



Universiteit
Leiden
The Netherlands

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

Sommer, F.

Citation

Sommer, F. (2020, October 15). *Chemokine signaling mechanisms underlying inflammation and infection control: Insights from the zebrafish model*. Retrieved from <https://hdl.handle.net/1887/137821>

Version: Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/137821>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/137821> holds various files of this Leiden University dissertation.

Author: Sommer, F.

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

Issue Date: 2020-10-15

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 G α subunit is bound to GDP (guanosine diphosphate). Ligand binding triggers the canonical G protein-dependent signaling pathway in which

the G α subunit exchanges the GDP molecule for GTP (guanosine triphosphate), the GTP-G α subunit complex uncouples from the receptor and the G β - 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 (*Cxcl11*aa, 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 Cxcr3.3 have opposite effects on macrophage behavior during wound-induced inflammation and infection, strengthening the hypothesis that Cxcr3.3 is an ACKR.

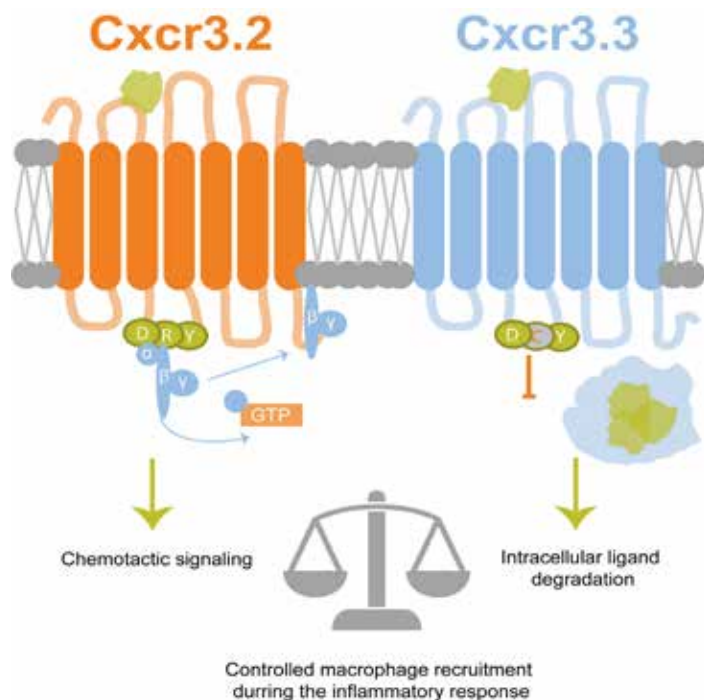


Figure 1. Antagonistic interaction between Cxcr3.2 and Cxcr3.3 regulates macrophage recruitment during the inflammatory response. After binding to its cognate ligand, Cxcr3.2 (orange) signals in the canonical G protein-dependent manner to mediate macrophage recruitment to inflammatory sites. Cxcr3.3 (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 back that leads to the polarized phenotype of migrating macrophages during chemotaxis (**Figure 2**).

