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Chemokine signaling in innate immunity of zebrafish embryos

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Chapter 5

General discussion

The zebrafish embryo model has emerged as an ideal platform to study the function of the innate immune system in inflammation and infectious diseases (Renshaw and Trede, 2012; Meijer and Spaink, 2011). The clear temporal separation of development of the innate and adaptive immune system in zebrafish makes the embryo model a convenient system to study the contribution of innate immune cells to host-pathogen interactions. In recent years, the zebrafish has been employed to model diseases caused by a variety of human pathogens or closely related fish pathogens (Phelps and Neely, 2005; Meeker and Trede, 2008; Meijer and Spaink, 2011; Ramakrishnan, 2012). In this thesis, we have used *Salmonella typhimurium* and *Mycobacterium marinum* infection models to study CXC chemokine signaling, particularly focusing on the identification of bacterial-induced chemokines and the functional analysis of a chemokine receptor gene *cxcr3.2*. Here in this chapter, we summarize the main results and insights that we obtained from the experiments described in the previous chapters. In addition, we used recent data from RNA deep sequencing analysis of leukocyte populations of zebrafish embryos to provide a broader overview of chemokine and chemokine receptor gene expression in the immune system of zebrafish.

Expression of CXCR3 family members in zebrafish leukocytes

In chapter 2, we identified a leukocyte-specific CXC chemokine receptor gene *cxcr3.2* by a double microarray strategy. Our approach was to look for genes that were down-regulated in zebrafish embryos upon knockdown of the hematopoietic transcription factor Pu.1/Spi1, and that simultaneously showed enriched expression in GFP-positive myeloid cells obtained by fluorescence-activated cell sorting (FACS) from *spi1:egfp* transgenic embryos. Double fluorescent *in situ* hybridization studies with known markers for distinguishing myeloid cell types showed that *cxcr3.2* is expressed specifically in macrophages in early zebrafish embryos (1-2 dpf). Since *cxcr3.2* expression was below the detection limit of *in situ* hybridization after 2 dpf, other experimental approaches were required to gain insight in the cell type specificity of *cxcr3.2* at later stages. Transient expression of a fluorescent reporter gene driven by the *cxcr3.2* promoter region revealed that by 3dpf *cxcr3.2* expression labeled a population of neutrophils in addition to macrophages (Chapter 4). We then investigated the expression of *cxcr3.2* as well as other zebrafish CXC chemokine receptor genes by RNA sequencing (RNA-seq) analysis of macrophage, neutrophil, and early T-cell populations obtained by FACS sorting of 5-6 days old larvae from different transgenic reporter lines (Chapter 4). These included a novel macrophage marker line, *mpeg1:egfp* (Ellett *et al.*, 2010) that became available during the course of this work, and *mpx:egfp* and *lck:egfp* lines for neutrophils and T-cells, respectively. RNA-seq analysis of GFP-positive cell fractions from these lines confirmed that *cxcr3.2* is predominantly expressed in zebrafish embryonic macrophages, but is also expressed in neutrophils at later stages of development. Expression of *cxcr3.1*, the second homolog of human CXCR3 in zebrafish, was

more than 20-fold lower in *mpeg1:egfp*-positive macrophages compared with *cxcr3.2* expression, and not detectable in *mpx:egfp*-positive neutrophils. In contrast, *cxcr3.1* expression was detectable in *lck:egfp*-positive cells, while expression of *cxcr3.2* was not. These data suggest that *CXCR3* functions in myeloid and lymphoid cells may have been divided over the two duplicated genes, *cxcr3.1* and *cxcr3.2* in zebrafish.

