Supplementary materials



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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General discussion

Zebrafish as a model to assess chemokine signaling axes

Zebrafish are increasingly used as a vertebrate model to study mechanisms of development and to model diseases of different natures [1]. The popularity of this model species is primarily due to the accessibility of embryos and larvae for genetic manipulation, microscopic imaging, and drug screening. Moreover, the zebrafish model is being increasingly used in research because of the wide range of readily available molecular tools and its cost-effective housekeeping requirements. Over 80% of all human disease-associated genes identified so far have at least one putative functional homolog in zebrafish [2]. This is true also for genes of the chemokine receptor family, which play fundamental roles in the immune system [3, 4, 5]. During fish evolution, extensive gene duplications occurred in two rounds of whole-genome duplication, which were followed by duplication of single genes in different lineages. On the one hand, these duplications complicate the study of zebrafish homologs of human chemokine receptors, but at the same time, they provide a useful experimental system to explore chemokine receptor sub-functionalization and loss of function events [6, 7, 8]. Recent findings in zebrafish significantly contributed to our understanding of the role of chemokine receptors and their ligands in homeostatic and pathological conditions. Non-invasive *in vivo* imaging in zebrafish contributed to clarify the role of different chemokine signaling axes in the migration and function of immune cells in various inflammatory contexts and to unveil regulatory processes intrinsic to the chemokine system [9, 10, 11, 12, 13]. At the same time, it also provides a useful experimental system to explore chemokine receptor sub-functionalization and loss of function events [6, 7, 8]. This thesis is focused on the zebrafish homologs of the human CXCR3 and CCR2 receptors, two important targets for anti-inflammatory therapies.

Atypical chemokine receptors are a recent and fundamental addition to the chemokine receptor family

Chemokine receptors play major roles during embryonic development, cell differentiation, cell proliferation, and are essential for immunity [14, 15]. They are amongst the largest subfamilies within class A (rhodopsin-like) G protein-coupled receptors (GPCRs). Their structure consists of an intracellular COOH terminus, an extracellular NH2 terminus, and seven transmembrane domains connected by three extracellular and intracellular loops [5, 16]. The cognate ligands of these receptors are small proteins called chemokines. In resting conditions, chemokine receptors are coupled to heterotrimeric G proteins and the Gα subunit is bound to GDP (guanosine diphosphate). Ligand binding triggers the canonical G protein-dependent signaling pathway in which

the Gα subunit exchanges the GDP molecule for GTP (guanosine triphosphate), the GTP-Gα subunit complex uncouples from the receptor and the Gβ-γ heterodimer triggers the activation of various downstream effectors [14, 16]. Chemokine receptors can also signal independently of G proteins through β-arrestin or by directly activating the JAK/STAT (Janus kinase /Signal transducer and activator of transcription) signaling pathway [17, 18]. The chemokine system is highly pleiotropic [19]. Most chemokine receptors can bind multiple chemokines, and chemokines can also bind to numerous receptors. This seemingly redundant system, however, requires tightly regulated mechanisms to confer specificity to the response resulting from receptor-ligand interactions [5, 20]. Therefore, chemokine signaling and signal integration require to be regulated at the genetic, functional, spatial, and temporal levels. Atypical chemokine receptors (ACKRs) are unable to signal in the canonical G-protein dependent manner and act as functional regulators of conventional chemokine receptors by scavenging shared ligands and shaping chemokine gradients [21, 22, 23].

Both conventional chemokine receptors and ACKRs are highly conserved in humans and zebrafish, which is best illustrated by the conserved interaction between CXCR4 and ACKR3 (CXCR7), whereby CXCR7 antagonizes the function of CXCR4 in several developmental processes and tumor progression in zebrafish and mammalian models [7, 17, 13]. The chemokine receptors CXCR3 and CCR2 (two primary players in inflammatory responses) and their ligands are also well conserved in zebrafish [24, 25]. In addition to functions on other leukocyte cell types, both CXCR3 and CCR2 function as macrophage chemoattractant receptors. Macrophages together with neutrophils are the main immune cell type in zebrafish embryos and larvae, which have not yet developed adaptive immune cells [26, 27]. We have therefore used this model to explore the innate immune functions of the CXCR3 and CCR2 chemokine receptors.

Previous work of our group has shown that the zebrafish Cxcr3.2 chemokine receptor is a functional homolog of human CXCR3 and potentially interacts with seven Cxcl11- like chemokines (Cxcl11aa, ac, ad, ae, af, ag, and ah) that share common ancestry with the human CXCR3 ligands CXCL9-10-11 to mediate macrophage recruitment to inflammatory foci [25]. Likewise, zebrafish Ccr2 is a functional counterpart of human CCR2 and can be activated both by human CCL2 and by zebrafish Ccl2, also to mediate macrophage recruitment [24, 28]. A third chemokine receptor expressed by macrophages is Cxcr3.3, which (like Cxcr3.2) resulted from duplication of the Cxcr3 gene in zebrafish [25]. In **Chapter 2** we explored the interplay between the *cxcr3.2* and *cxcr3.3* zebrafish paralogs to regulate macrophage migration during mycobacterial infection and wound-induced inflammation. Our work illustrates the sub-functionalization of the Cxcr3.3 chemokine receptor which acquired a regulatory function by antagonizing Cxcr3.2 through a ligand-scavenging function typical for ACKRs (**Figure 1**). The AKCKR group of chemokine receptors was only recently identified [29] and remains

largely uncharacterized because of its structural diversity and distant phylogenetic relatedness. Although Cxcr3.2 and Cxcr3.3 are closely related, the latter displays an altered DRY-motif (DCY in Cxcr3.3) and differences in microswitch elements indicative of its inability to signal in a G-protein-dependent fashion (**Chapter 2, Figure 1**). Chemokine receptors can also signal through β-arrestin to internalize and degrade ligands during chemotaxis. While such molecular mechanism remains to be shown for Cxcr3.3, we showed that Cxcr3.2 and Cxcr3.2 have opposite effects on macrophage behavior during wound-induced inflammation and infection, strengthening the hypothesis that Cxcr3.3 is an ACKR.

