Exploitation of host chemokine signalling by pathogenic mycobacteria

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Chapter 8

General discussion and final conclusions

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Rationale of using a non-mammalian host to model mycobacterial diseases

In this thesis we applied the *Danio rerio* (zebrafish)-*Mycobacterium marinum* (*Mm*) infection model to obtain novel insights into how chemokines orchestrate the response of immune cells to mycobacterial infection. *Mm* is a natural pathogen of zebrafish and is phylogenetically very close to *Mycobacterium tuberculosis* (*Mtb*)[1], the causative agent of human tuberculosis (TB). The *Mtb* bacillus is carried by one third of the world population and remains the most severe global health problem of bacterial entity since its emergency and adaptation to humans in prehistorical eras, before the out-of-Africa emigration of the *Homo sapiens* species (~70.000 years ago)[2,3,4]. *Mtb* mainly provokes a lung disease[5], although it is able to colonise extrapulmonary tissues of the host (including the central nervous system and meninges[6,7], the eye[8], the breast[9], the liver[10], the kidney[11], the gastrointestinal tract[12], the genitourinary tract[13], the skin[14], the bones[15] and the lymph nodes[16]). In all these tissues *Mtb* infection can lead to the formation of granulomas, a hallmark of local inflammation. Granuloma formation is initiated by infected macrophages that subsequently attract new macrophages and other immune cells that confine the pathogen and the necrotising tissue[17]. Since *Mtb* can persist in granulomas for many years, the host-pathogen interplay that drives granuloma formation is key for our understanding of TB pathogenesis.

In comparison to *Mtb*, *Mm* represents a less host-specialised pathogen that can infect an expanded niche of ectothermic species, including fresh- and salt-water fish, amphibians, but also invertebrates and protists[18,19,20,21,22,23,24,25,26]. Furthermore, in sporadic cases, *Mm* can infect endothermic animals, including humans, where it is generally restricted to the dermis, since it grows very poorly at human internal body temperature[27,28]. *Mm*, in its multicellular hosts can induce formation of granulomatous aggregates, similar to human-*Mtb* granulomas[27,28]. Almost two decades of use of the zebrafish model have proven that the two pathogens in their respective natural hosts rely on the activation of specific and evolutionary conserved disease-causing programmes, in order to induce granuloma aggregation[20,29,30,31,32]. This is also illustrated by the fact that in the rare cases of *Mm* infection in humans, this pathogen can still induce formation of dermal granulomas, that are phenotypically indistinguishable from those that *Mtb* would form in the same tissue[27,33].

The ability to grow within host macrophages is the key virulence attribute of pathogenic mycobacteria. This is well exemplified by studies in macrophage-like models, such *Dictyostelium discoideum* amoebas, where *Mm* can establish lasting intracellular parasitosis, similarly to those evoked *in vivo* in vertebrates and in macrophage cell cultures[25,34,35]. Interestingly, the pathogenesis in the monocellular models (including macrophage cultures) substantially relies on the capability to escape to the cytosol and on non-lytic ejection, which are important virulence traits that pathogenic mycobacteria

maintain also when infecting vertebrate host macrophages *in vivo*[25,36,37,38,39,40]. Despite this, in complex animal models, additional mechanisms of pathogenesis add to these core mechanisms of disease. These include, for example, the formation of distinct cell aggregates (the granulomas)[17], the manipulation of host angiogenesis[41] (**Chapter 6**) and the control of the inflammatory process via intricate cross talks that can solely occur in multicellular hosts[42]. Notably both aspects of mycobacterial pathogenesis that act at cellular and multicellular level are largely driven by virulence factors encoded by the RD1 (Region of Difference 1) locus, a genomic region conserved among tubercular bacilli (including *Mm*) and remarkably absent in environmental mycobacteria (e.g. *M. smegmatis*) and in the non-pathogenic BCG (bacillus of Calmette–Guérin) *M. bovis* strain, used to provide immunisation against *Mtb*.