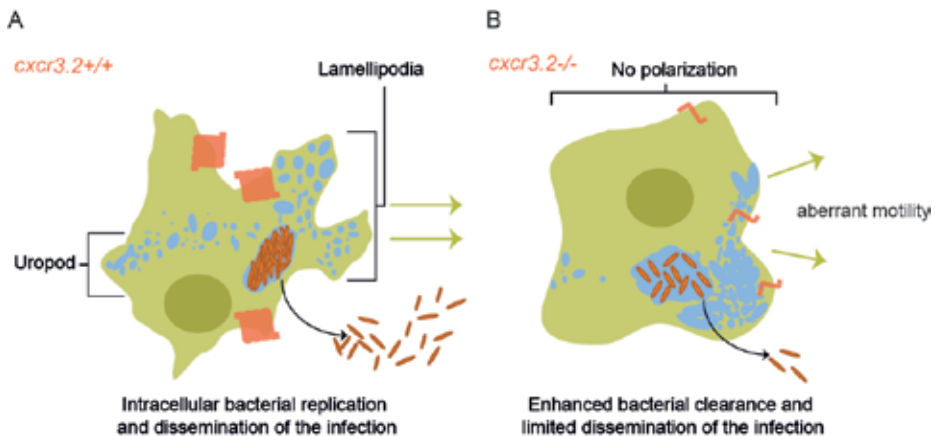


Figure 2. Disruption of *Cxcr3.2* signaling reduces macrophage motility and affects lysosomal function and intracellular vesicle trafficking, rendering cells more microbicidal. Chemotactic signaling contributes to cell polarization during chemotaxis by sustaining the cycling of lysosomal contents from 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 C (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 Ca^{2+} -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 *cxc3.2* and *cxc3.3* 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.

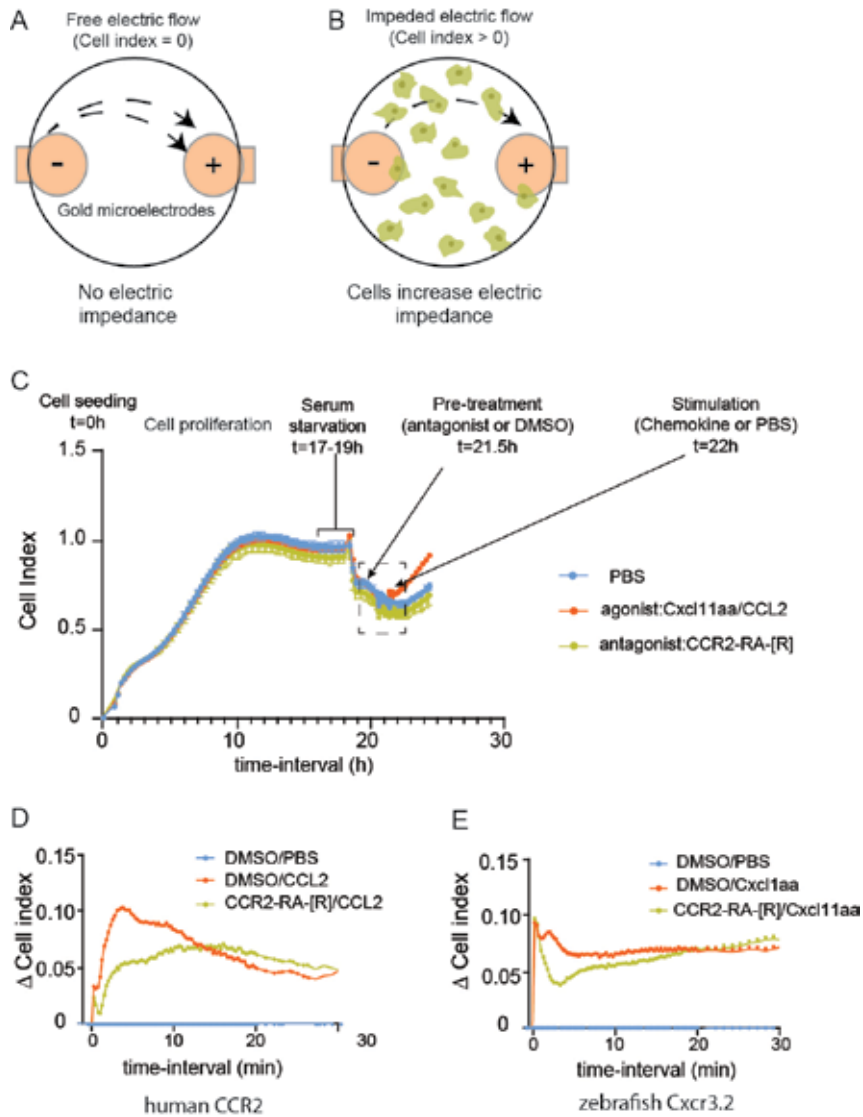


Figure 3. The xCELLigence Real-Time Cell Analysis (RTCA) assay principle and set-up. The gold microelectrodes attached to the bottom of each well of the plate generate an electric flow through the administration of a small voltage and its interaction with the salt-containing culture medium. The system 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 37°C + 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 Cxcl1 Iaa (**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 Cxcl1 Iaa 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.