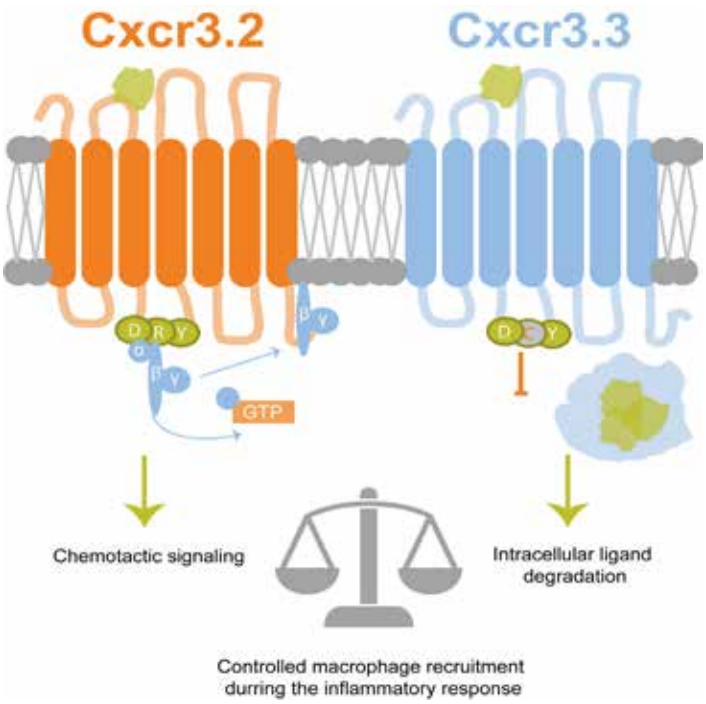

Figure 1. Anagonistic interaction between Cxcr3.2 and Cxcr3.3 regulates macrophage recruitment during the inflammatory response. After binding to its cognate ligand, Cxcr3.2 (orange) signals in the canonical G protein-dependent manner to mediate macrophage recruitment to inflammatory sites. Cxcr3.3 (blue) can bind the same ligands as Cxcr3.2 but is unable to signal through G proteins and acts as a ligand scavenger that regulates Cxcr3.2-mediated macrophage recruitment by limiting the ligand bioavailability. Both receptors are required for balanced macrophage recruitment and the absence of either of them results in reduced or enhanced macrophage recruitment.

Our analysis of a *cxcr3.3* zebrafish CRISPR mutant showed that the loss of this receptor brings macrophages in an activated state and stimulates their recruitment to sites of inflammation (**Chapter 2**). We propose that this phenotype is due to exacerbated Cxcr3.2 signaling and that this Cxcr3.2-Cxcr3.3 antagonism is mediated by competition for the same ligands (**Figure 1**). In addition, our results suggest that Cxcr3.2-dependent signaling may affect *cxcr3.3* transcription, potentially serving as a feedback mechanism to indirectly regulate Cxcr3.2 function. While Cxcr3.3 suffered significant structural changes that ultimately led to the loss of its original function, the high degree of conservation of the critical amino acids in the ligand-binding pockets of Cxcr3.2 and Cxcr3.3 throughout evolution allowed the interplay between them through their shared ligands (**Chapter 2**, **Figure 3**). This mechanism could have evolved because inflammation-driven induction of Cxcl11-like ligands is independent of the expression of both *cxcr3* paralogs and therefore, ligand availability had to be regulated at the post-transcriptional level. The Cxcr3.2-Cxcr3.3 antagonism highlights how a specific mechanism for chemokine signaling regulation can develop as a result of gene duplication and subsequent selection for functionally specialized receptor variants. An analogous antagonistic mechanism exists between the human CXCR3-A and CXCR3B splice variants. Upon binding to CXCL9-11 chemokines CXCR3-A induces chemotaxis and proliferation, whereas CXCR3-B inhibits cell migration and proliferation, and induces cell death [30, 31]. The evolution of different mechanisms to regulate CXCR3 signaling suggests a form of convergent evolution in zebrafish and humans.

Using the *zebrafish-Mycobacterium marinum* infection model, which mimics aspects of human tuberculosis, we show how the absence of Cxcr3.3 results in enhanced macrophage recruitment to mycobacteria and the subsequent dissemination of the infection, arguably because of exacerbated Cxcr3.2 signaling and increased macrophage motility. In agreement, loss of *cxcr3.2* has been shown to reduce infection levels and macrophage motility [25]. The results of *cxcr3.2* and *cxcr3.3* mutations are also in line with other studies showing that macrophages facilitate the spreading of mycobacterial infection and that dampening the migratory activity of macrophages improves host resistance [25] The ligand-scavenging activity of Cxcr3.3 modulates the processing of inflammatory cues as shown by the up-regulation of pro-inflammatory markers and the branched and elongated shape of *cxcr3.3* mutant macrophages and their migration speed (**Chapter 2, Figure 6**). These findings underscore the importance of ACKRs in fine-tuning chemokine signaling networks and the immune response as a whole.

In conclusion, the results of **Chapter 2** add to the recently recognized roles of CXCR3 signaling on macrophages. Together with CCR2, this chemokine receptor is critical for determining macrophage polarization and a better understanding of the role in this process could aid the development of novel therapies against infections, inflammatory disorders, and favor tissue regeneration as well as unveil developmental processes [32,

33]. GPCRs are the largest protein family targeted by approved drugs [34]. Therefore, it is important to further our understanding of the fundamental mechanistic principles of GPCR-related pathways to open up new therapeutic options against multidrug-resistant strains of *M. tuberculosis*, for example. A deeper knowledge of the role of ACKRs in different homeostatic and pathogenic contexts is required to characterize the extensive crosstalk among chemokine signaling axes and to better understand this highly complex system. ACKRs could be also pharmacologically targeted to develop host-directed therapies aimed at modulating the inflammatory response.

Chemotactic signaling drives cell motility and affects lysosomal properties and macrophage immune competence

The integration of extracellular chemotactic signals and intracellular pathways driving cell motility, metabolism, and the immune response remain largely uncharacterized despite the growing interest in the topic [35, 36]. In **Chapter 3** we showed that the depletion of the chemokine receptor Cxcr3.2 is associated with the overall deregulation of lysosomal and Golgi-related genes in which several of them were upregulated. Macrophages depleted in Cxcr3.2 had a rounded shape, highly acidic and enlarged lysosomes, and enhanced bactericidal properties. We also showed that the chemokine receptor is required for the proper cycling of lysosomes from the cell front to the rear that leads to the polarized phenotype of migrating macrophages during chemotaxis (**Figure 2**).