Table 1. Chemokine receptor gene expression levels in RNA-seq data of FACS-sorted macrophages, neutrophils, and immature T-cells¹

Gene group	Gene symbol	Ensembl ID	<i>mpeg1</i> ⁺	<i>mpx</i> ⁺	<i>lck</i> ⁺	GFP ⁻
cell lineage markers	<i>mpeg1</i>	ENSDARG0000005290	126	12	0	5
	<i>mpx</i>	ENSDARG00000019521	37	662	0	2
	<i>lck</i>	ENSDARG00000059282	1	0	237	3
CXC motif chemokine receptors	<i>cxcr1</i>	ENSDARG00000054975	0	0	0	0
	<i>cxcr3.1</i>	ENSDARG00000007358	3	0	7	1
	<i>cxcr3.2</i>	ENSDARG00000041041	62	26	0	3
	<i>cxcr4a</i>	ENSDARG00000057633	6	4	9	1
	<i>cxcr4b</i>	ENSDARG00000041959	889	344	830	74
	<i>cxcr5</i>	ENSDARG00000010514	0	0	0	0
	<i>cxcr7b</i>	ENSDARG00000058179	7	11	7	49
	<i>cxcr7 (2 of 2)</i>	ENSDARG00000062478	1	2	9	5
CC motif chemokine receptors	<i>ccr6a</i>	ENSDARG00000087474	1	0	1	1
	<i>ccr6b</i>	ENSDARG00000038968	0	0	3	0
	<i>ccr7</i>	ENSDARG00000044561	1	0	1	2
	<i>ccr8.1</i>	ENSDARG00000095789	0	0	0	0
	<i>ccr9a</i>	ENSDARG00000055186	633	305	1187	83
	<i>ccr9b</i>	ENSDARG00000068310	0	0	664	5
	<i>ccr9 (2 of 3)</i>	ENSDARG00000053943	0	0	0	0
	<i>ccr10</i>	ENSDARG00000040643	9	7	9	10
	<i>ccr12.2</i>	ENSDARG00000026417	189	83	0	2
	<i>ccr12.3</i>	ENSDARG00000038541	6	4	0	2
C motif chemokine receptors	<i>xcr1a</i>	ENSDARG00000054847	4	0	0	0
	<i>xcr1 (1 of 4)</i>	ENSDARG00000052988	0	0	0	0
	<i>xcr1 (3 of 4)</i>	ENSDARG00000054846	0	0	0	0
	<i>xcr1 (4 of 4)</i>	ENSDARG00000087978	1	0	0	0

chemokine-like receptors	<i>ccl1a</i>	ENSDARG00000078729	0	0	4	1
	<i>ccl1b</i>	ENSDARG00000040133	0	1	1	2
	<i>cmklr1</i>	ENSDARG00000090890	327	74	1	7
other G-protein coupled receptors	<i>gpr34b</i>	ENSDARG00000002959	0	0	18	1
	<i>gpr55</i>	ENSDARG00000043447	1	7	63	2
	<i>gpr56</i>	ENSDARG00000027222	31	26	85	53
	<i>gpr82</i>	ENSDARG00000093418	0	0	11	1
	<i>gpr84</i>	ENSDARG00000077308	122	341	2	6
	<i>gpr89B</i>	ENSDARG00000077983	3	2	7	2
	<i>gpr97</i>	ENSDARG00000074782	435	337	18	11
	<i>gpr132 (1 of 2)</i>	ENSDARG00000057195	54	9	4	3
	<i>gpr141 (1 of 2)</i>	ENSDARG00000057619	40	23	2	2
	<i>gpr174</i>	ENSDARG00000079826	9	12	2	1
	<i>gpr183</i>	ENSDARG00000010317	32	78	60	13
	<i>gpr183 (2 of 3)</i>	ENSDARG00000074302	16	93	60	3
	<i>gpr183 (3 of 3)</i>	ENSDARG00000069756	1	5	2	0

¹ RNA-seq libraries were constructed by Illumina sequencing of RNA from GFP-positive (+) and negative (-) cell fractions obtained by dissociating and FACS-sorting 5-6 dpf larvae of transgenic lines expressing enhanced GFP under control of the *mpeg1*, *mpx*, or *lck* promoters (Kanwal, 2012). RPKM values (read counts per kilobase per million mapped reads) are averaged from 2-4 biological replicates of GFP-positive cells per line. The RPKM value for GFP-negative cells is the average of all libraries. RPKM values of different classes of chemokine receptor genes are shown in comparison to those of *mpeg1*, *mpx*, and *lck*, as markers for macrophages, neutrophils, and immature T-cells, respectively. The table also includes other G-protein coupled receptor genes that showed significantly enriched expression in one or more of the GFP⁺ cell fractions with an RPKM of at least 5. Enriched expression in macrophages, neutrophils, or T-lymphoblasts is indicated in orange, green, and blue, respectively. Darker shades of color mark RPKM values above 50 and lighter shades of color mark RPKM values in the range of 5-50.

