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Exploitation of host chemokine signalling by pathogenic mycobacteria

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Chapter 1

Introduction and thesis outline

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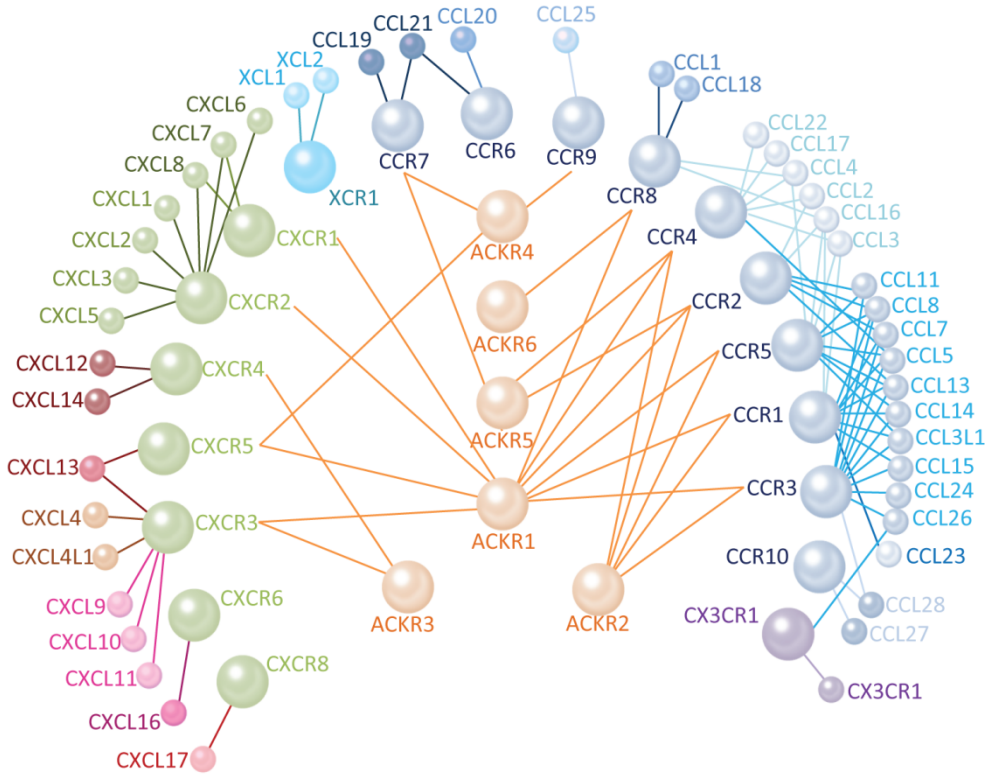
Bacterial infections remain a major cause of morbidity and mortality for mankind. In particular, the WHO Global Tuberculosis report (2015) estimated that one third of the world population is infected with *Mycobacterium tuberculosis* and that 9.6 million people have contracted tuberculosis (TB) in 2014 alone. An emergent problem is the prominence of multidrug and extensively drug-resistant strains. The evolution of antibiotic resistance is inevitable and requires continuous development of alternative therapeutic strategies. Therefore, a deeper understanding of the host-pathogen interface is needed. If the molecular mechanisms underlying host-pathogen interactions will be better understood, it may become possible to treat intracellular infections by targeting host cell functions that support bacterial growth, rather than targeting the pathogen itself. An important advantage of this approach is that it is more difficult for the pathogen to develop resistance to a host-targeted therapy. In this thesis, we investigated the host-pathogen dynamics during mycobacterial infection and we focused on the mechanisms by which mycobacteria can hijack chemokine signalling. Chemokines are small secreted signalling proteins that play multiple functions in a vast range of diseases. Chemokine receptors are highly expressed by immune cells and chemokine ligands are largely induced by inflammatory processes, including mycobacterial infections. However, since chemokine ligands and receptors are largely redundant in function and expression patterns, it has been difficult to address the relevance of different chemokine signalling axes for the onset and the progression of inflammatory diseases. Research on chemokine functions in TB has been particularly complicated in the most widely studied murine model. In this thesis, we alternatively applied the zebrafish (*Danio rerio)-Mycobacterium marinum* model to dissect how chemokines orchestrate the response of immune cells to mycobacterial infection. Several chemokine axes are conserved from fish to mammals and the similarities between human-*M. tuberculosis* and zebrafish-*M. marinum* pathology are remarkable. In this introduction, we briefly describe these aspects, which, together with the suitability of the zebrafish for microscopic analysis and genetic manipulations, have made us choose this model to investigate chemokine function in mycobacterial disease.

GRAPHICAL ABSTRACT



The human chemokine network as currently known consists of 25 receptors and 45 ligands. Ligand-receptor connecting lines indicate binding specificity. The AKCR family comprises atypical receptors that lack the motif for signalling through G-proteins and can antagonise the activities of other chemokine receptors (connected with orange lines) by binding one or more of their ligands. The chemokine receptor families include CXCR (green), XCR (cyan), CCR (blue), CX3CR (violet), ACKR (orange). Chemokine ligand families include CXCL (ELR+ chemokines in shades of green, homoeostatic chemokines in shades of red, platelet chemokines in shades of orange, dual function chemokines in shades of pink), XCL (cyan), CCL (shades of blue) and CX3CL (violet).

The zebrafish model system for biomedical research

Zebrafish initially emerged as a powerful vertebrate model organism to study embryonic development and morphogenesis[1,2,3,4,5] and this model species is now used to study the mechanisms of a wide variety of human diseases[5]. Due to their optical accessibility, the early embryonic and larval stages are ideally suited for non-invasive intravital imaging and longitudinal *in vivo* studies[1]. The short generation time (3-4 months) together with the external fertilisation and the possibility to daily obtain a large number of fertilised eggs from a zebrafish adult colony has permitted the efficient application of genetic tools, thereby providing the zebrafish field with transgenic lines that fluorescently label distinct tissues, organs, cell types, but also the cellular ultrastructural components, such as the nucleus, autophagosomes and the cytoskeleton[6].

The genome of zebrafish has been sequenced and forward genetics approaches have been successfully applied to mutate a vast array of genes[3,4]. Additionally, reverse genetic approaches, including morpholino knockdown and mRNA injection-based overexpression, are widely used in zebrafish embryos and have permitted rapid screening of gene functions. The zebrafish model also keeps up with state-of-the-art technologies, such as novel genome editing techniques (e.g. TALENS and CRISPR/Cas9)[7,8,9] and the application of cell/tissue-specific RNA-sequencing analysis[10], which are contributing to exponentially increase the availability of tools and data for this model system.

Due to their continuous exchanges with the surrounding water, embryos and larvae are easily penetrated by a variety of drugs and vital dyes and are therefore chemically and pharmacologically tractable. With the increase of tools and successes in using zebrafish, we have gained a grounded knowledge on how to exploit this model to study diverse biological processes. Therefore, not surprisingly, the zebrafish field has promptly expanded from the initial niche in developmental research to other scientific branches, spanning from eco/toxicology to neuronal physiology, behavioural studies and immunology. The potential of zebrafish research and its translational value to develop new treatments for human diseases is also underscored by new therapeutics that emerged from initial studies in zebrafish. In particular, a milestone for the zebrafish translational research was achieved when the first drug developed through the use of zebrafish has passed phase 1 trial and advanced to phase 2 clinical studies[11,12].