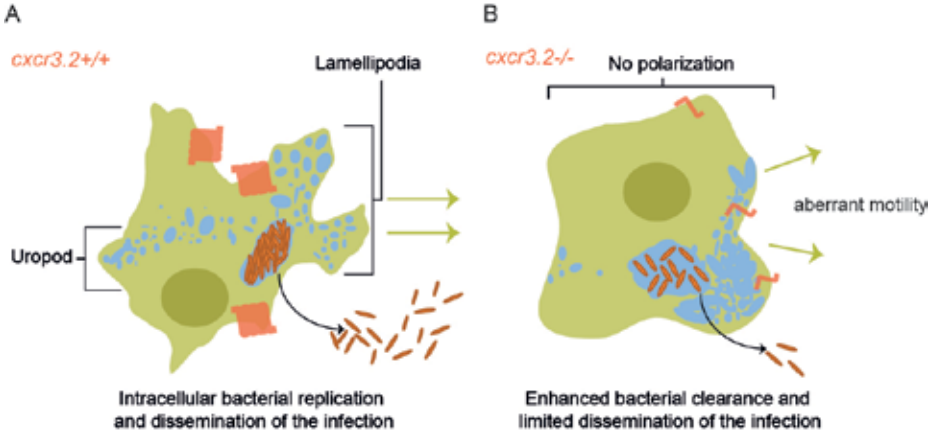

Figure 2.Disruption of Cxcr3.2 signaling reduces macrophage motility and affects lysosomal function and intracellular vesicle trafficking, rendering cells more microbicidal. Chemotactic signaling contributes to cell polarization during chemotaxis by sustaining the cycling of lysosomal contents from the front of the cell (lamellipodia) to the back (uropod) (**A**). In *cxcr3.2* mutants, macrophages are not polarized, they have a rounded shape, accumulate lysosomal contents in the front of the cell during chemotaxis and clear bacteria more efficiently (**B**).

The hallmark of chemotactic signaling is the rapid increase in intracellular calcium, which allosterically regulates different enzymes and proteins to orchestrate the cytoskeletal rearrangements, adhesion-de-adhesion cycles, and membrane remodeling processes required for cell motility [15, 35]. The binding of a chemokine receptor and its cognate ligand activates the membrane-associated phosphoinositide-specific phospholipase Ć (PLC) which in turn generates IP3 that binds to IP3 receptors on the ER and lysosomes that open up calcium channels and increase the intracellular concentration of free Ca^{2+} [15]. Although the essential role of PLCs during chemotaxis has been challenged [37], cells treated with an intracellular calcium chelator showed reduced chemotaxis, supporting that Ca^{2+} release is required for the process [38, 39]. Calcium release and its intracellular distribution and concentration are central to complex interchained processes such as cell motility, lysosomal function, cell metabolism, and immunity. Transgenic zebrafish reporter lines have been generated that allow *in vivo* measurement of Ca^{2+} waves [40, 41]. It would be of great interest to employ such Ca^{2+} indicator lines in future work to study the molecular mechanism that links activation of Cxcr3.2 to the trafficking of lysosomes and polarization of macrophages.

It is likely that small GTPases play a role in the lysosomal phenotype of *cxcr3.2* mutants. Rho GTPases link chemokine signaling to cytoskeleton remodeling and cell polarization [42, 43], and Rab GTPases regulate membrane trafficking, vesicle formation, and mobilization through actin and tubulin networks [35, 44]. The small GTPases Rab27a and Rab3a have been shown to interact with Ca2+-sensing synaptotagmin (SYT) 7 and SYT5L that mediate lysosomal vesicle fusion with the plasma membrane and recycle receptors back to the plasma membrane to regulate lysosomal exocytosis during chemotaxis [35, 44]. Small GTPases and guanine nucleotide exchange factors (GEFs) are also known to play a fundamental role in bridging the regulation of lysosomal biogenesis master genes TFEB/TFE3 with cell metabolism and cell motility [45, 46, 47, 48]. Furthermore, the nutrient-sensitive Rag-Ragulator complex subunits associate with TFEB/TFE3 on the lysosomal membrane to modulate cell metabolism and immunity [45, 47]. We found that blocking the zebrafish homolog of TFEC, a regulator of TFEB/TFE3 resulted in similarly enhanced microbicidal properties of macrophages as observed upon disruption of Cxcr3.2 chemotactic signaling. To better understand the regulatory mechanisms governing the activity of the TFEB/TFEC family and its associated GTPases in *cxcr3.2* mutant and wild type zebrafish will contribute to a more comprehensive understanding of chemotaxis and its functional link to other cellular processes and immunity. **Chapter 3** reflects how chemotaxis, cell metabolism, and immunity are deeply intertwined and provides evidence on a direct effect of chemokine signaling on lysosomal function *in vivo*. Unlike *in vitro* experiments, the zebrafish model allowed us to assess the effect of the *cxcr3.2* mutation in macrophage migration and lysosomal function in real-time and during the inflammatory response at the whole organism level. Studies focusing on calcium-dependent intracellular pathways and the regulatory role of small

GTPases in such pathways would contribute to bridging gaps in our understanding of chemotaxis as a complex process that incorporates various physiological processes and integrates different extracellular cues.

Zebrafish model as an *in vivo* screening platform for chemokine receptor inhibitors

In our studies of the *cxcr3.2* and *cxcr3.3* receptor mutants, we took advantage of the power of zebrafish for non-invasive *in vivo* imaging of a whole organism due to its optical transparency at early developmental stages. The use of zebrafish larvae also enables the high throughput assessment of candidate anti-inflammatory drugs [49, 50]. Since specific cell types can be fluorescently labeled using available molecular tools and tracked in real-time the physiological effect of a chemical compound can be evaluated in a tissue-specific manner. As a proof-of-principle that drugs targeting human chemokine receptors can be assessed in zebrafish, our group previously showed a human CXCR3 inhibitor to interfere with macrophage recruitment in zebrafish [25]. Here, we extend these studies to inhibitors of CCR2.

Chapter 4 confirms the compatibility of the CXCR2-CXCL2 and CXCR3-CXCL11 signaling axes between zebrafish and humans and supports that zebrafish can be used to screen human CCR2 inhibitors in a fast and robust manner (**Chapter 4, Figure 2**). Different concentrations of existing orthosteric and allosteric CCR2 inhibitors reduce macrophage recruitment to inflamed tissue in zebrafish after bath-exposure (**Chapter 4, Figure 3-4**). Using a live organism allowed us to simultaneously assess compound toxicity through survival curves and choose highly efficient compounds with low toxicity. Given its many advantages, we propose that screening methods using zebrafish could be implemented at the early stages of the drug-discovery process to speed up the development of anti-inflammatory drugs.

The CCR2 and CXCR3 signaling axes have been implicated in multiple inflammatory diseases. In secondary multiple sclerosis, CCR2 and CXCR3 can be found in demyelinating plaques as hypertrophic astrocytes secrete both CCL2 and CXCL10 [51]. CCR2-expressing macrophages phagocytose axonal debris and drive the axonal damage that characterizes this pathology [51]. In atherosclerotic lesions, CCR2-depleted mice show a striking reduction in the recruitment of macrophages and the formation of atherosclerotic lesions [52]. Interestingly, in mice with induced colitis the simultaneous blockade of CCR2 and CXCR3 with a non-peptide chemokine receptor antagonist (TAK-779), reduced monocyte/macrophage infiltration into the colonic mucosa and the secretion of pro-inflammatory cytokines [53]. Novel allosteric inhibitors targeting both CCR2 and CXCR3 could be tested using zebrafish and expand anti-inflammatory treatment options in a fast and reliable manner.