Expression patterns of other CXCR family members

Homologs of other human CXCR receptors have also been identified in zebrafish, with the exception of CXCR6. CXCR1 and CXCR2 are responsible for chemotactic responses of neutrophils based on *in vivo* studies in mice and *in vitro* studies using human cells. The chemotactic activity of neutrophils could be blocked by antibodies to CXCR1 and CXCR2 *in vivo* and *in vitro*, and neutrophils failed to accumulate in *Cxcr2*^{-/-} mutant mice after Prolyl-Glycyl-Proline (PGP) peptide challenge (Weathington *et al.*, 2006). *Cxcr2* was dramatically down-regulated in neutrophils of wild type mice with severe sepsis, which correlated with reduced chemotaxis to

Cxcl2 *in vitro* (Alves-Filho *et al.*, 2009). Apart from CXCL2, CXCR2 also interacts with IL8 (CXCL8), which also is a ligand for CXCR1. For example, IL8 promoted killing of *Pseudomonas aeruginosa* through CXCR1 (Hartl *et al.*, 2007). In zebrafish, the genes for *il8*, *cxcr1* and *cxcr2* are conserved with the human counterparts (Oehlers *et al.*, 2010), but three other genes closely related to *il8* are also present (van der Aa *et al.*, 2012; Chapter 3). Expression of *cxcr1*, *cxcr2* and *il8* in gut epithelial cells and induction of *il8* expression by inflammatory insults suggested that *il8* signaling plays a role during gut inflammation (Oehlers *et al.*, 2010). We have also observed strong induction of *il8* during bacterial infections (Stockhammer *et al.*, 2009; Chapter 3). Furthermore, we have shown that purified zebrafish IL8 protein injected into the otic vesicle of zebrafish larvae has chemoattractant properties on neutrophils (Chapter 3). The expression of *cxcr1* was not detectable in our RNA-seq analysis of macrophages, neutrophils, and T-cell fractions of zebrafish larvae (Table 1). *Cxcr2* is therefore the likely candidate that mediates neutrophil chemotaxis toward IL8 in the otic vesicle assay. However, expression of *cxcr2* on neutrophils could not yet be confirmed because the gene is not annotated in Ensembl Zv9 and therefore RNA-seq read counts mapping to this gene were missed in our initial data analysis.

CXCR4/CXCR7 signaling is involved in many immunologically important processes. CXCR4 was shown to function as a cofactor for entry of human immunodeficiency virus (HIV)-1 into CD4-expressing T-cells (Feng *et al.*, 1996). SDF1 (CXCL12), the ligand of CXCR4, is a potent inhibitor of infection by lymphocyte-tropic HIV-1 strains (Bleul *et al.*, 1996; Oberlin *et al.*, 1996). As a co-receptor for T-cell-tropic viruses, the function of CXCR4 is complementary to that of CCR5, the major macrophage-tropic co-receptor for HIV-1 (Xiao *et al.*, 2000; Yeaman *et al.*, 2004). CXCR4 has been shown to activate a cluster of monocytes after lipopolysaccharide (LPS) ligation and is involved in LPS signal transduction (Triantafyllou *et al.*, 2001). It has also been demonstrated that the CXCR4/SDF1 pathway regulates trafficking of hematopoietic progenitor cells (HPCs) in the bone marrow (Levesque *et al.*, 2003). CXCR7 has been identified as an alternative receptor for SDF1, and was shown to act cooperatively with CXCR4 in mediating tumor metastasis in several types of cancer (Burns *et al.*, 2006; Sun *et al.*, 2010). *CXCR4*, *CXCR7*, and *SDF1* all have been duplicated in zebrafish. Most studies in the zebrafish model have focused on the *Cxcr4b/Cxcr7b-Sdf1a* (*Cxcl12a*) axis. This chemokine signaling pathway plays critical roles in axon guidance, germ cell migration, and neuromast primordium migration (Miyasaka *et al.*, 2007; Dumstrei *et al.*, 2004; Knaut and Schier, 2008; Boldajipour *et al.*, 2008; Mahabaleshwar *et al.*, 2008; Haas and Gilmour, 2006; Dambly-Chaudière *et al.*, 2007; Valentin *et al.*, 2007). Its involvement in tolerance to LPS-induced inflammation has also been proposed (Novoa *et al.*, 2009). Furthermore, recent live imaging studies in zebrafish showed a role for the *Cxcr4b-Sdf1a* axis in regulation of neutrophil motility (Walters *et al.*, 2010; Deng *et al.*, 2011). By RNA-seq analysis, we detected high levels of