Despite the many advantages of using the zebrafish as a model for human diseases, there are also several limitations that should be taken into account when extrapolating conclusions from experiments in this animal model. The first important aspect to consider is the evolutionary divergence. There are about 445 millions of years of evolutionary divergence between the zebrafish and the human species, which is approximately 5-fold higher than the rodents-human divergence (93 millions of years). While key aspects of the immune signalling architecture are maintained between human and zebrafish, some significant differences exist between fish and mammalian immunology. An example is the fact that the Toll-like receptor Tlr4, differently than in mammals, is not involved in bacterial lipopolysaccharide sensing in zebrafish and many other fish species, which explains also the high tolerance of fish to this compound[13]. Another difficulty when working with zebrafish, resides in the fact that the teleost genome has undergone genome duplication, which means that a variety of genes are present in two copies in these species. Some gene families have also experienced species-specific expansions in zebrafish *per se*

(e.g. the chemokine ligand families[14]). Adding to this, the different gene copies have sometimes undergone functional specialisation and have diverged to assume slightly different roles[15].

Oftentimes regarded as an advantage, the small size of the zebrafish can be, in some circumstances, a limiting factor. In fact, the reduced dimension of zebrafish makes it difficult (in juveniles and adults) or nearly impossible (in embryos and larvae) to collect body fluids or organs, and therefore to precisely quantify bioactive molecules and chemicals in the blood or specific tissues. This has been a relevant limit, especially for the application of zebrafish larvae for high-throughput screening of drugs via bath exposure, including anti-tubercular therapeuticals[16,17]. In fact, not all the drugs can infiltrate through the skin and the impossibility of blood and tissue sampling made it impossible to distinguish inactive compounds from non-permeable false negatives. However, protocols for sampling the yolk sac have been described and it has been shown that dosage of compounds from these specimens can be used as an indication for the compound uptake, enabling therefore, to discriminate ineffective compounds from those that could not cross the skin barrier[18].

The zebrafish community additionally suffers from the poor availability of immunological reagents, and from the inefficiency of site-directed genome manipulation strategies, which effectively apply to the murine model. However, the lack of antibodies is well compensated by the expanding availability of transgenic reporter lines and the possibility to apply whole mount RNA *in situ* hybridisation. Additionally, the recent advances in genome editing technology (e.g. CRISPR/Cas9 editing) has enabled precise gene disruptions and generation of conditional knockout, at par of those available in mice[19,20].

Finally, another limit of the zebrafish model consists in the fact that, differently from rodents, strict breeding and husbandry protocols are not widely applied in the zebrafish community and true inbred zebrafish lines are not adopted in the majority of studies. However, generation of clonal zebrafish lines is possible, either by conventional repeated sibling incrosses or even directly via gynogenesis[21]. Therefore, it is expected that the zebrafish community will increasingly adopt these lines and more standardised culturing protocols for future research.

Taken together, despite some drawbacks, the zebrafish model holds great promise for translational biomedical research as it concentrates in a single vertebrate system several useful features, including remarkable homology to mammals in biological processes, optical transparency, availability of genetic tools, chemical tractability, adaptability to high-throughput research methods and finally, suitability to address research questions at multiple levels, from the molecular level to the whole organism level.

Tuberculosis and mycobacterial infection in the zebrafish-*M. marinum* surrogate model

Mycobacterium tuberculosis (*Mtb*) is a respiratory pathogen responsible for tuberculosis (TB). The main pathologic feature of mycobacterial infection is the formation of granulomas, which are inflammatory collections of immune cells that contain the infection and that are often chronically maintained[22]. TB is a life threatening and global disease, with high prevalence in all human populations[23]. Although many organs and tissues can be

infected with *M. tuberculosis*, the disease most often affects the lungs, with 75-80% of TB cases where the infection is exclusively pulmonary[24]. Approximately 1.5 million people die of tuberculosis each year, and there are over nine million new cases annually[23]. Although drug treatments are available, the therapeutic regimen is long and requires a combination of several pharmaceuticals. In addition, drug-resistant strains have evolved that do not respond to the currently available treatments. One third of the world population is estimated to be infected with *Mtb*. Only about 10% of infected individuals will manifest active TB disease while, in the vast majority of cases, the pathology is contained although not eliminated (latent TB). In case of latency, people are generally not contagious, and may never develop active TB. However, the infection can reactivate even many years later, especially in immune-suppressed/compromised patients, for example people subjected to chemotherapies or carrying HIV infection.

The granuloma, the histopathological hallmark of TB disease, is composed mostly of infected and non-infected immune cells that have migrated to the infectious focus. The signals responsible for granuloma formation and maintenance are only partly elucidated and it is clear that both host and pathogen signals integrate together to initiate the cellular and molecular programme that leads to granuloma formation[22]. As further elaborated on in **Chapter 2**, *M. marinum* (*Mm*) is a closely related species to *Mtb* that is a natural pathogen of zebrafish and other ectothermic animals. *Mm* causes necrotic granulomatous lesion formation in host tissues and these lesions are histologically very similar to those generated by *Mtb* in TB patients[25,26,27]. Additionally, the *Mm*-zebrafish surrogate model has been proven to recapitulate key aspects of TB, including bacterial dormancy/reactivation, the coordination of host and pathogen components in the formation of granulomas, the dynamicity of these structures and the key role of the inflammatory status in controlling the infection[28,29,30,31]. The striking conservation of molecular mechanisms, cellular dynamics and histopathological features between human and fish tuberculosis, together with the unique accessibility of the early stages of granuloma formation in zebrafish larvae, made the zebrafish-*Mm* host-pathogen pair a valuable platform to unravel the core pathogenic processes of mycobacterial infections. However, it must be taken into account that experimental fish embryo infections are generally performed using non-natural infection routes. Most commonly, the bacteria are delivered directly into the blood and generate a systemic infection that anatomically poorly represents the lung disease that is evoked in *M. tuberculosis*-infected patients. While use of a fish model makes it obviously impossible to model the organ-specific effects of TB pathology, a variety of alternative routes can be used to mimic and study specific aspects of the disease. For example, the importance of the epithelium for the initial steps of macrophage recruitment and granuloma formation has been addressed by delivering the bacteria into the hindbrain cavity[32], which is delimited by an epithelial barrier that resembles the alveolar lumen. Similarly, the relevance of the vasculature response could be addressed by delivering the pathogens in the poorly vascularised trunk tissue, where progression of the infection substantially depends on the induction of local angiogenesis[33].

Human infections with *Mm* are rare and generally limited to granulomatous nodules in the tissues of the skin, owing to the optimal growth of this pathogen at lower temperatures than that of the human body. Hence, *Mm* does not represent a serious risk for health, when compared to *Mtb*. The limited safety concern and the possibility to handle *Mm* under biosafety level 2 instead of level 3 conditions greatly expands the research opportunities that can be explored using the zebrafish-*Mm* surrogate model.