Rodents and lagomorphs have been largely applied as *in vivo* models to study the histological aspects of mycobacterial disease. However, mice granulomas generally do not caseate[43] (necrotising granulomas, a typical characteristic of tuberculosis granulomas in humans) and the limited genetic tools available for the other rodents and lagomorphs that do form caseating granulomas (e.g. guinea pigs and rabbits[44]) make these models less attractive, due to restricted research opportunities. To date, the animal model that most closely resembles human tuberculosis is the macaque-*Mtb* model, that in terms of histopathology, physiopathology and disease progression/manifestation (including the existence of active and latent forms of TB) is near-identical to the human disease[45,46]. However, for obvious ethical, economical and practical restrictions, the use of this model is very limited. In contrast to the mice-*Mtb* model, caseating granulomas, wasting syndrome effects (another common symptom of TB displayed in primates) and latency have been observed when fish are infected with *Mm*[19,20,21,47].

Evolutionary, *Mtb* and *Mm* are very close and share about 85% of genome identity[1]. The genome of *Mm* is ~1.5 fold larger than the *Mtb* genome, which is likely related with the ubiquitous distribution of *Mm* in waters of different ecological niches and to the capability of *Mm* to infect a larger spectrum of hosts[1,28,48]. However, there is also a 14% of *Mtb*-specific genome sequence, which does not have orthologues in *Mm*, and which has likely arisen after their evolutionary divergence. About 8% of this *Mtb*-specific genetic material was estimated to have derived from horizontal gene transfer from other microbes that share a similar niche (e.g. respiratory microflora)[1,49,50,51,52] and is essentially related to niche adaptation (differences in temperature, organ-specificity, host-to-host transmission mechanisms), rather than to the central mechanisms of virulence.

Taken together, the *Mm* and *Mtb* species have most likely diverged from an *Mm*-like common ancestor, which was already adapted to the intra-macrophage life in vertebrate animals, was able to induce granuloma aggregation with a wide tissue tropism, and was able to alternate phases of life inside the host with phases of environmental life. This hypothesis is also justified by the fact that intramacrophage-specific and granuloma-specific genes exist in both *Mm* and *Mtb*, which are activated when the pathogens resides in phagocytic cells and when the granuloma aggregation is initiated[53,54].

Leprosy, the disease caused by *Mycobacterium leprae,* represents a severe cause of deformity and life-long disability in developing countries[55]. Unfortunately, the pathology of leprosy remains still poorly characterised, partly because culturing this pathogen axenically is near-impossible[56,57,58], and partly because there is a limited availability of

animal models[59,60,61]. The main experimental model for leprosy consists of the murine footpad infection[59] where the temperature of approximately 30°C mirrors the cooler tissues (skin and peripheral nerves) preferentially infected in humans. However, immunologically competent mice develop a poor infection with *M. leprae* and, apart from humans, armadillos are the only other known hosts of *M. leprae*, which represents the sole animal model to study the pathology of *M. leprae* infection in a natural host[60].

The evidence that leprosy predominantly affects peripheral tissues reflects the fact that, similarly to *Mycobacterium marinum*, the optimal growth temperature of this pathogen is lower than the human body temperature. Noteworthy, it has been described that *M. leprae* can infect and replicate in a variety of cold-blooded experimentally-injected species, including several fish, such as goldfish (*Carassius auratus*), spots (*Leiostomus xanthurus*), spotted sea-trout (*Cynoscion nebulosus*) and croakers (*Micropogon undulates*)[62,63]. Not only could *M. leprae* persist and replicate in these heterologous hosts, but the bacilli could also establish a distinctive type of intracellular parasitism of *M. leprae* infections, referred to as "lepra cells", large foamy macrophages containing numerous intracellular bacilli. These evidences suggest that the zebrafish model might be explored also as an experimental model to study *M. leprae* infection and, in particular, its use might help to elucidate the function of the innate immune cells and of immune signalling pathways in the onset of different manifestations of leprosy.