expression of *cxcr4b* in macrophages, neutrophils, and T-cells, as well as in the GFP-negative background of the transgenic lines (Table 1), which is consistent with its broad functions in multi-tissue involved biological processes. Expression of *cxcr4a*, the second *CXCR4* homolog in zebrafish, was very low as compared to that of *cxcr4b*, but also detectable in all leukocyte cell fractions. Expression of *cxcr7b* in leukocytes was in a similar range as that of *cxcr4a*, but *cxcr7b* showed higher expression in other tissues. The second homolog of *CXCR7* (annotated in Ensembl as *cxcr7 (2 of 2)*) was also expressed at a low level, but most enriched in T-cells.

In human, *CXCR5*, also known as Burkitt lymphoma receptor 1 (*BLR1*) is expressed in B-lymphocytes and functions as a receptor for *CXCL13*. *CXCR5* was shown to be involved in B-cell movements between different zones of secondary lymphoid organs (Reif *et al.*, 2002). Altered expression of *CXCR5* and *CXCL13* may be involved in B-cell dysfunction during HIV-1 infection, since *CXCL13*-positive B cells were present in lymph nodes of HIV-1-seropositive patients, but not in control tissue (Cagigi *et al.*, 2008). Expression of zebrafish *cxcr5* was not observed in our RNA-seq data of zebrafish larval leukocyte populations. Recently, it was shown that *cxcr5* is expressed in the brain of adult zebrafish (Kizil *et al.*, 2012). Following injury of the telencephalon, expression of *cxcr5* was transiently increased in radial glial cells and neurons, while no overlap was observed with L-plastin-positive leukocytes. Overexpression and knockdown of *cxcr5* affected proliferation of the radial glial cells, indicating a function of this gene in regenerative neurogenesis (Kizil *et al.*, 2012).

Expression and function of CXC chemokine ligands

We also investigated the expression of CXC chemokine genes in RNA-seq data from larval leukocyte populations. Out of 17 identified chemokines (Chapter 3), only 10 genes were annotated in the Ensembl Zv9 database. Expression levels of *cxcl11* (two near-identical genes encoding the same protein) and *cxcl-c5g* were on average more than 3-fold higher in macrophages compared with neutrophils (Table 2), while their expression was not clearly detectable in early T-cells. The *SDF1/CXCL12* homologs *cxcl12a* and *cxcl12b* were detectable in different populations of leukocytes, but showed higher expression in cells from the background tissues, consistent with previous reports in functions of *cxcl12* signalling in many biological processes. As compared to *cxcl12a* and *cxcl12b*, the expression levels of *cxcl-c1c* and *cxcl14* were much higher in all the cell populations. Furthermore, *cxcl-c5c* showed relatively high expression in neutrophils, whereas *cxcl-c24e* was clearly enriched in T-cells. In chapter 3, we used qPCR analysis to investigate the expression of all 17 reported *cxcl* chemokine genes during *S. typhimurium* and *M. marinum* infections. These studies showed that many of the *cxcl* chemokine genes are infection inducible. In particular, *il8*, *cxcl-c1c*,

and *cxcl11-like* (*cxcl11*) were inducible at multiple stages of *S. typhimurium* and *M. marinum* infections, and additionally *cxcl11* was inducible at the later stage of *M. marinum* infection. While purified I18 protein had a chemoattractant function on neutrophils, we found that Cxcl11 protein attracted macrophages in zebrafish embryos.