Chemokines and chemokine receptors, the portrait of an "extended" family

Chemokines are chordate-specific signalling molecules. Their primary function is to control cell movements by activating specific heterotrimeric G-protein coupled receptors (GPCRs), a class of 7 transmembrane (7TM) receptors (**Figure 1**, **Table 1**). Chemokines and chemokine receptors play critical roles in a wide context of biological processes, both in physiological and pathological conditions, which include development[34], ontogenesis and homing of immune cells[35], angiogenesis[36], organogenesis[37], autoimmune reactions[38], infectious diseases[39] and cancer[40,41]. These activities are not exclusively related to chemokine function in chemotaxis and cell migration, since multiple signal transduction pathways can be activated by GPCRs, including cell proliferation and differentiation[42].

GPCRs are the largest and most diverse group of membrane receptors in eukaryotes[43] and their pleiotropic functions, despite similar protein and signalling architecture, have greatly influenced modern medical research. In fact, GPCRs mainly signal via well-described pathways, which has facilitated drug discovery and testing. Additionally, they are involved in a variety of biological processes and therefore therapeutic targeting of GPCRs is relevant for many diseases. It has been recently estimated that between 1/3 and 1/2 of all the drugs available on the market target GPCRs[44,45].

At the intracellular side of the plasma membrane, inactive GPCR/chemokine receptors constitutively interact with a three subunit G-protein complex, also known as heterotrimeric G-proteins. G-proteins are able to bind and exchange guanosine triphosphate (GTP) with guanosine diphosphate (GDP). Specifically, it is the Gα subunit of the heterotrimer that exchanges GDP/GTP in response to conformational changes of the receptor[46]. Receptor conformational switches are evoked by binding of cognate chemokine ligands to their receptors. In the inactive form, Gα is bound to GDP. Activation of the receptor evokes physical replacement of GDP with GTP. This affects the conformation of the Gα subunit, which in turn dissociates from both the receptor and the Gβ-γ heterodimer. Both Gα-GTP and Gβ-γ possess lipid anchors and therefore diffuse laterally on the plasma membrane, where they interact with their downstream signalling partners[46]. This phenomenon is transient and, as soon as Gα-GTP decays to Gα-GDP, it re-associates to the GPCR receptor and to Gβγ. Despite this highly conserved signalling mechanism, vertebrate genomes encode multiple variants of G-proteins, which therefore can transduce diverse downstream signalling[47]. Some chemokine receptors have also shown selectivity for coupling with G-protein complexes containing specific G isoforms[47].

The cascade initiated by GPCR/chemokines receptors commonly includes the activation of phosphoinositide 3-kinase (PI3K), which leads to changes in intracellular phosphatidylinositol-3,4,5-triphosphate, and mediates the recruitment of pleckstrin homology (PH) domain–containing proteins to the cell membrane[48]. Several components of the Rho family of small GTPases, as well as their GTP/GDP exchange factors (GEFs) and kinases, also contain PH domains. Activation of the small GTPases (e.g. RAC1, CDC42, RHOA) is critical to link chemoattractant receptors to cytoskeletal changes and the formation/maintenance of cell polarity[49].

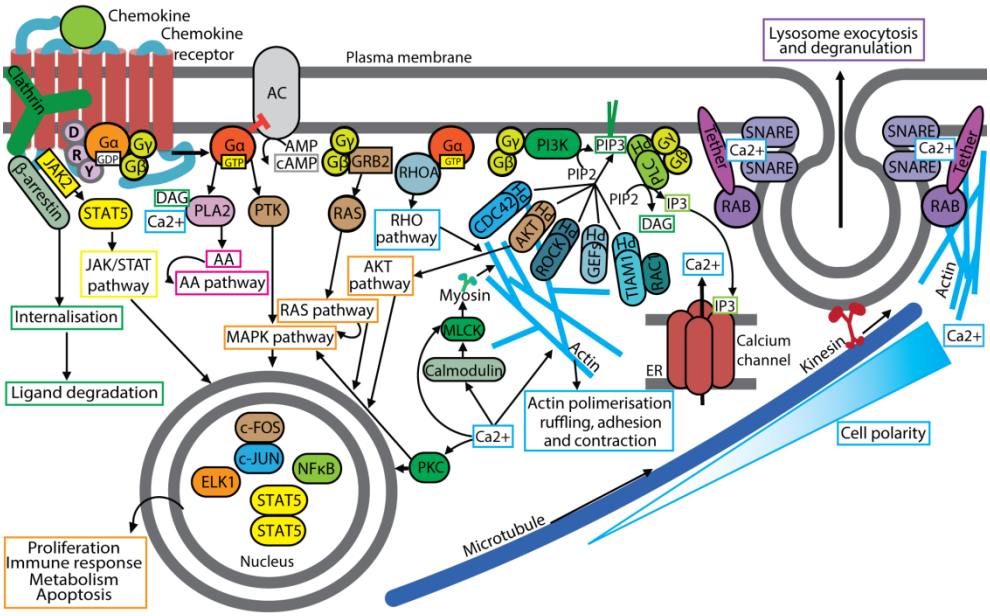


Figure 1. Downstream pathways activated by chemokine signalling. Most of the downstream signals evoked by chemokine ligand/receptor binding depend on receptor dissociation from heterotrimeric G-proteins (Gα-β-γ) and on the GDP-GTP (guanosine diphosphate and triphosphate) exchange in the Gα subunit. Different families of Gα-proteins exist (Gs, Gi, and Gq), which can activate RHOA (RAS homologue A), PLC or PLA2 (phospholipase C or A2) and PTK (protein tyrosine kinase). Gi (shown in figure) can additionally inhibit AC (adenylyl cyclase), while Gs can stimulate this enzyme and production of cAMP (cyclic adenosine monophosphate). PLA2 controls AA (arachidonic acid) release and affects the synthesis of pro/anti-inflammatory derivatives. PTK activates the MAPK (mitogen-activated protein kinases), while RHOA evokes actin polymerisation and sustains cell migration. Gβ-γ modulates GRB2 (growth factor receptor-bound protein 2), which via activation of RAS proteins, leads to downstream effects synergistic with the MAPK. Gβ-γ also activates PI3K (phosphoinositide 3-kinase) and production of PIP3 (phosphatidylinositol-3,4,5-triphosphate). PIP3 is key to maintain cell asymmetry by mediating cortical recruitment and activation of PH (pleckstrin homology) domain-containing proteins, including small GTPases and their regulators (e.g. TIAM1/RAC1, GEFs, ROCK, CDC42). PIP3 also activates AKT and PLC. AKT controls a phosphorylation cascade while PLC leads to IP3 (inositol-1, 4, 5-triphosphate) and DAG (diacylglycerol) production. IP3 mobilises Ca^{2+} from the ER (endoplasmic reticulum) and evokes profound changes in the cell, as Ca^{2+} controls cytoskeleton dynamics and modulates a variety of targets, such as PLA2 and PKC (protein kinase C). Calmodulin and MLCK (myosin light chain kinase) control contractility of actin fibres in a Ca^{2+}-dependent manner. Additionally, microtubule elongation depends on Ca^{2+} gradients. As a consequence, lysosomes and secretion granules that are trafficked on microtubule tracks via the motor protein kinesin accumulate towards the cell periphery. Ca^{2+} also controls the activity of RABs and synaptotagmins, which are involved in the formation of the SNARE (Soluble NSF attachment protein receptor) complexes, evoking membrane fusion and exocytosis. G-protein signalling terminates with the modulation of gene expression via an array of transcription factors, including NFκB (nuclear factor kappa B) and others (e.g. c-FOS/c-JUN, ELK1). The effects are pleiotropic and span from proliferation/apoptosis to immune defence and metabolism. Chemokines can also induce G-protein independent signalling, for example via direct activation of JAK2/STAT5 (Janus kinase 2/signal transducer and activator of transcription 5) or via β-arrestin. The latter is also involved in clathrin-dependent internalisation of chemokine ligand/receptor complex, which ultimately leads to ligand degradation and recycling of the receptor to the plasma membrane.