References

- [1] E. E. Patton and D. M. Tobin, Spotlight on zebrafish: the next wave of translational research, The Company of Biologists Ltd, 2019.
- [2] K. Howe, M. D. Clark, C. F. Torroja, J. Torrance, C. Berthelot, M. Muffato, J. E. Collins, S. Humphray, K. McLaren, L. Matthews and others, "The zebrafish reference genome sequence and its relationship to the human genome," *Nature*, vol. 496, pp. 498-503, 2013.
- [3] J. Bussmann and E. Raz, "Chemokine-guided cell migration and motility in zebrafish development," *The EMBO journal*, vol. 34, pp. 1309-1318, 2015.
- [4] E. Raz and H. Mahabaleshwar, "Chemokine signaling in embryonic cell migration: a fisheye view," *Development*, vol. 136, pp. 1223-1229, 2009.
- [5] S. Bird and C. Tafalla, "Teleost chemokines and their receptors," *Biology*, vol. 4, pp. 756-784, 2015.
- [6] H. Nomiyama, N. Osada and O. Yoshie, "Systematic classification of vertebrate chemokines based on conserved synteny and evolutionary history," *Genes to Cells*, vol. 18, pp. 1-16, 2013.
- [7] B. Boldajipour, M. Doitsidou, K. Tarbashevich, C. Laguri, S. R. Yu, J. Ries, K. Dumstrei, S. Thelen, J. Dörries, E.-M. Messerschmidt and others, "Cxcl12 evolution--subfunctionalization of a ligand through altered interaction with the chemokine receptor," *Development*, vol. 138, pp. 2909-2914, 2011.
- [8] F. Sommer, V. Torraca, S. M. Kamel, A. Lombardi and A. H. Meijer, "Frontline Science: Antagonism between regular and atypical Cxcr3 receptors regulates macrophage migration during infection and injury in zebrafish," *Journal of leukocyte biology*, vol. 107, pp. 185-203, 2020.
- [9] D. Malhotra, J. Shin, L. Solnica-Krezel and E. Raz, "Spatio-temporal regulation of concurrent developmental processes by generic signaling downstream of chemokine receptors," *eLife*, vol. 7, p. e33574, 2018.
- [10] A. Marchese, "Endocytic trafficking of chemokine receptors," *Current opinion in cell biology*, vol. 27, pp. 72-77, 2014.
- [11] L. Ramakrishnan, "Revisiting the role of the granuloma in tuberculosis," *Nature Reviews Immunology*, vol. 12, pp. 352-366, 2012.
- [12] E. Donà, J. D. Barry, G. Valentin, C. Quirin, A. Khmelinskii, A. Kunze, S. Durdu, L. R. Newton, A. Fernandez-Minan, W. Huber and others, "Directional tissue migration through a self-generated chemokine gradient," *Nature*, vol. 503, pp. 285-289, 2013.
- [13] C. Dambly-Chaudière, N. Cubedo and A. Ghysen, "Control of cell migration in the development of the posterior lateral line: antagonistic interactions between the chemokine receptors CXCR4 and CXCR7/RDC1," *BMC developmental biology*, vol. 7, p. 23, 2007.
- [14] I. F. Charo and R. M. Ransohoff, "The many roles of chemokines and chemo-

kine receptors in inflammation,” *New England Journal of Medicine*, vol. 354, pp. 610-621, 2006.