Table 2. CXC chemokine gene expression levels in RNA-seq data of FACS-sorted macrophages, neutrophils, and immature T-cells¹

Gene group	Gene symbol	Ensembl ID	mpeg1 ⁺	mpx ⁺	lck ⁺	GFP ⁻
cell lineage markers	<i>mpeg1</i>	ENSDARG00000055290	126	12	0	5
	<i>mpx</i>	ENSDARG00000019521	37	662	0	2
	<i>lck</i>	ENSDARG00000059282	1	0	237	3
CXC motif chemokines	<i>cxcl11</i> (1 of 4)	ENSDARG00000093779	6	2	1	0
	<i>cxcl11</i> (2 of 4)	ENSDARG00000094706	9	3	0	1
	<i>cxcl-c5g</i>	ENSDARG00000092423	3	0	0	0
	<i>cxcl12a</i>	ENSDARG00000037116	4	16	7	76
	<i>cxcl12b</i>	ENSDARG00000055100	1	5	3	31
	<i>cxcl14</i>	ENSDARG00000056627	37	79	29	114
	<i>cxcl-c1c</i>	ENSDARG00000075045	42	75	244	356
	<i>cxcl-c5c</i>	ENSDARG00000075163	1	5	0	1
	<i>cxcl-c24e</i>	ENSDARG00000071499	0	0	16	3
	<i>cxcl-c64</i>	ENSDARG00000095747	0	0	0	0

¹RNA-seq libraries were constructed by Illumina sequencing of RNA from GFP-positive (+) and negative (-) cell fractions obtained by dissociating and FACS-sorting 5-6 dpf larvae of transgenic lines expressing enhanced GFP under control of the *mpeg1*, *mpx*, or *lck* promoters (Kanwal, 2012). RPKM values (read counts per kilobase per million mapped reads) are averaged from 2-4 biological replicates of GFP-positive cells per line. The RPKM value for GFP-negative cells is the average of all libraries. RPKM values of different CXC chemokine receptor genes are shown in comparison to those of *mpeg1*, *mpx*, and *lck*, as markers for macrophages, neutrophils, and immature T-cells, respectively. Enriched expression in macrophages, neutrophils, or T-lymphoblasts is indicated in orange, green, and blue, respectively. Darker shades of color mark RPKM values above 50 and lighter shades of color mark RPKM values in the range of 5-50.

CCR and other chemokine receptor families

In humans, in addition to CXC chemokine pathways, CCR and other chemokine receptors are also playing critical roles in many immune related responses. Similar as for *cxcr3.2*, we investigated which members of the zebrafish *CCR* receptor gene

family are dependent on the hematopoietic transcription factor Pu.1/Spi1 in zebrafish embryos. Among 10 known CCR family members (Table 1), only *ccr12.3* showed clear down-regulation at different time points upon Pu.1/Spi1 morpholino knockdown by qRT-PCR (Fig 1A). We then analyzed the spatial expression of *ccr12.3* by whole-mount *in situ* hybridization and found it to be restricted to single cells in the hematopoietic region of the tail (Fig 1B). These results strongly indicate that *ccr12.3* is a leukocyte-specific chemokine receptor in zebrafish embryos. By RNA-seq analysis, it was confirmed that expression of *ccr12.3* is enriched in macrophage and neutrophil populations of zebrafish larvae. Interestingly, expression of a closely related receptor, *ccr12.2*, is also specifically expressed in larval macrophages and neutrophils, at 30-fold and 20-fold higher levels, respectively, than *ccr12.3*. The zebrafish *ccr12.2* and *ccr12.3* receptors are most closely related to human CCR2 and CCR5, which have important functions in monocyte and macrophage migration and have also been linked to the pathogenesis of immunologic diseases (Zhao *et al.*, 2010).