Chemokine sensing also activates phospholipase C (PLC)[47], which hydrolyses phosphatidylinositol-(4,5)-bisphosphate to produce inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 induces the mobilisation of intracellular calcium stores, which in turn has effects on calmodulin and myosin light-chain kinase (MLCK). Increased intracellular calcium is a hallmark of chemoattractant stimulation as this mediates, for example, cytoskeleton redistribution and relocation of focal adhesions[50]. Ca^{2+} also controls microtubule stability and their directional elongation in the direction of the cell movement. Secretion granules and lysosomes, which are trafficked in the cells via kinesin (a microtubule-associated motor protein), follow microtubule tracks and tend to accumulate at the cortex of the leading edge. Here the coordinated activity of Ca^{2+}, RABs and synaptotagmins – the main components of the SNARE (Soluble NSF attachment protein receptor) complex – will mediate fusion and exocytosis[51]. This process will, in turn, deliver phospholipids to the plasma membrane to sustain emission of protrusions and will determine secretion of enzymes and signalling mediators into the extracellular space, to facilitate locomotion and to transmit signals to neighbouring cells.

Another target of G-proteins is adenylyl cyclase (AC), which catalyses synthesis of cyclic adenosine monophosphate (cAMP). Some versions of Gα can activate this signalling while others (called inhibitory Gα or Gi) can instead negatively control this enzyme. Most chemokine receptors seem to associate with Gi complexes and therefore tend to antagonise this signalling pathway[52]. The activated G-proteins can also have a variety of other effects. These include activation of RAS/mitogen-activated protein kinase (MAPK) pathways[53,54] and modulation of phospholipase A2 (PLA2)[55], with downstream effects as diverse as apoptosis and cell proliferation[54], immune responses[56,57], arachidonic acid (AA) signalling[55] and metabolic control[58] (**Figure 1**).

Chemokine receptors were originally thought to act entirely through G-protein-mediated processes. However, we now know that some chemokine receptors can also initiate alternative signalling pathways, that do not require G-protein-coupling. For example, some chemokines can directly activate the JAK-STAT (Janus kinase-signal transducer and activator of transcription) signalling[59] or β-arrestin signalling[60,61], with the latter being associated with clathrin-mediated ligand/receptor complex internalisation and desensitisation. There is also a class of these receptors that are completely incapable of transmitting any G-signalling, known as atypical chemokine receptors which are further discussed below. Notably, their ligand-mediated internalisation/recycling remains intact (or even enhanced), since this requires only direct β-arrestin coupling. This aberrant signalling mechanism is responsible for the role of these receptors as ligand scavengers, as the internalisation of the ligand/receptor complex mediates degradation of the cargo and recycling of the empty receptor to the plasma membrane, where new cycles of scavenging can take place[62,63].

Chemokine ligands are classified into the CXC, CC, XC (or C) and CX3C subfamilies, according to the arrangement of four conserved cysteine residues, which are important for maintenance of their tridimensional structure[64,65]. In mammals as in teleost fish, the CXC and the CC families are the two largest subfamilies, whereas the CX3C and C families only contain few members (**Table 1**). In zebrafish, another fish-specific chemokine subfamily was also identified, namely CX chemokines. These CX chemokines lack one of the two conserved cysteine residues in the N-terminus but retain the third and the

fourth. This distinguishes them from the XC subfamily chemokines, which only retain the second and fourth of the signature cysteine residues[14].

CCR	CCL	Function	Specificity	Significance of the axis in TB
CCR1	CCL3-3L1-5-6*-7-8-9*-13-14-15-16-23	Inflammatory	M(Φ) , DC, B, T, mast cells, basophils, eosinophils	-
CCR2	CCL2-7-8-11-12*-13-16	Inflammatory	M(Φ), DC, T , NK	Controversial. Recruitment of M(ϕ) and DCs via CCR2 permitted better containment of high *Mtb* doses when injected intravenously. However, *CCR2* KO mice were indistinguishable from wt when exposed to lower doses, most likely due to compensations.
CCR3	CCL3L1-5-7-8-11-13-14-15-24-26-28	Inflammatory	T, eosinophils	-
CCR4	CCL2-4-3-5-17-22	Dual	M(Φ), DC, T, NK, NKT, basophils	Controversial. Differential recruitment/priming of T cells and iNKT evoked increased susceptibility of *CCR4* KO mice to *M. bovis*-BCG. However, no impairment was found in *CCR4* KO animals when exposed to low *Mtb* doses.
CCR5	CCL3-3L1-4-5-8-11-13-14-16	Inflammatory	M(Φ), microglia, DC, eosinophils,	Controversial. Pathology of *CCR5* KO mice was indistinguishable from that of wt, most likely due to compensation by CCR1. However, studies performed with *CCL5* KO animals suggest a beneficial effect of this axis early after exposure with low infection dose.
CCR6	CCL20-21	Dual	DC, B, T, NKT	Impaired recruitment of iNKT and T cells in *CCR6* KO mice increased susceptibility to *M. bovis*-BCG. Follow-up experiments with *Mtb* still necessary.
CCR7	CCL19-21	Homoeostatic	DC, B, T	Beneficial. Reverse migration of DCs to the draining lymph nodes is delayed in *CCR7* KO animals, which delays activation of T cell-mediated response against *Mtb*.
CCR8	CCL1-4-16-18	Dual	DC, B, T, NK	-
CCR9	CCL25	Homoeostatic	T	-
CCR10	CCL27-28	Homoeostatic	B, T, eosinophils	-

Table 1. Mammalian chemokine axes, and their roles in tuberculosis as emerging from murine knockout (KO) studies *(continued on the next page)*. B: B-lymphocytes; T: T-lymphocytes; M(Φ): monocyte/macrophages; NΦ: neutrophils; DC: dendritic cells, NK: natural killers; NKT: natural killer T cells. Underlined names indicate the existence of zebrafish orthologues, based on synteny and sequence alignment. * CCL6-9-12 and CXCL15 are absent in humans but present in rodents.