Most human CCR2 inhibitors also target closely related CCR1 and CCR5 receptors. Closely related Ccr2 homologs can also be found in zebrafish. Therefore, developing mutants of the relevant *ccr* genes could provide new tools to study the specificity of CCR2 inhibitors *in vivo*. However, the considerable amount of duplicated chemokine and chemokine receptor genes in zebrafish may also complicate the identification of single chemokine-receptor-ligand interactions. The assessment of the chemokine receptor inhibitors on cells transfected with a single zebrafish receptor would help to pinpoint and characterize specific chemokine receptor-inhibitor interactions and support the comparison with the human homologs. Based on the *in vivo* data obtained from the CCR2 inhibitors screening in **Chapter 4**, we developed an *in vitro* assay using Chinese hamster ovary (CHO) cells transfected with zebrafish and human CCR2 and CXCR3 chemokine receptors and used the xCELLigence Real-Time Cell Analysis (RTCA) Instruments technology to assess cell response upon ligand binding and exposure to CCR2 inhibitors. The xCELLigence technology uses electronic microtiter 96 well-plates (E-Plate® 96) with gold microelectrodes embedded in the bottom of each well to detect changes in cell shape size and adherence in a highly sensitive and non-invasive manner [54]. The system measures the impedance of the electric current created by the electrodes and the cell-culture medium. The impedance is measured in a unitless parameter called "cell-index", which reaches a plateau when cells entirely cover the bottom of the well (100% confluence) and sets the starting point for the real-time assessment of cell behavior (**Figure 3 A-B**). One of the main advantages of this system is that it does not require radioligands or any other labels so that purified CCR2 and ligands CXCR3 (CCL2 and Cxcl11aa, respectively) can be directly administered and that the response can be recorded in real-time [54].

As a first step to applying xCELLigence technology to the study of zebrafish chemokine receptors, we transfected plasmids containing the protein-coding sequences of human CCR2 and zebrafish Cxcr3.2 in CHO cells. Transfection was performed using the cationic polymer polyethyleneimine (PEI), which binds and condenses DNA into positively charged molecules that are endocytosed by the cells and release the DNA into the cytoplasm [55]. Human CCR2 served as a positive control since its expression and function using the xCELLigence technology had been previously standardized. We pre-treated cells with either vehicle (DMSO) or with the CCR2 inhibitor/antagonist CCR2-RA-[R] and then added either vehicle (PBS) or the relevant chemokine ligand (CCL2 or Cxcl11aa) (**Figure 3C**). The baseline-corrected results for CCR2 show an increase in Cell index upon stimulation with CCL2 when treated with vehicle compared to the response in antagonist-treated cells showing that CCL2 induces a response that is counteracted by CCR2-RA-[R] (**Figure 3D**). Preliminary data on Cxcr3.2-expressing cells treated with vehicle and stimulated with Cxcl11aa show a clear but short-lived response, and in line with observations *in vivo*, CCR2-RA-[R] did not lower the mag-

nitude of this response (**Figure 3E**). This preliminary data shows the potential of the xCELLigence technology, but further work is required to establish the conditions for studying the zebrafish receptors.

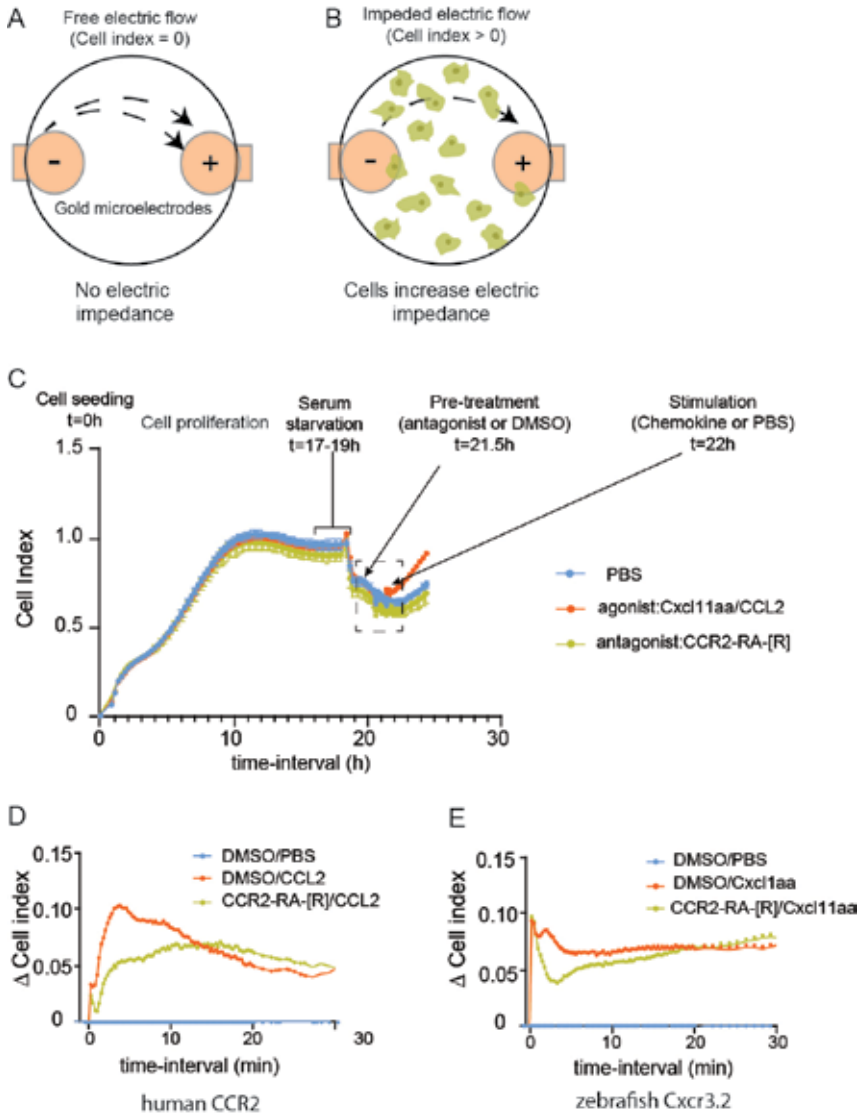

Figure 3. The xCELLigence Real-Time Cell Analysis (RTCA) assay principle and set-up. The gold microelectrodes attached to the bottom of each well of the plate generate an electric flow through the administration of a small voltage and its interaction with the salt-containing culture medium. The system measures the impedance of the electric flow resulting from changes in cell number, shape, adherence and permeability. Impedance is reported as a measure of cell adhesion to the bottom