[15] R. Bonecchi, E. Galliera, E. M. Borroni, M. M. Corsi, M. Locati and A. Mantovani, “Chemokines and chemokine receptors: an overview,” *Front Biosci*, vol. 14, pp. 540-551, 2009.

[16] A. Zlotnik and O. Yoshie, “The chemokine superfamily revisited,” *Immunity*, vol. 36, pp. 705-716, 2012.

[17] B. Boldajipour, H. Mahabaleshwar, E. Kardash, M. Reichman-Fried, H. Blaser, S. Minina, D. Wilson, Q. Xu and E. Raz, “Control of chemokine-guided cell migration by ligand sequestration,” *Cell*, vol. 132, pp. 463-473, 2008.

[18] H. Mahabaleshwar, K. Tarbashevich, M. Nowak, M. Brand and E. Raz, “ β -arrestin control of late endosomal sorting facilitates decoy receptor function and chemokine gradient formation,” *Development*, vol. 139, pp. 2897-2902, 2012.

[19] X.-F. Zhang, J.-F. Wang, E. Matczak, J. Proper and J. E. Groopman, “Janus kinase 2 is involved in stromal cell--derived factor-1 α --induced tyrosine phosphorylation of focal adhesion proteins and migration of hematopoietic progenitor cells,” *Blood, The Journal of the American Society of Hematology*, vol. 97, pp. 3342-3348, 2001.

[20] A. Rot and U. H. Von Andrian, “Chemokines in innate and adaptive host defense: basic chemokine grammar for immune cells,” *Annu. Rev. Immunol.*, vol. 22, pp. 891-928, 2004.

[21] R. Bonecchi and G. J. Graham, “Atypical chemokine receptors and their roles in the resolution of the inflammatory response,” *Frontiers in immunology*, vol. 7, p. 224, 2016.

[22] M. Massara, O. Bonavita, A. Mantovani, M. Locati and R. Bonecchi, “Atypical chemokine receptors in cancer: friends or foes?,” *Journal of leukocyte biology*, vol. 99, pp. 927-933, 2016.

[23] A. Vacchini, M. Locati and E. M. Borroni, “Overview and potential unifying themes of the atypical chemokine receptor family,” *Journal of leukocyte biology*, vol. 99, pp. 883-892, 2016.

[24] C. J. Cambier, K. K. Takaki, R. P. Larson, R. E. Hernandez, D. M. Tobin, K. B. Urdahl, C. L. Cosma and L. Ramakrishnan, “Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids,” *Nature*, vol. 505, pp. 218-222, 2014.

[25] V. Torraca, C. Cui, R. Boland, J.-P. Bebelman, A. M. Sar, M. J. Smit, M. Siderius, H. P. Spaink and A. H. Meijer, “The CXCR3-CXCL11 signaling axis mediates macrophage recruitment and dissemination of mycobacterial infection,” *Disease models & mechanisms*, vol. 8, pp. 253-269, 2015.

[26] R. Lesley and L. Ramakrishnan, “Insights into early mycobacterial pathogenesis from the zebrafish,” *Current opinion in microbiology*, vol. 11, pp. 277-283, 2008.

[27] S. K. Yoo, Q. Deng, P. J. Cavnar, Y. I. Wu, K. M. Hahn and A. Huttenlocher, “Differential regulation of protrusion and polarity by PI (3) K during neutrophil motil-

ity in live zebrafish,” *Developmental cell*, vol. 18, pp. 226-236, 2010.

[28] C. J. Cambier, S. M. O’Leary, M. P. O’Sullivan, J. Keane and L. Ramakrishnan, “Phenolic glycolipid facilitates mycobacterial escape from microbicidal tissue-resident macrophages,” *Immunity*, vol. 47, pp. 552-565, 2017.

[29] R. J. B. Nibbs, D. S. Gilchrist, V. King, A. Ferra, S. Forrow, K. D. Hunter and G. J. Graham, “The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumors,” *The Journal of clinical investigation*, vol. 117, pp. 1884-1892, 2007.