CXCR	CXCL	Function	Specificity	Significance of the axis in TB
CXCR1	CXCL6-7-8	Inflammatory	NΦ,	-
CXCR2	CXCL1-2-3-5-6-7-8	Inflammatory	NΦ, NK	Detrimental. *CXCR2* KO animals presented reduced lung pathology, due to contained Nϕ infiltration, which limited inflammation.
CXCR3	CXCL4-4L1-9-10-11-13	Dual	M(Φ), microglia, T, NK	Detrimental. *CXCR3* KO mice displayed attenuated lung pathology, attributed to differential T cell priming.
CXCR4	CXCL12-14	Homoeostatic	M(Φ), B, T	KO not available, since embryonic lethal.
CXCR5	CXCL13	Homoeostatic	B, T	Beneficial. Aberrant recruitment of lymphocytes limited *Mtb* control in *CXCR5* KO mice.
CXCR6	CXCL16	Dual	B, NKT	-
-	CXCL15*	Inflammatory	NΦ	-
CXCR8 (GPR35)	CXCL17	Homoeostatic	M(Φ), DC	-

CX3CR/XCR	CX3CL/XCL	Function	Specificity	Significance of the axis in TB
CX3CR1	CX3CL1, CCL26	Inflammatory	M(Φ), microglia, DC, T	Dispensable. Following low-dose aerosol challenge with *Mtb*, *CX3CR1* KO mice were indistinguishable from wt in terms of survival and infection burden.
XCR1	XCL1-2	Dual	T	-

ACKR	ACKRL	Function	Specificity	Significance of the axis in TB
DARC (ACKR1)	CCL1-2-5-7-8-11-13-14-16-17-18, CXCL1-2-3-5-6-7-8-9-10-11-13	Atypical	ligand scavenger	-
D6 (ACKR2)	CCL2-3-3L1-4-4L1-5-7-8-11-12-13-14-17-22-23-24-26	Atypical	ligand scavenger	Beneficial. *D6* KO mice are highly susceptible to low doses of *Mtb*. The phenotype is linked to an increased number of M(Φ)s, DCs and T cells infiltrating the infected tissues.
CXCR7 (ACKR3)	CXCL11-12	Atypical	ligand scavenger/ gradient control	KO not available, since lethal *in utero*.
CCRL1 (ACKR4)	CCL19-21-25, CXCL13	Atypical	ligand scavenger	-
CCRL2 (ACKR5)	CCL2-19	Atypical	ligand scavenger	-
PITPNM3 (ACKR6)	CCL18	Atypical	ligand scavenger	-

Table 1. Mammalian chemokine axes and their implication in tuberculosis as emerging from murine knockout (KO) studies *(continued from the previous page)***.**

In mammals, CXC chemokines can be further divided into ELR and non-ELR chemokines, based on the presence of the ELR (Glu-Leu-Arg) motif upstream of their CXC motif[64]. In these species, the presence of the ELR motif distinguishes neutrophil-competent chemokines (ELR-positive) from non-neutrophil-competent chemokines (ELR-negative), which attract other myeloid and lymphoid cells (monocyte/macrophages, lymphocytes, dendritic cells and natural killer cells)[66,67] (**Table 1**). Mammalian ELR+ chemokines are CXCL1-2-3-5-6-7-8-15. These act mostly through receptors CXCR1-2. ELR− chemokines include CXCL4-9-10-11-12-13-14-16-17 and interact with receptors CXCR3-4-5-6-8. In fish, the ELR motif is not conserved and is not essential for the attraction of neutrophils by fish neutrophil-chemotactic chemokines[68].

The chemokine receptors are also classified in subgroups, namely CXCR, CCR, XCR or CX3CR, depending on the ligands that they can bind (**Table 1**)[64,65]. A CXR family (which should bind the zebrafish-specific CX chemokines) has not been identified and the interacting partners of CXL ligands remain unknown[14]. In addition to classical chemokine receptors, a new group of receptors (or more precisely, interceptors) has been introduced to include non-classical chemokine receptors. This group phylogenetically belongs to the same protein family, but differs functionally, due to the inability to mediate classical GPCR signal transduction by coupling with G-proteins. This heterogeneous group of "decoy" receptors mostly act as broad spectrum ligand scavengers and are designated as atypical chemokine receptors or ACKR[69]. Most atypical receptors lack or display an altered E/DRY (Glu/Asp-Arg-Tyr) motif, which is key to couple 7TM receptors with G-proteins and mediate classical signalling (**Figure 1**).

Chemokines can be functionally classified as homoeostatic (or constitutive) or inflammatory according to the regulation and pattern of their expression[65]. The homoeostatic chemokines are generally expressed by a variety of tissues in physiological situations, while the expression of inflammatory chemokines requires inflammation/exposure to pathogens. However, this classification is not strict and many chemokines (e.g. CXCL9-10-11) exert a dual function, by controlling both physiological and pathological responses (**Table 1**).

The number of chemokines identified and characterised has expanded rapidly in the past decades, both for human and animal models[65]. In humans, 25 chemokine receptors (including ACKRs, **Table 1**) and 45 chemokine ligands have been identified, while in zebrafish at least 36 putative chemokine receptors and 75 putative chemokine ligands have been mapped[65]. Studies have revealed high levels of redundancy in the function and expression patterns of both chemokines and chemokine receptors[41]. Several receptors bind multiple chemokines and several chemokines can also bind to more than one receptor (**Table 1**). During infection/inflammation and development, a variety of chemokine ligands can be expressed at a specific site and multiple chemokine receptors can be expressed by the chemoattracted cells. Taken together, chemokine ligands and receptors can be highly synergistic, complementary or, in some cases, even antagonistic[41,42]. Due to this complexity, it is very difficult to accurately assess to what extent the chemokine axes are really redundant. Current understanding of the roles and dynamics of each chemokine in the complexity of an *in vivo* system is also incomplete. Therefore, the evolutionary, functional and regulative forces, that permitted expansion and radiation of diverse chemokine ligand and receptor lineages, remain largely unknown[65].

Homologies between the chemokine signalling axes of human and zebrafish

While most of the ligand-receptor interacting partners have been identified in human and mammalian models, functional roles in zebrafish are only beginning to be examined[70]. At transcriptional level, several zebrafish chemokines have been found to be expressed in discrete areas/organs and tissues (homoeostatic) and others were found to be highly responsive to inflammation (inflammatory), like in humans[14,71,72,73,74]. In a recent review on the translational value of zebrafish and other teleosts to study chemokine functions[70], it was reported that only one CCL chemokine axis has been currently shown to have clear *in vivo* functional homologies with the human and mammalian system. In this study, the zebrafish Ccl25a gene was found essential for normal T cell lymphotaxis to the thymus[73].

While the CCL ligand family is more divergent between mammals and teleosts, the CXCL family is much better conserved. For example, 11 canonical CXCRs and 20 CXCLs can be currently identified in zebrafish, versus 7 CXCRs and 17 CXCLs described in humans[14]. Several true homologies between zebrafish and mammals (corroborated by sequence alignments, synteny studies and functional analyses) have been demonstrated for CXC axes. As described in this thesis (**Chapter 3**), the CXCR3/CXCL9-10-11 axis exists in zebrafish. On the ligand side, a cluster of seven CXCL11-like tandem-duplicated genes have been identified. Some of these genes are infection-inducible, similar to their mammalian counterpart, and we showed that two of the CXCL11-like chemokines encoded by these infection-inducible genes signal via an orthologue of CXCR3, the zebrafish receptor Cxcr3.2 (**Chapter 3-4**). The *CXCR3* gene is also duplicated in zebrafish and two paralogues of *cxcr3.2* exist, namely *cxcr3.1* and *cxcr3.3*. In zebrafish embryos and larvae the *cxcr3.2* and *cxcr3.3* genes are expressed by macrophages and neutrophils (**Chapter 3**), while the expression of *cxcr3.1* is very low in these cells. Preliminary data suggest that *cxcr3.1* is detectable at higher levels than the other two orthologues in *lck*$^{+}$ cells (immature T cells)[75]. Therefore, it is possible that the three isoforms regulate chemotaxis of different cell types and represent sub-functionalisation of the mammalian CXCR3 that is expressed by both myeloid and lymphoid leukocytes (T cells, natural killer cells, dendritic cells, macrophages and microglial cells). However, this remains speculative, since the expression patterns remain to be further examined in adult zebrafish. The function of Cxcr3.3 has not been yet elucidated. However, as described in **Chapter 5**, *cxcr3.3* genes exist exclusively in fish and in most cases they present peculiar sequence adaptations, including disruption of the E/DRY-motif consensus, that suggest atypical functions.