Among the other CCR receptors of zebrafish, only the expression of *ccr10*, *ccr9a*, *ccr9b* was detectable in larval leukocyte populations (Table 1). Both *ccr10* and *ccr9a* were expressed in all myeloid and lymphoid cell fractions, but the expression level of *ccr9a* was 40 to 70 fold higher in neutrophils and macrophages, and over a hundred fold higher in T-cells. The other CCR9 homolog, *ccr9b* was also highly expressed in T-cells and appeared to be entirely lymphocyte specific. This observation is in line with the function of human CCR9 in T cell recruitment and development (Mora *et al.*, 2003, Eksteen *et al.*, 2004).

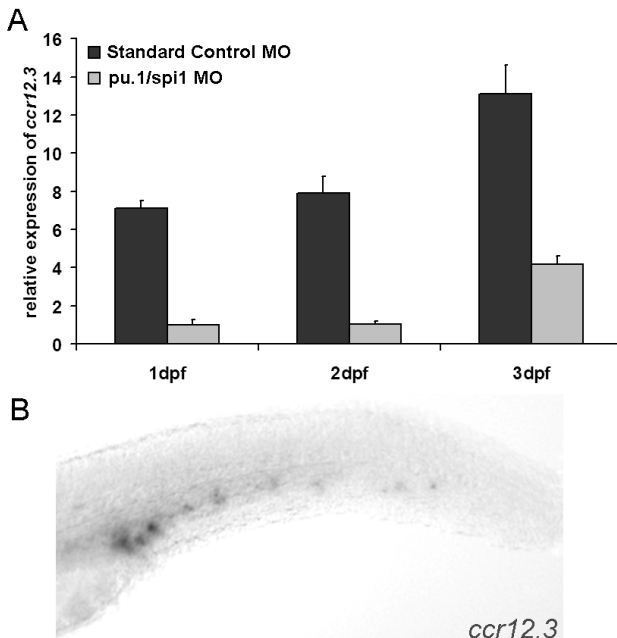


Figure 1. Pu.1/Spi1-dependency and leukocyte specificity of chemokine receptor gene *ccr12.3* (A) qRT-PCR data showing down-regulation of *ccr12.3* in pu.1/spi1 knockdown embryos at 1, 2 and 3 days post fertilization (dpf). (B) Whole mount *in situ* hybridization of a 1 dpf embryo showing expression of *ccr12.3* in the hematopoietic region of the tail, a temporary location of leukocyte differentiation in zebrafish embryos.

Notably, one chemokine-like receptor gene, namely *cmklr1*, showed enriched expression in macrophages and neutrophils. In humans *CMKLR1* is also known as the chemerin receptor (ChemR23), which acts as a chemotactic mediator for leukocyte populations, particularly immature plasmacytoid dendritic cells, but also immature myeloid dendritic cells, macrophages and natural killer cells (Bondue *et al.*, 2011). Given the high expression of *cmklr1* in zebrafish embryonic myeloid cells it would be interesting to further address the inflammatory roles of chemerin signaling in leukocyte migration in zebrafish.

Overall, our RNA-seq data have provided a clear view on the primary candidate genes for functional studies of the role of chemokine receptors in coordinating immune cell responses in zebrafish embryo and larval models. The efficient tools for reverse genetics in zebrafish will be instrumental in establishing clear roles for individual chemokine signalling pathways in this model.