Function of chemotactic cues in driving granuloma aggregation

Mtb strongly induces chemokine expression and both TB patients and cell/animal models infected with *Mtb* exhibit a rapid induction of many of these chemotactic peptides[64]. We and others have found that also zebrafish infected with *Mm* displays large induction of chemokines, which include the mammalian counterparts of CXCL9-10-11 (CXCL11aa-ae-af-ag, **Chapter 3-4**), CXCL1-2-3-5-6-7-8 (Cxcl8a-8bb-18b[65,66,67] **Chapter 7**) and CCL2[68] (**Figure 1**). Since mycobacteria mostly reside in macrophages, these cells experience profound transcriptomic changes and represent a primary source of chemokine ligands[69,70,71,72,73,74,75,76] (**Chapter 1**). In agreement with this, we have found here that infected macrophages inside the zebrafish host largely upregulate *cxcl11aa*, the ligand of Cxcr3.2 (**Chapters 3-4**). However, also non-infected cells participate in the production of chemokines. Our studies, for example, show that Cxcl18b, a neutrophil-specific zebrafish chemokine, functionally similar to the ELR+ chemokines of mammals (**Chapters 1,7**), does not derive from infected or uninfected phagocytic cells (macrophages and neutrophils), but from stromal cells that reside within the granuloma microenvironment (**Figure 1D**). Similarly, it was suggested that one of the putative zebrafish orthologues of CCL2, which is able to induce macrophage chemotaxis, is mostly expressed by epithelial cells (**Figure 1A**)[68].

The initial stages of granulomas are characterised by a continuous, bidirectional trafficking of innate immune cells[22,41] (**Figure 1**, **Chapter 2**). We and others have shown that, since most macrophages are unable to readily eradicate the intracellular mycobacterial infection, the pathogen can take advantage of this in/out trafficking that guarantees a continuous supply of the mycobacterial infection niche (**Figure 1B**) and generates a mechanism for secondary dissemination[41,77] (**Figure 1B,E**, **Chapters 2-3**).