Two clear orthologous CXCL12/CXCR4 axes have also been reported in zebrafish and extensive functional studies have been performed for both systems[72,76,77,78,79] (**Chapter 6**). Both CXCL12 and CXCR4 are in fact duplicated in fish (Cxcl12a/b and Cxcr4a/b). Cxcr4a and Cxcr4b are mostly expressed in separate tissues and cell types, which corroborates the hypothesis that after gene duplication the two receptors have divided functions between the two copies[80]. It appears that Cxcr4a evokes more potent responses via Cxcl12b while Cxcr4b predominantly signals via Cxcl12a, however, cross-reactivity has also been described[81]. Importantly, it has been demonstrated that cross-communication between the zebrafish and human ligands and receptors takes place, supporting the translational value of the zebrafish model[40]. The mammalian CXCL12 and either one or both of the zebrafish counterparts, Cxcl12a/b, are chemotactic for endothelial cells, haematopoietic progenitor cells, lymphocytes and several

other types of leukocytes. In agreement with their more homoeostatic function, these axes were implicated in the development of morphogenetic/organogenetic programs, such as the formation of blood and lymphatic vessels, the migration of germ cells and of the lateral line primordium, the organisation and patterning of the nervous system and the homing of haematopoietic stem cells[72,76,79,82,83]. The axis (mostly the Cxcr4b/Cxcl12a paralogue axis) has also been shown to play a crucial function during inflammatory responses, for example, promoting the metastatic behaviour of CXCR4-positive cancer human-xenotransplants[40]. Notably in both mammalian models and in zebrafish, this axis is regulated by the activity of the atypical chemokine receptor CXCR7/ACKR3, which acts as a Cxcl12 ligand scavenger to maintain an efficient formation of ligand gradient and facilitate cell migration[76].

At least 2 *cxcl8* genes were identified and characterised in zebrafish, which are both induced by inflammation, wounding and infection. Similar to the human CXCL8 (IL8), the zebrafish paralogues are potent neutrophil chemoattracts via the Cxcr2 receptor, although in contrast to humans, expression of Cxcr1 alone does not seem to permit any significant Cxcl8-dependent recruitment[84,85,86,87]. In mammals, CXCR2 is highly promiscuous and can respond to a range of CXC-chemokines (**Table 1**). Therefore, it is likely that multiple ligands also exist for this receptor in zebrafish. As indicated in this thesis (**Chapter 7**), the zebrafish-specific chemokine Cxcl18b also partly requires signalling via Cxcr2, and not Cxcr1, to evoke optimal recruitment of neutrophils.

Chemokine function in mycobacterial diseases

Since chemokines and chemokine receptors direct cells to specific sites within the tissues, these molecules may be highly relevant for the progression of TB and granuloma formation, for example by directly controlling the cellular trafficking at the granuloma. There have been a variety of studies aimed at characterising expression of chemokines in response to *Mtb in vitro* and *in vivo*. *Mtb* is a strong inducer of chemokine expression. Following *Mtb* infection of human or mice macrophages *in vitro,* these cells exhibit rapid expression of many chemokines, detectable as early as 2 hours post-infection[88,89]. Mammalian macrophages can produce the chemokines CCL2-3-4-5-7-12, CXCL2-9-10-11, and CX3CL1 with a response that, in many cases, depends on the pathogen virulence[90,91,92,93,94,95,96,97]. The cell wall components of mycobacteria are considered crucial for chemokine induction, as the production of several chemokines is significantly reduced following exposure to wall-deficient mycobacteria[97]. Macrophages are not the only cells inducing expression of chemokines upon *Mtb* infection. Chemokine expression is also induced in cells intrinsic to tissue surrounding the infection and other attracted leukocytes[88,98]. Expression of CCL2 and CXCL5-8-9-10-11 was detected in human or murine epithelial cells in response to virulent strains of *M. tuberculosis*[98,99,100,101]. Circulating granulocytes from TB patients produced high levels of CCL2-3 and CXCL1-8[102,103], bronchoalveolar lavage of patients displayed elevated levels of CCL2-5-7-12, CXCL8-10[90,104,105] and plasma/serum level of CCL4-11-24-26 and CXCL10 were also found increased[106,107,108]. Two other studies that profiled gene expression of *Mtb*-infected murine and non-human-primate lungs described overall upregulation of CXCL1-2-3-5-9-10-11-13 and CCL2-3-4-5-7-8-12-19-20[98,109,110]. Recent studies also revealed that, in TB of the central nervous system (TB meningitis), microglia (specialised macrophages residing in the brain) can also produce several chemokines, including CCL2-5 and CXCL10[111]. These studies indicate that a variety of cells participate in generating chemotactic/chemokinetic

cues in response to mycobacteria (**Figure 2**). Regarding the specificity of recruited cells, the chemokines reported are known to induce mobilisation of diverse types of cells (monocyte/macrophages, neutrophils, eosinophils, T cells, natural killer cells, dendritic cells, and others) according to the expression pattern of their receptor counterparts on target cells (**Table 1**, **Figure 2**)[41,42,65].

Regulation of the expression of signals inducing chemokine ligands still requires complete characterisation, as much in TB as in other physiological and pathological contexts. Chemokines are regulated by many cytokines and infection-related products. There is evidence for example that IFNγ, TNFα and *Mtb*-components can induce expression of chemokines (**Figure 2**). Expression of chemokines by macrophages appears to be highly influenced by TNFα production, which controls CCL2-5 and CXCL9-10-11[112.] TNFα is produced by macrophages themselves in response to *Mtb* infection and blockade of TNFα signalling can attenuate chemokine production. Notably, CXCL9-10-11, which are generally regarded as IFNγ inducible chemokines, were also found to be induced by TNFα during mycobacterial infection[112,113]. However, IFNγ itself is also found increased in TB patients and TNFα/IFNγ may synergise to induce these and other chemokines.

While expression of chemokines is increased by *Mtb* infection, the expression of chemokine receptors remains much more stable. However, transcriptional profiling of the murine lung revealed a general upregulation of CXCR3-6 and of CCR1-5-9[98,110]. Clinically relevant might be also the fact that monocytes increase expression of CCR5 and CXCR4 (HIV co-receptors) in response to intracellular *Mtb*, which may result in increased infectivity of HIV in cases of *Mtb*/HIV co-infections[114,115]. In another study, dendritic cells having taken up *Mtb* were found to upregulate CCR7, which induces their reverse migration from the infection focus to the draining lymph nodes (expressing its ligands CCL19-21) and permits a faster priming of the T cell-mediated adaptive immunity[116,117]. Similarly, upon recruitment to the lung, T cells upregulate CXCR3 and CCR5 receptors. However, this upregulation maintains them at the infected locus, since high levels of CXCR3 and CCR5 ligands are produced at the lesion[118].