called "cell index". In the absence of cells, there is no electric impedance and the cell index = zero (**A**). When cells proliferate and attach to the bottom the electric flow is impeded and cell index is > zero (**B**). Panel **C** provides an overview of the single steps of the xCELLigence assay. Chinese hamster (CHO) cells were transfected with human CCR2 or zebrafish Cxcr3.2 and expression was verified by ELISA. Transfected cells were seeded in an E-Plate® 96 at a density of 50,000 cells/well and incubated for 17h at 37oC + 5% CO2. During this time, cells proliferated and the cell index gradually increased until growth reached a plateau (**cell proliferation**). The cell culture medium was exchanged for fetal calf serum- free medium and cells were incubated for two more hours (**serum starvation**). Cells were subsequently incubated with either DMSO or the CCR2 antagonist CCR2-RA-[R] (**pre-treatment**) and stimulated after 30 min with PBS, CCL2 or Cxcl11aa (**stimulation**). Data were baseline corrected (DMSO pretreatment and PBS stimulation = 0). Cells transfected with CCR2 and incubated with DMSO showed a clear response upon stimulation with CCL2 and a reduced response when incubated with CCR2-RA[R] (**D**). Cxcr3.2-expressing cells showed a clear but transient response upon stimulation with Cxcl11aa and were not affected by CCR2-RA[R] incubation (**E**).

Taken together, combining *in vivo* and *in vitro* data on the efficacy and binding selectivity of CCR2 inhibitors could provide valuable insight into the cell- and tissue-specific effect of these compounds, their potential side effects and assist the design of inhibitors targeting single or multiple chemokine receptors to develop novel anti-inflammatory drugs.

Concluding remarks

The present work provided new insight into one of the multiple regulatory mechanisms underlying chemokine signaling axes by taking advantage of the duplication of the CXCR3 gene in zebrafish. It showed that macrophage recruitment to sites of infection requires the interplay between the conventional Cxcr3.2 receptor and the atypical chemokine receptor Cxcr3.3 to exert a balanced inflammatory response during mycobacterial infection and tissue wounding. It also shows that chemotactic signaling directly affects lysosomal function *in vivo*, highlighting the deep connection between chemotactic signaling and intracellular processes involving metabolism and immunity, and suggests that the chemokine system should be studied in a wider context instead of focusing on chemotaxis alone. This work also illustrates how the zebrafish model constitutes a fast and robust screening platform for chemokine receptor inhibitors aimed at reducing inflammation and proposes to complement *in vivo* observations with *in vitro* assay to offer a comprehensive view on how inhibitors act on specific chemokine receptors. Altogether, the work included in this thesis emphasizes the essential role of chemokine receptors in immunity and calls for a better characterization of the chemokine system. It also serves as a proof of principle that the zebrafish model can be used as an *in vivo* screening platform using zebrafish for the identification of chemokine receptor inhibitors to assist the development of anti-inflammatory drugs.

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Summary

Macrophages are immune cells that express multiple receptors on their membrane to sense and process external signals required to mount an effective immune response against pathogens and upon tissue damage. Chemokine receptors direct macrophages to inflamed or infected tissue by following and binding to a gradient of small proteins called chemokines. The chemokine receptors CXCR3 and CCR2 guide macrophages to infectious and inflammatory foci, where these cells engulf and degrade bacteria, modulate inflammation and support the repair of injured tissue. Chemokine receptors and their functions on macrophages are therefore considered central elements of the innate immune response and form the main subject of this work.

Due to the easy optical access and the wide variety of readily available molecular tools, the zebrafish model enables the live tracking and visualization of fluorescently labeled macrophages lacking or overexpressing chemokine receptors during bacterial infection and injury. We exploited these advantages to study the zebrafish homologs of CXCR3 and CCR2, addressing three main research questions: i) Is macrophage migration regulated by the interplay between closely related chemokine receptors? ii) Does chemokine signaling link the migration of macrophages to their microbicidal activity? and iii) What is the potential of the zebrafish model for screening anti-inflammatory drugs that target chemokine receptors?

In **Chapter 1**, we provide an overview of chemokine signaling principles and review the role of chemokine receptors in different inflammatory contexts and zebrafish development. The chapter focuses on macrophages and neutrophils and their ability to clear microbial invaders, resolve inflammation, and tune the activation status of macrophages. It briefly describes the link between chemokine signaling and cytoskeletal rearrangements required for cell movement and phagocytosis (ingestion of pathogenic particles and dying cells) and emphasizes that human and zebrafish chemokine systems are highly conserved despite the extensive gene duplication in the latter. The chapter compiles the most relevant contributions of the zebrafish model to the characterization of fundamental mechanisms in chemokine signaling networks and their role during cancer progression and wound- and pathogen-induced inflammation. It underscores the versatility and robustness of the zebrafish model to assess developmental processes and early innate responses in real-time.

Chapter 2 describes the antagonistic interplay between the *cxcr3.2* and *cxcr3.3* paralogs in zebrafish macrophages. The Cxcr3.2 chemokine receptor is homologous to human CXCR3 and drives macrophage movement towards sites of infection and inflamma-

tion. Cxcr3.3 has structural and phylogenetic features of so-called atypical chemokine receptors, which are unable to signal but scavenge chemokines shared with conventional receptors to regulate their function. In this chapter, we explore the functional antagonism of these receptors in the context of mycobacterial infection and wound-induced inflammation. The depletion of the Cxcr3.2 receptor by mutation leads to reduced macrophage motility, while the loss of Cxcr3.3 enhances cell motility. The reduced motility of *cxcr3.2* mutant macrophages limits the dissemination of intracellular bacteria and has a host-protective effect, while enhanced motility in *cxcr3.3* mutants leads to bacterial spreading and poor control of the infection. Also, fewer macrophages are recruited to sites of injury in *cxcr3.2* mutants, while more cells are recruited in *cxcr3.3* mutants. These observations and the structure of Cxcr3.3 suggest that this receptor regulates Cxcr3.2 function by limiting the availability of shared ligands. Therefore, the absence of Cxcr3.3 results in enhanced Cxcr3.2-driven macrophage recruitment. This antagonistic regulatory mechanism resulting from the diversification of conventional and atypical receptor variants in zebrafish is analogous to the role of antagonistic splice variants CXCR3A and CXCR3B in humans. Our findings highlight the usefulness of the zebrafish to study core mechanisms of chemokine networks and the translatability to humans.