Other leukocyte-specific G-protein-coupled receptors

Apart from the chemokine receptor families, we also investigated the cell specific expression of other G-Protein Coupled Receptors (GPCRs) in RNA-seq data. From a total of 108 annotated *GPCRs* in Ensembl, 13 genes show significantly enriched expression in larval leukocyte cell fractions. Among these 13 genes, *gpr84*, and *gpr97* showed highest expression levels in macrophage and neutrophil populations. In addition, *gpr56*, *grp132* (1 of 2), *gpr141*, *grp174*, *gpr183*, and *gpr183* (2 of 3) were clearly detectable in myeloid cells. Among the human homologs of these genes, *GPR84* is known as an inflammation-related receptor EX33 (Venkataraman and Kuo, 2005; Bouchard *et al.*, 2007), and *GPR132* is known as a high-affinity receptor for lysophosphatidylcholine (LPC) and may react to LPC levels at sites of inflammation (Yang *et al.*, 2004; Radu *et al.*, 2004; Frasch *et al.*, 2007,2008; Kabarowski 2009). *GPR56* expression was found to be a common trait of cytotoxic T-lymphocytes, possibly affecting their migration properties (Peng *et al.*, 2011), and indeed, this gene was also highly expressed in zebrafish T-cells. Other genes that were abundant in zebrafish T-cells included *gpr55*, *gpr183* and *gpr183* (2 of 3). A T-cell-specific function of human *GPR55* has not been reported, but this gene has been implicated in recruitment of neutrophils to inflammatory cannabinoids (Balenga *et al.*, 2011). In agreement, we also detected expression of *gpr55* in zebrafish neutrophils. Human *GPR183* was identified by the up-regulation of its expression upon Epstein-Barr virus infection of primary B lymphocytes, but its function is unknown (Craig *et al.*, 2007). It is expressed in B-lymphocyte cell lines and lymphoid tissues but was not detected in T-lymphocyte cell lines or peripheral blood T lymphocytes. Expression of *gpr34b*, *gpr82* *gpr89b*, and *gpr97* was also clearly detectable in zebrafish T-cells. In humans, *GPR89b* has been reported to function as an anion channel critical for acidification and functions of the Golgi apparatus, and has also been linked with activation of mitogen-activated protein

kinases and nuclear factor kappa-B (Maeda *et al.*, 2008). Finally, like in zebrafish, human *GPR97* is also expressed in T-cells (Peng *et al.*, 2011). Knockdown or knockout studies of these myeloid and lymphoid specific GPCRs in the zebrafish model will be a useful approach to gain further understanding of their functions in the vertebrate immune system.

Functional analysis of *Cxcr3.2*

To perform the first functional analysis of chemokine signaling in bacterial-induced inflammatory responses in zebrafish embryos, we focused on the role of *Cxcr3.2* in macrophages. Using morpholino knockdown and analysis of a knockout mutant we could demonstrate a role for *Cxcr3.2* in migration of macrophages towards local sites of infection with *S. typhimurium* and *M. marinum* (Chapter 2 and 4). As a likely consequence of the migration defect, bacterial burden of local infections was enhanced in *cxcr3.2* mutants. In particular, in *M. marinum* infection experiments with wild type and attenuated strains we also observed that higher numbers of bacteria were present extracellular in *cxcr3.2* mutants (Chapter 4). Based on phylogeny analysis, a group of six chemokines showed closest relationships with the human CXCL9/10/11 group, which includes the ligands of human CXCR3 (Chapter 3). Two of the members of this group, *cxcl11* and *cxcl11l* showed inducible gene expression during systemic bacterial infection. It remains to be further investigated whether these chemokine genes are also locally induced around bacterial infection sites. A *Pichia pastoris* expression system proved very useful for the purification of zebrafish chemokine proteins. We used *in vivo* chemotaxis assays in zebrafish embryos to test the biological activities of *Cxcl11* and *Il8* proteins that were purified using the *Pichia pastoris* system. These chemotaxis assays showed that *Cxcl11* attracted macrophages, while *Il8* attracted neutrophils. The ability of *Cxcl11* to attract macrophages was lost in *cxcr3.2* mutants, strongly suggesting that *Cxcl11* is a ligand for the *Cxcr3.2* receptor. It remains to be studied if *Cxcr3.2* may have additional ligands. In particular, the gene annotated in NCBI as *cxcl11l* showed high induction at different stages of *S. typhimurium* and *M. marinum* infection, and might also interact with the *Cxcr3.2* receptor.

In summary, our results have demonstrated the essential role of *Cxcr3.2* in bacterial-induced macrophage migration and control of local infections. In addition to demonstrating a function for *Cxcr3.2* in the zebrafish innate immune system, we discovered a putative ligand of this receptor. *Cxcl11-Cxcr3.2* is the first ligand-receptor pair with a proposed function in migration of zebrafish macrophages in response to bacterial infection, and therefore its identification is an important step towards understanding the chemokine signaling network underlying innate immune responses in the zebrafish model.

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