Leprosy represents another relevant mycobacterial disease, caused by the species *Mycobacterium leprae*. The disease, which essentially affects the skin and the peripheral nervous system, is still endemic in several developing countries, where it represents a relevant cause of permanent disability[119]. Leprosy manifests itself in two main polarised forms, namely lepromatous leprosy and tuberculoid leprosy. Lepromatous leprosy is characterised by exuberant bacillary replication and failure of T-helper 1 activation (which is essential to counteract the pathogen). Conversely, tuberculoid leprosy, is characterised by reduced count of bacilli, expression of T-helper 1 cytokines and is generally restricted to delimited skin lesions. Compared to TB, leprosy still remains poorly understood, for several limitations, which include the scarce cultivability in axenic conditions[120,121,122] and the limited availability of animal models[123,124]. However, several studies have demonstrated that expression levels of different chemokines, including CXCL8-9-10 and CCL2-3-7, are sensitive to leprosy infection[125,126,127,128,129]. Notably, recent studies have also demonstrated that the expression of several chemokine ligands and receptors can be specifically correlated with the different manifestations of leprosy.

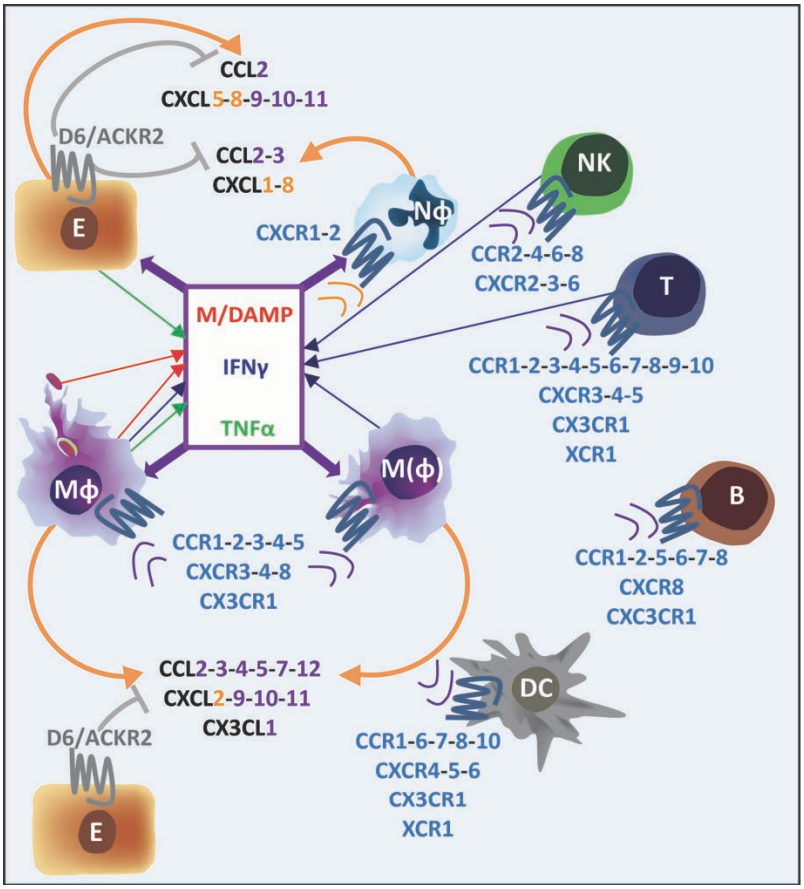


Figure 2. Schematic representation of main chemokine ligands and receptors implicated in the response to mycobacterial infection. Infected macrophages (MΦ) and epithelial/endothelial cells (E) respond to microbe/damage-associated molecular patterns (M/DAMPs and red arrows) by producing cytokines and several chemokines, including interferon γ (IFNγ and blue arrows), tumour necrosis factor α (TNFα and green arrows), CCL2-3-4-5-7-12, CXCL1-2-5-8-9-10-11 and CX3CL1 (orange arrows). TNFα and IFNγ can act on other cells in the surrounding microenvironment (initially, mainly monocyte/macrophages, epithelial, endothelial, stromal cells and neutrophils) and stimulate the production of more chemokines. Different types of leukocytes express different kinds of chemokine receptors (blue names) and, depending on their ligand specificity, these receptors mediate chemotaxis of leukocytes to the initial infection focus. CXCL1-2-5-8 (orange names) are mostly chemotactic towards neutrophils (NΦ), while other chemokines, such as CCL2-3-4-5-7-11, CXCL9-10-11 and CX3CL1 (violet names) are chemotactic towards monocyte/macrophages (M(Φ)), dendritic cells (DCs), T, B and/or natural killer (T, B, NK) cells. Antigen presenting activity by M(Φ) and DCs, will support activation of adaptive B and T cell-mediated immune responses. Additionally activated NK and T cells will produce high levels of inflammatory cytokines (especially IFNγ), which will sustain further stimulation of the innate immune cells and additional release of inflammatory chemokines, which in turn will support supplementary infiltration of leukocytes. The atypical chemokine receptor D6/ACKR2 (grey) is also upregulated in the cells surrounding the infection site and exerts a scavenging activity against a wide spectrum of CC-cytokines, contributing to tight control of chemokine levels. Ultimately these forces permit aggregation of immune cells at the infection focus and contribute to the formation of tubercular granulomas.

In particular, it was identified that expression of CCL17 or CCL18 are either correlated to the lepromatous (CCL18) or to the tuberculoid (CCL17) form[130], and that the expression levels of both CXCR3 and its ligand CXCL10 can be used as indicator of leprosy type 1 reaction, a severe complication of leprosy, characterised by acute activation of the cell-mediated immune response with consequent inflammation and nerve damage[131].

In vivo analysis of chemokine axes in murine TB models

While large amounts of data are available from *in vitro* studies, the expression and relevance of chemokines have only been characterised to a limited degree *in vivo* and the real meaning of these molecules for the pathology of TB remains elusive and speculative in several cases (**Table 1**). Using knockout murine models it was shown that CCR2 and its ligand CCL2 are essential for migration of macrophages and dendritic cells to the lungs and to control infection[92,94]. However, the susceptibility of these mice was found to be dose-dependent, since these animals were highly susceptible to a moderate or high dose of *Mtb* administered intravenously, but did not have increased bacterial loads compared to wildtypes (wt) when exposed to low dose aerosol or intravenous infection[132,133]. Another controversial finding came from the study of a *CCR4* knockout (KO). In a model of acute *M. bovis*-BCG pulmonary infection, these animals exhibited higher susceptibility and increased bacterial burden. However, a follow-up study showed that *CCR4*$^{-/-}$ mice were not more susceptible to low dose of *Mtb* infection[110,134]. *CCR6* mutants also displayed increased susceptibility to *M. bovis*[135], but studies with *Mtb* are still necessary. The function of CCR5 signalling is also controversial; an initial study in *CCR5*$^{-/-}$ mice reported no difference in the bacterial burden evoked by *Mtb*[136] and suggested that another receptor, such as CCR1 (which can bind the same ligands of CCR5, including CCL3-5), could compensate for CCR5 loss. Another study performed with *CCL5* mutant mice with low dose aerosol infection, revealed instead an increased bacterial burden early after infection. In this study, recruitment of immune cells via CCL5 was predominantly evoked by CCR5, suggesting that compensation may occur at later time points but that CCR5 and CCR1 are not entirely redundant early after infection[137]. Furthermore, in the *CCR2*$^{-/-}$ mice studies described above, it was reported that while the number of macrophages migrating to the infection is initially lower, new cohorts of monocyte/macrophages can still be recruited at later time points. It was hypothesised that the initial increased burden in these animals evokes a higher upregulation of other chemokines in a second phase, which still sustains infiltration of immune cells and formation of granulomas[92,94]. These examples illustrate well the difficulties that can be experienced when addressing chemokine function *in vivo*.