Cell movement requires the integration of multiple extracellular and intracellular cues that remain largely elusive. A recently characterized mechanism involved in chemotaxis links chemokine receptors with the cycling of lysosomes (primary degradative cell organelles) from the front to the rear of migrating cells. **Chapter 3** examines the link between lysosomal function, directed cell movement (chemotaxis), and immunity. It shows that several lysosomal genes are upregulated in macrophages lacking the Cxcr3.2 chemokine receptor, which also display an accumulation of highly acidic lysosomes in the front of the cell, which rarely shuttle to the rear of the cell during chemotaxis. This defect in the cycling of lysosomes and their altered properties confer an increased capacity to degrade intracellular bacteria to *cxcr3.2* mutant macrophages. To confirm the link between lysosome function and *cxcr3.2* mutation, we used molecular tools that enable us to overexpress or block Transcription factor EC (Tfec), an inhibitor of the lysosomal regulator complex. Tfec overexpression strongly inhibited lysosomal function and resulted in poor intracellular clearance of bacteria. Tfec blockade enhanced lysosomal function and boosted bacterial clearance like the *cxcr3.2* mutation. Furthermore, Tfec overexpression in Cxcr3.2-deficient macrophages counteracted the protective effect of the mutation, confirming that the lack of the chemokine receptor alters the function of lysosomes. Finally, to assess the link between the altered lysosomal function and cell motility, we tracked lysosomes of *cxcr3.2* mutant macrophages during chemotaxis. Lysosomes concentrated in the front and rarely went to the back of the cell, suggesting that the absence of Cxcr3.2 disrupts the lysosome trafficking required for chemotaxis while rendering these cells more efficient at clearing bacteria. This chapter illustrates the complex network of processes that need to be integrated during cell movement and

immunity and links chemotactic signaling with lysosomal function in the context of wounding and infection.

Zebrafish larvae are increasingly used as a platform for chemical screens because they are highly permeable to chemical substances, but also due to their fast development, small size, and cost-effective housekeeping requirements. Adult zebrafish lay large amounts of eggs granting representative sample sizes and robust statistical analyses. The transparency of larvae enables the real-time visualization of fluorescently labeled cells during inflammation. A further advantage of using live zebrafish larvae is that compound toxicity can be easily evaluated by assessing survival, development, and morphological abnormalities. Previous work had shown that a human CXCR3 inhibitor reduces macrophage recruitment similar to *cxcr3.2* mutation. **Chapter 4** focuses on another key receptor for macrophage recruitment, CCR2, and assesses the viability of using the zebrafish model to robustly screen human CCR2 inhibitors in a whole organism. CCR2 is involved in multiple inflammatory disorders and therefore an important drug target. CCR2 inhibitors aimed at reducing inflammation exist but none of them has made it to clinical trials. Demonstrating the high degree of conservation across human and zebrafish chemokine signaling axes, human chemokines effectively induced macrophage migration in zebrafish. Furthermore, macrophage migration in zebrafish was reduced by human CCR2 inhibitors, belonging to either the orthosteric class (blocking the chemokine binding site) or the allosteric class (blocking receptor function via an interaction outside the chemokine binding site). These results serve as proof-of-principle for screening CCR2 inhibitors using the zebrafish model. We suggest a workflow to evaluate the efficacy of human CCR2 inhibitors by quantifying macrophages recruited to inflamed tissue and simultaneously assess toxicity to find compounds and concentrations with high efficacy and low toxicity. Implementing the zebrafish model to the CCR2 inhibitors testing scheme could speed-up the development of anti-inflammatory drugs to treat multiple diseases.

Chapter 5 discusses the results of the previous chapters and the broader perspective of chemokine signaling research using the zebrafish model. It discusses the relevance of characterizing and understanding regulatory mechanisms underlying the chemokine system and calls for a better classification of atypical chemokine receptors and their roles. It contextualizes our findings on the link between chemotactic signaling and lysosomal function during wounding and infection in relation to current advances in related fields and explores areas that could further our understanding of chemokine signaling in a broader context. Finally, it provides preliminary data of *in vitro* assays that could be refined and implemented to develop a comprehensive screening platform for chemokine receptor inhibitors that uses the combination of zebrafish and cell culture systems to enable robust, fast, and detailed efficacy and toxicity assessments.

In conclusion, the work in this thesis highlights the relevance of chemokine signaling axes in the immune response, their link to other cellular processes besides chemotaxis, and shows that zebrafish can be used as a robust and fast screening platform for chemokine receptor inhibitors. Taking advantage of the extensive duplication of chemokine receptor genes, it demonstrates that two copies of the *cxcr3* gene evolved to antagonize each other to balance macrophage recruitment to inflammatory foci. Live imaging of migrating macrophages lacking the functional homolog of human CXCR3 showed that lysosomal properties, numbers, and localization were affected in these cells and that they degraded intracellular bacteria more efficiently despite having aberrant motility. It also demonstrates that it is feasible to use the zebrafish model as a screening platform for inhibitors of chemokine receptors and assess their efficacy and toxicity simultaneously at the early stages of the drug testing process to speed up the development of novel anti-inflammatory drugs.

Samenvatting

Macrofagen zijn immuuncellen die verschillende receptoren op hun oppervlakte dragen waarmee zij signalen uit de omgeving kunnen waarnemen en verwerken tot een effectieve reactie op ziekteverwekkende micro-organismen of weefselschade. Immuuncellen worden naar ontstoken en geïnfecteerde weefsels geleid via een gradiënt van kleine signaaleiwitten die chemokinen genoemd worden. De chemokinereceptoren CXCR3 en CCR2 zijn belangrijk voor de migratie van macrofagen naar plaatsen van infectie en ontsteking, waar deze cellen bacteriën opnemen en afbreken, de ontstekingsreactie moduleren en het herstel van beschadigde weefsels bevorderen. Chemokinereceptoren en hun functies in macrofagen spelen daarom een centrale rol in het aangeboren immuunsysteem en vormen het hoofdonderwerp van het onderzoek in dit proefschrift.

De bijzonder goede toegankelijkheid voor microscopie en een scala aan moleculaire gereedschappen maken de zebravis bij uitstek geschikt als model om de reactie van macrofagen op infectie en verwonding in beeld te brengen en te onderzoeken in een levend organisme. Hierbij kunnen de macrofagen met fluorescerende eiwitten worden gemarkeerd en kunnen de chemokinereceptoren worden uitgeschakeld of tot overexpressie gebracht. Wij hebben van deze technische mogelijkheden gebruik gemaakt om de zebravishomologen van de humane CXCR3- en CCR2-receptoren te bestuderen met drie overkoepelende onderzoeksvragen: i) Wordt de migratie van macrofagen gereguleerd door een samenspel van nauwverwante chemokinereceptoren? ii) Verbinden chemokinesignaalroutes de migratie van macrofagen met hun antimicrobiële capaciteit? en iii) Welk potentieel heeft het zebravismodel voor het identificeren van nieuwe ontstekingsremmende medicijnen die aangrijpen op chemokinereceptoren.