[30] C. Billottet, C. Quemener and A. Bikfalvi, “CXCR3, a double-edged sword in tumor progression and angiogenesis,” *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, vol. 1836, pp. 287-295, 2013.

[31] S. D. Chakravarty, J. Xu, B. Lu, C. Gerard, J. Flynn and J. Chan, “The chemokine receptor CXCR3 attenuates the control of chronic *Mycobacterium tuberculosis* infection in BALB/c mice,” *The Journal of Immunology*, vol. 178, pp. 1723-1735, 2007.

[32] X.-J. Lu, Q. Chen, Y.-J. Rong, F. Chen and J. Chen, “CXCR3. 1 and CXCR3. 2 differentially contribute to macrophage polarization in teleost fish,” *The Journal of Immunology*, vol. 198, pp. 4692-4706, 2017.

[33] M. Nguyen-Chi, B. Laplace-Builhe, J. Travnickova, P. Luz-Crawford, G. Tejedor, Q. T. Phan, I. Duroux-Richard, J.-P. Levraud, K. Kissa, G. Lutfalla and others, “Identification of polarized macrophage subsets in zebrafish,” *Elife*, vol. 4, p. e07288, 2015.

[34] R. A. Bond and A. P. IJzerman, “Recent developments in constitutive receptor activity and inverse agonism, and their potential for GPCR drug discovery,” *Trends in pharmacological sciences*, vol. 27, pp. 92-96, 2006.

[35] R. A. Colvin, T. K. Means, T. J. Diefenbach, L. F. Moita, R. P. Friday, S. Sever, G. S. V. Campanella, T. Abraszinski, L. A. Manice, C. Moita and others, “Synaptotagmin-mediated vesicle fusion regulates cell migration,” *Nature immunology*, vol. 11, pp. 495-502, 2010.

[36] M. Bretou, P. J. Sáez, D. Sanséau, M. Maurin, D. Lankar, M. Chabaud, C. Spanpanato, O. Malbec, L. Barbier, S. Muallem and others, “Lysosome signaling controls the migration of dendritic cells,” *Science Immunology*, vol. 2, 2017.

[37] A. Sumoza-Toledo, I. Lange, H. Cortado, H. Bhagat, Y. Mori, A. Fleig, R. Penner and S. Partida-Sánchez, “Dendritic cell maturation and chemotaxis is regulated by TRPM2-mediated lysosomal Ca²⁺ release,” *The FASEB Journal*, vol. 25, pp. 3529-3542, 2011.

[38] T. L. Bach, Q.-M. Chen, W. T. Kerr, Y. Wang, L. Lian, J. K. Choi, D. Wu, M. G. Kazanietz, G. A. Koretzky, S. Zigmond and others, “Phospholipase C β is critical for T cell chemotaxis,” *The Journal of Immunology*, vol. 179, pp. 2223-2227, 2007.

[39] C. Wei, X. Wang, M. Chen, K. Ouyang, L.-S. Song and H. Cheng, “Calcium flickers steer cell migration,” *Nature*, vol. 457, pp. 901-905, 2009.

[40] R. Ashworth and C. Brennan, “Use of transgenic zebrafish reporter lines to

study calcium signalling in development,” *Briefings in Functional Genomics*, vol. 4, pp. 186-193, 2005.

[41] J. Chen, L. Xia, M. R. Bruchas and L. Solnica-Krezel, “Imaging early embryonic calcium activity with GCaMP6s transgenic zebrafish,” *Developmental biology*, vol. 430, pp. 385-396, 2017.

[42] M. Gao and C. A. Kaiser, “A conserved GTPase-containing complex is required for intracellular sorting of the general amino-acid permease in yeast,” *Nature cell biology*, vol. 8, pp. 657-667, 2006.