A murine model was also employed to study the function of CXCR3 and its ligands CXCL9-10-11 during *Mtb* infection. A beneficial effect of *CXCR3* mutation was observed, with associated decrease in number, size and density of granulomas[110,138]. Unexpectedly, the recruitment of T cells was increased, rather than diminished in *CXCR3* mutants, and the improved resistance of *CXCR3*-deficient mice was attributed to the function of CXCR3 in T-cell priming, rather than T-cell recruitment[138,139]. Mice deficient for either *CXCR2* or its ligand *CXCL5* had reduced lung pathology in comparison to their wt counterparts, and this phenotype was linked to a reduced accumulation of neutrophils in the mutants and to decreased detrimental neutrophil-dependent inflammation[101]. CXCL8, one of the most potent chemoattractants for neutrophils in humans, which is abundantly expressed upon *Mtb* infection, is however absent in mice[140], which makes it difficult to exactly address the role of CXCR2-signalling using this model. Finally, a study of *Mtb* infection in a

CX3CR1$^{-/-}$ mouse model, showed that this mutation, while affecting infiltration of different leukocyte subsets, did not lead to protection or increased susceptibility to the pathogen, indicating that this receptor is dispensable[141].

In few cases, specific chemokine axes have been shown to play unique, non-redundant, functions *in vivo*. For example, both receptor and ligand mutants of the *CXCR5*/*CXCL13* pair were shown to display decreased control of *Mtb*, linked to aberrant recruitment of a specific set of CXCR5^{+} lymphocytes[114,115,142]. Similarly, the function of CCR7 in dendritic cell reverse migration to the draining lymph nodes to prime the adaptive immune response was also found to be unique[116,117].

Regarding the relevance of atypical chemokine receptors for the response to mycobacterial infection, a recent study on *D6*/*ACKR2* KO mice showed that these animals are highly susceptible to low dose aerosol exposure to *Mtb*, owing to the increased number of monocyte/macrophages, dendritic cells and T cells infiltrated in the lesioned tissues. Blockade of a wide spectrum of chemokines with a cocktail of antibodies could rescue the phenotype of *D6* deficiency, which suggests that the activity of *D6*/*ACKR2* as a chemokine scavenger receptor is fundamental to maintain a balanced activation of leukocyte recruitment via chemokine signalling[143].

Despite the seemingly large redundancy in function exerted by chemokines in TB suggested by *in vitro* and *in vivo* models, human studies have still shown associations between mutations of multiple chemokine ligands (CCL2-5, CXCL8-10) and TB, indicating that besides the apparent redundancy, several chemokines may have defined roles to play in the pathogenesis of TB which we still do not entirely comprehend[144,145,146,147,148,149,150,151].

Taken together, it has been difficult to dissect the function of chemokine signalling in TB using the mice as an *in vivo* model. This situation may reflect, at least partly, the fact that mice do not represent an ideal model for TB, since *Mtb* is not a natural murine pathogen and infection in mice does not recapitulate the primate pathology. Additionally, the use of the murine-*Mtb* infection model leads to restrictions in terms of research opportunities. *In vivo* imaging and longitudinal studies in rodents require invasive and technically challenging procedures. Furthermore, work with the mouse model imposes severe ethical restrictions, since adult animals must be sacrificed for the experiments or for intra-uterine collection of embryos/foetuses. Moreover, research prospects are severely limited by the use of the *Mtb* pathogen, which represents a serious health risk for the researchers. To circumvent all these difficulties, in this thesis we have exploited the zebrafish-*Mm* natural infection model to evaluate the role of chemokines in mycobacterial infection. Using this surrogate system, we took advantage of its attractive optical and genetic tools to obtain a more dynamic representation of how the chemokine axes calibrate the immune response to mycobacteria.

Contribution of this thesis: a brief outline

In this thesis, we applied the zebrafish (*Danio rerio)-Mycobacterium marinum* natural infection model to obtain novel insights into the dynamic processes underlying host-pathogen interactions and, in particular, to dissect how chemokines, a class of small chemotactic proteins, orchestrate the response of immune cell types to mycobacterial

infection. Several chemokine axes are conserved from fish to mammals and the similarities between human-*M. tuberculosis* and zebrafish-*M. marinum* pathology are remarkable. These two aspects, together with the excellent possibilities for intravital imaging provided by zebrafish transparency, have enabled us to study more dynamically chemokine-dependent processes in an *in vivo* context and allowed us to dissect how these axes are implicated in defined spatio-temporal windows of mycobacterial disease.

The work presented in this thesis has helped to elucidate the function of several chemokine axes in mycobacterial disease, and contributed to interpret the translational impact of the zebrafish model for biomedical research. This chapter (**Chapter 1**) introduces the chemokine signalling system and the current knowledge of its role in tuberculosis from *in vitro* and murine studies. **Chapter 2** elaborates on the translational value of the zebrafish model and reviews how the application of this model has advanced our understanding of host-pathogen interactions for different infectious diseases. **Chapter 3** and **Chapter 4** dissect the function of the chemokine receptor Cxcr3.2 and of its ligands Cxcl11aa and Cxcl11af during the response to mycobacterial infection, expanding on the effects of this signalling system for both direct chemotaxis of macrophages and control of their intrinsic microbicidal competence against mycobacteria. **Chapter 5** describes the generation and initial characterisation of a *cxcr3.3* mutant zebrafish line and explores the possibility of atypical receptor crosstalk at play between Cxcr3.3 and Cxcr3.2. **Chapter 6** presents the importance of the homoeostatic chemokine receptor Cxcr4b in order to sustain a pro-granuloma angiogenetic programme. In **Chapter 7** we characterised the function of the chemokine ligand Cxc18b, a fish-specific inflammatory chemokine that, similarly to Cxcl8, exerts a neutrophil-specific chemotactic activity, at least partly via Cxcr2. Finally, **Chapter 8** summarises and contextualises the findings of this thesis in relation to the current scientific literature.

Due to the high redundancy of chemokines and to the practical limitations when attempting to study tuberculosis in a murine system, the use of a zebrafish platform to address chemokine signalling during mycobacterial pathogenesis promises to greatly contribute to our understanding of the actual *in vivo* significance of these multifaceted signalling systems.

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