In Hoofdstuk 1 geven wij een overzicht van de principes van signaaltransductie door chemokinereceptoren en bespreken de huidige kennis van de rol van deze receptoren onder verschillende ontstekingscondities en tijdens de ontwikkeling van zebravisembryo's. De nadruk ligt hierbij op macrofagen en neutrofielen en hun rol bij verschillende processen zoals het opruimen van microbiële indringers, de resolutie van ontstekingsreacties en het fijnregelen van de activeringsstatus van macrofagen. We beschrijven het verband tussen chemokinesignaaltransductie en de veranderingen in het cytoskelet van immuuncellen die nodig zijn voor migratie en fagocytose (opname van micro-organismen en celresten). Verder laten we zien hoe sterk de chemokinesystemen van mens en zebravis geconserveerd zijn, ondanks dat er tijdens de evolutie van vissen meerdere genduplicaties hebben plaatsgevonden. Ten slotte beschrijft dit hoofdstuk de meest relevante bijdragen van het zebravismodel aan de karakterisering van fundamentele mechanismen in de signaalnetwerken van chemokinereceptoren en hun functies tijdens tumorpro-

gressie en ontstekingsreacties opgewekt door verwonding of infectie. We onderstrepen de veelzijdigheid en robuustheid van het zebravismodel om ontwikkelingsprocessen en vroege stadia van de aangeboren immuunrespons in "real time" te bestuderen.

Hoofdstuk 2 behandelt het antagonistische samenspel tussen twee paraloge genen, *cxcr3.2* en *cxcr3.3*, in macrofagen van de zebravis. De chemokinereceptor Cxcr3.2 is homoloog aan de humane CXCR3-receptor en stuurt de migratie aan van macrofagen naar plaatsen van infectie of ontsteking. Cxcr3.3, daarentegen, heeft structurele en fylogenetische kenmerken van de zogenoemde atypische chemokinereceptoren die geen signalen kunnen doorgeven, maar wel chemokinen kunnen wegvangen. Daarmee reguleren ze de functie van conventionele receptoren die diezelfde chemokinen binden. In dit hoofdstuk hebben wij het functionele antagonisme van deze receptoren uitgezocht in de context van mycobacteriële infectie en wond-geïnduceerde ontsteking. Uitschakeling van de Cxcr3.2-receptor door mutatie vermindert de motiliteit van macrofagen, terwijl mutatie van Cxcr3.3 de motiliteit juist verhoogt. De verminderde motiliteit van macrofagen in *cxcr3.2*-mutanten beperkt de verspreiding van intracellulaire bacteriën en heeft een beschermend effect tegen de infectie, terwijl de verhoogde motiliteit in *cxcr3.3*-mutanten de verspreiding van de bacteriën bevordert met als gevolg dat de zebravisembryo's de infectie slecht onder controle kunnen houden. Ook bleek dat er bij *cxcr3.2*-mutanten minder macrofagen worden aangetrokken naar verwondingen, terwijl dat er bij *cxcr3.3*-mutanten juist meer zijn. Deze observaties, samen met de structuur van de receptor, suggereren dat Cxcr3.3 de functie van Cxcr3.2 reguleert door de beschikbaarheid van gedeelde liganden te beperken. Daarom resulteert de afwezigheid van Cxcr3.3 in een toename van Cxcr3.2-gestuurde macrofaagmigratie. Dit antagonistische regulatiemechanisme, voortgekomen uit diversificatie van conventionele en atypische receptorvarianten in de zebravis, is analoog aan de rol die de antagonistische splicing-varianten CXCR3A en CXCR3B spelen in de mens. Onze resultaten onderstrepen het nut van het zebravismodel voor onderzoek naar centrale mechanismen van chemokinenetwerken en de vertaling daarvan naar humane ziekten.

Celmigratie vereist de integratie van diverse extracellulaire en intracellulaire signalen die nog voor een groot deel onbekend zijn. Recent onderzoek heeft een verband gelegd tussen de functie van chemokinereceptoren tijdens chemotaxis en de cyclische beweging van lysosomen (verteringsblaasjes) van de voorzijde naar de achterzijde van migrerende cellen. **Hoofdstuk 3** belicht de relatie tussen lysosomale functie, chemotaxis en immuniteit. Het laat zien dat verschillende lysosomale genen verhoogd tot expressie komen in macrofagen met een mutatie in de chemokinereceptor Cxcr3.2. Deze macrofagen bevatten bovendien sterk zure lysosomen, die accumuleren in het voorste gedeelte van de cel en die zich tijdens chemotaxis maar zelden naar het achterste gedeelte verplaatsen. Deze veranderde eigenschappen van de lysosomen verlenen aan macrofagen van de *cxcr3.2*-mutant een verhoogde capaciteit om intracellulaire bacteriën af te breken. Om

het verband tussen lysosomale functie en *cxcr3.2*-mutatie te bevestigen hebben we moleculaire gereedschappen gebruikt om Transcriptiefactor EC (Tfec, een repressor van het lysosomale regulatiecomplex) te blokkeren of tot overexpressie te brengen. Overexpressie van Tfec gaf een sterke remming van de lysosomale functie en resulteerde in verminderde afbraak van intracellulaire bacteriën. Het blokkeren van Tfec, daarentegen, verbeterde de lysosomale functie en versterkte de afbraak net zoals de *cxcr3.2*-mutatie. Bovendien kon het beschermende effect van de *cxcr3.2*-mutatie teniet worden gedaan door overexpressie van Tfec, wat bevestigt dat het gemis van Cxcr3.2 de functie van lysosomen verandert. Ten slotte hebben wij met microscopische analyse de beweging van lysosomen gevolgd tijdens chemotaxis om het verband tussen de veranderde lysosomale functie en de motiliteit van macrofagen nader te onderzoeken. De observatie dat lysosomen bij de *cxcr3.2*-mutant accumuleren in het voorste gedeelte van migrerende cellen, suggereert dat de afwezigheid van Cxcr3.2 de voor chemotaxis noodzakelijke verplaatsing van lysosomen verstoort, waarbij de efficiëntie van deze cellen om bacteriën af te breken juist toeneemt. Dit hoofdstuk legt daarmee een verband tussen chemotactische signaaltransductie en lysosomale functie in de context van verwonding en infectie. Dit illustreert het complexe netwerk van processen die geïntegreerd moeten worden ten behoeve van celmigratie en cellulaire immuniteit.