[43] M. A. Lawson and F. R. Maxfield, “Ca²⁺-and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils,” *Nature*, vol. 377, pp. 75-79, 1995.

[44] Y. Dou, H.-j. Wu, H.-q. Li, S. Qin, Y.-e. Wang, J. Li, H.-f. Lou, Z. Chen, X.-m. Li, Q.-m. Luo and others, “Microglial migration mediated by ATP-induced ATP release from lysosomes,” *Cell research*, vol. 22, pp. 1022-1033, 2012.

[45] N. Pastore, O. A. Brady, H. I. Diab, J. A. Martina, L. Sun, T. Huynh, J.-A. Lim, H. Zare, N. Raben, A. Ballabio and others, “TFEB and TFE3 cooperate in the regulation of the innate immune response in activated macrophages,” *Autophagy*, vol. 12, pp. 1240-1258, 2016.

[46] K. Shen, H. Sidik and W. S. Talbot, “The Rag-Ragulator complex regulates lysosome function and phagocytic flux in microglia,” *Cell reports*, vol. 14, pp. 547-559, 2016.

[47] L. El-Houjeiri, E. Possik, T. Vijayaraghavan, M. Paquette, J. A. Martina, J. M. Kazan, E. H. Ma, R. Jones, P. Blanchette, R. Puertollano and others, “The transcription factors TFEB and TFE3 link the FLCN-AMPK signaling axis to innate immune response and pathogen resistance,” *Cell reports*, vol. 26, pp. 3613-3628, 2019.

[48] J. D. Schilling, H. M. Machkovech, L. He, A. Diwan and J. E. Schaffer, “TLR4 activation under lipotoxic conditions leads to synergistic macrophage cell death through a TRIF-dependent pathway,” *The Journal of Immunology*, vol. 190, pp. 1285-1296, 2013.

[49] J. R. Mathias, M. T. Saxena and J. S. Mumm, “Advances in zebrafish chemical screening technologies,” *Future medicinal chemistry*, vol. 4, pp. 1811-1822, 2012.

[50] J. L. Tan and L. I. Zon, “Chemical screening in zebrafish for novel biological and therapeutic discovery,” in *Methods in cell biology*, vol. 105, Elsevier, 2011, pp. 491-516.

[51] N. Tanuma, H. Sakuma, A. Sasaki and Y. Matsumoto, “Chemokine expression by astrocytes plays a role in microglia/macrophage activation and subsequent neurodegeneration in secondary progressive multiple sclerosis,” *Acta neuropathologica*, vol. 112, pp. 195-204, 2006.

[52] N. R. Veillard, S. Steffens, G. Pelli, B. Lu, B. R. Kwak, C. Gerard, I. F. Charo and F. Mach, “Differential influence of chemokine receptors CCR2 and CXCR3 in development of atherosclerosis in vivo,” *Circulation*, vol. 112, pp. 870-878, 2005.

[53] H. Tokuyama, S. Ueha, M. Kurachi, K. Matsushima, F. Moriyasu, R. S. Blum-

berg and K. Kakimi, "The simultaneous blockade of chemokine receptors CCR2, CCR5 and CXCR3 by a non-peptide chemokine receptor antagonist protects mice from dextran sodium sulfate-mediated colitis," *International immunology*, vol. 17, pp. 1023-1034, 2005.

[54] J. M. Hillger, J. Schoop, D. I. Boomsma, P. E. Slagboom, A. P. IJzerman and L. H. Heitman, "Whole-cell biosensor for label-free detection of GPCR-mediated drug responses in personal cell lines," *Biosensors and Bioelectronics*, vol. 74, pp. 233-242, 2015.

[55] O. Boussif, F. Lezoualc'h, M. A. R. L. A. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix and J.-P. Behr, "A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine," *Proceedings of the National Academy of Sciences*, vol. 92, pp. 7297-7301, 1995.

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 *cxc3.2* 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 *cxc3* 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.