Zebravislarven worden in toenemende mate gebruikt als platform voor chemische screenings omdat ze goed doorlaatbaar zijn voor verschillende stoffen, maar ook vanwege hun snelle ontwikkeling, kleine omvang en kosteneffectieve huisvesting. Volwassen zebravissen produceren grote hoeveelheden embryo's, wat een representatieve steekproefomvang en robuuste statistische analyse garandeert. De transparantie van de embryo's en larven maakt het mogelijk om de migratie van fluorescent-gelabelde immuuncellen tijdens een ontstekingsreactie in beeld te brengen. Een bijkomend voordeel van het gebruik van zebravislarven is dat de toxiciteit van chemische stoffen eenvoudig beoordeeld kan worden op basis van overleving, ontwikkeling en morfologische afwijkingen. Eerder werk had laten zien dat een remmer van de humane CXCR3-receptor de macrofaagmigratie reduceert op eenzelfde manier als *cxcr3.2*-mutatie. **Hoofdstuk 4 r**icht zich op een andere sleutelfactor voor macrofaagmigratie, de CCR2-receptor. In dit hoofdstuk hebben wij het potentieel van zebravislarven onderzocht als organismaal model voor de screening van humane CCR2-remmers. CCR2 is betrokken bij verschillende ontstekingsziekten en is daarom een belangrijk doelwit voor de ontwikkeling van ontstekingsremmende medicijnen. Er bestaan al wel CCR2-remmers die ontsteking onderdrukken, maar geen van deze stoffen heeft de klinische testfase bereikt. Ons onderzoek laat zien dat humane chemokinen in staat zijn om macrofaagmigratie op te wekken na injectie in zebravislarven, wat aantoont dat er een grote mate van evolutionaire conservering is tussen de chemokinesignaalroutes van de mens en de zebravis. Deze macrofaagmigratie kon onderdrukt worden door humane CCR2-remmers behorende tot zowel de orthosterische klasse (remmers die de bindingsplaats van chemokinen blokkeren) als de allosterische

klasse (remmers die de functie van de receptor blokkeren via een interactie buiten de bindingsplaats van chemokinen). Deze resultaten dienen als een proof-of-principle voor de screening van CCR-remmers met behulp van het zebravismodel. Op basis van ons onderzoek stellen wij een procedure voor om humane CCR2-remmers te evalueren in zebravislarven, waarbij de accumulatie van macrofagen in ontstoken weefsel wordt gekwantificeerd en tegelijkertijd de toxiciteit beoordeeld om zodoende beter klinisch toepasbare stoffen te identificeren met een hoge werkzaamheid en een lage toxiciteit. Deze toepassing van het zebravismodel bij het testen van CCR2-remmers zou de ontwikkeling van ontstekingsremmende medicijnen kunnen versnellen.

Hoofdstuk 5 bediscussieert de resultaten van de voorgaande hoofdstukken en plaatst het onderzoek naar chemokinesignaalroutes met het zebravismodel in een breder perspectief. We onderstrepen het het belang van het beter karakteriseren en begrijpen van de regulerende mechanismen van het chemokinesysteem. Dit vraagt om een betere classificering van atypische chemokinereceptoren en hun functies. Onze resultaten over het verband tussen chemotactische signaaltransductie en de functie van lysosomen tijdens infectie en verwonding plaatsen wij in de context van recente ontwikkelingen in gerelateerde onderzoeksvelden en wij bespreken hoe verdere vooruitgang geboekt kan worden. Ten slotte presenteren wij in dit hoofdstuk preliminaire data van in vitro-assays die zouden kunnen worden verfijnd en toegepast om een uitgebreid screeningsplatform voor remmers van chemokinereceptoren te ontwikkelen. De combinatie van zebravis- en celcultuursystemen in een dergelijk screeningsplatform zou robuuste, snelle en gedetailleerde werkzaamheids- en toxiciteitsstudies mogelijk maken.

Concluderend, dit proefschrift onderstreept het belang van chemokinesignaalroutes bij de immuunrespons en hun verband met andere cellulaire processen naast chemotaxis. Gebruikmakend van genduplicaties die zijn opgetreden tijdens de evolutie van zebravissen, konden wij aantonen dat twee kopieën van het *cxcr3*-gen een antagonistische werking hebben. Hiermee is een regulatiemechanisme ontstaan waarmee de aantrekking van macrofagen naar ontstoken weefsel nauwkeurig kan worden afgestemd. Uit microscopische analyse van de migratie van zebravismacrofagen bleek dat een mutatie in de functionele homoloog van het humane CXCR3-gen niet alleen de celmigratie verstoort, maar ook de eigenschappen, aantallen en cellulaire locaties van lysosomen verandert. Dit nieuwe verband tussen CXCR3-gestuurde celmigratie en lysosomale functie is gekoppeld aan een verhoogde afbraakcapaciteit voor intracellulaire bacteriën. Ten slotte is het zebravismodel bruikbaar gebleken als een robuust en snel screeningsplatform voor remmers van chemokinereceptoren om tegelijkertijd de werkzaamheid en toxiciteit te beoordelen en daarmee de ontwikkeling van ontstekingsremmers efficiënter te maken.

List of Abbreviations

AB/TL: AB/Tupfel long fin
ACKR: atypical chemokine receptor
Cas9: CRISPR associated protein 9
CFU: colony forming units
CI: circularity index
CRISPR: Clustered regularly interspaced short palindromic repeats
DMSO: Dimethyl sulfoxide
dpf: days post fertilization
dpi: days post infection
EC: extracellular
eGFP: enhanced Green fluorescent protein
FACS: Fluorescence-activated cell sorting
GDP/GTP: Guanosine 5'- diphosphate/triphosphate
GEF: Guanine nucleotide exchange factor
GPCR: G-protein coupled receptor
Hb: hindbrain
hpa: hours post amputation
IC: intracellular
Mpeg1: macrophage expressed gene 1
Mpx: Myeloperoxidase
Mm/tb: *Mycobacterium marinum/ tuberculosis*
Mydd88: Myeloid differentiation primary response 88
PLC: Phospholipase
Ppiab: peptidylprolyl isomerase Ab
PTU: Phenylthiourea
qPCR: quantitative PCR
TFEB/C/E3: Transcription factor BC/EC/E3
Tg: transgenic
TM: transmembrane
WT: wild-type

Curriculum vitae

Frida Sommer was born in Mexico City, Mexico on May 13, 1987. After graduating from high school at the Alexander von Humboldt School, she started a Bachelor's program in general Biology at the Science Faculty of the National Autonomous University of Mexico (UNAM). She graduated after a two-year internship focused on HIV molecular epidemiology and HLA-associated HIV evolution in the Mesoamerican Region at the Infectious Diseases Research Centre (CIENI) of the National Respiratory Diseases Institute (INER) under the supervision of Dr. Santiago Avila Rios in Mexico City. After completion of her BSc degree, Frida was awarded a personal grant from the National Council of Science and Technology (CONACyT) to pursue a Master's degree. She was admitted to the MSc in Archaeological Sciences program at Durham University, UK. Her graduation project focused on rebuilding the phylogeny of domestic animals in Pakistan to infer human activity in the past and was supervised by Prof. Greger Larson. In 2015 she was awarded another CONACyT personal grant to join Prof.dr. Annemarie H. Meijer's lab at Leiden University to do a Ph.D. The work done during her time at Leiden University is presented in this thesis.

List of Publications

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