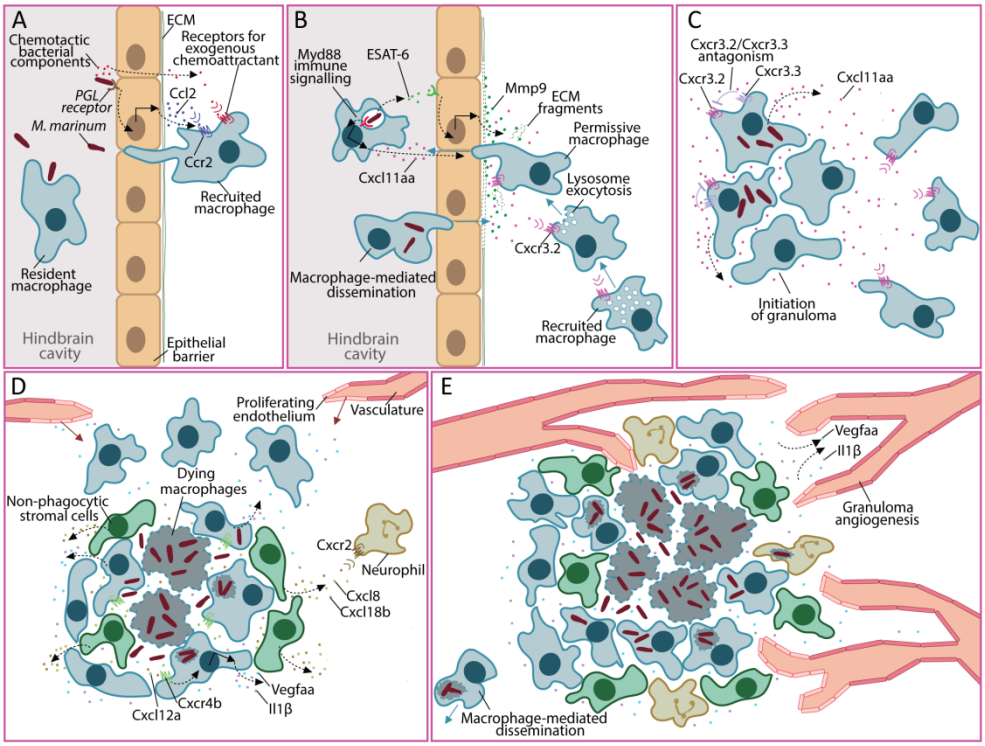

Figure 1. Host and pathogen signalling mechanisms that drive granuloma formation. A. Recruitment of macrophages to invading mycobacteria (e.g. injected in the hindbrain cavity) can be guided by several bacterial molecules. Additionally, it has been proposed that recognition of phenolic glycolipids (PGL) by the epithelium mediates induction of the macrophage chemokine CCL2. However, mycobacteria are also phagocytosed by tissue-resident macrophages and active chemotaxis may not be essential to establish the initial intracellular parasitosis. **B.** When resident in macrophages, mycobacteria produce ESAT-6, which induces expression of Mmp9 by the epithelium. By digesting the extracellular matrix (ECM), Mmp9 facilitates macrophage infiltration into the infected focus. Once the intramacrophage infection is established, this triggers expression of chemokines in the host macrophages. In particular, the macrophage chemokine Cxcl11aa is highly induced in infected macrophages, with a mechanism that requires Myd88-dependent pathogen sensing. Signalling of Cxcl11aa via its receptor Cxcr3.2 can control lysosomal function, for example via induction of exocytosis, therefore, this signalling not only controls macrophage recruitment, it might also contribute to generate and maintain an infection-permissive phenotype. Already from these very initial stages, some infected macrophages are seen to mediate mycobacterial dissemination to other tissues. **C.** The Mmp9 and Cxcl11aa signalling mechanisms continue to sustain macrophage recruitment and the aggregation of macrophages indicates the initiation of granulomas. Although mechanistically still unclear, the atypical Cxcr3.3 receptor exerts a host protective function and limits infection, most likely by antagonising Cxcr3.2. **D.** Macrophages that do not contain infection die releasing the bacteria, most of which will be re-phagocytosed when still encapsulated in cell debris. The inflammatory properties of the granuloma progressively increase. Neutrophil chemokines, including Cxcl8a and Cxcl18b, are also locally released. Induction of local hypoxia determines the production of Vegf and primes neutrophil protective functions. Cxcr4b, which can control the level of inflammation by modulating production of Il1β, cooperates with the Vegfaa signalling to support inflammation-associated angiogenesis. **E.** The vascularisation of the granuloma further supports its expansion and departure of infected cells from the lesion can seed new granulomas.

Despite their capability to persist in macrophages, pathogenic mycobacteria grow very slowly in their host, when compared to non-pathogenic environmental mycobacterial strains, such as *M. smegmatis*[78,79]. These two opposing forces, together with the capability of macrophages to contain the infection to a certain extent in healthy situations, generate a balanced dynamic equilibrium. This equilibrium is characterised by granuloma lesions that expand slowly or do not expand at all, and by a disease that develops into its active form only in about 10% of cases[80,81]. This balance of host and pathogen responses probably emerged from host-pathogen reciprocal adaptations and likely represents an important aspect of evolutionary fitness, as both the host and the pathogen survive when the infection is contained by granulomas[80,81,82]. The zebrafish model has been used to show that bacterial persistence in granulomas is accomplished by the spread of bacteria from dying macrophages to newly arriving ones[22]. When a macrophage can no more contain the intracellular bacterial replication, it undergoes cell death and leaves viable bacteria still encapsulated within the cell debris. Simultaneously, new uninfected macrophages are recruited to the granuloma, which engulf the bacteria and the remains of dead macrophages (**Figure 1D**).

The concomitant activity and the integration of host and pathogen factors plays an important role in the process of granuloma formation. Previous studies implicated the ESX-1 secretion system (one of the virulence determinants encoded by the RD1 locus) into driving macrophage aggregation during the initial stages of granuloma formation, most likely via the release of the virulence factor ESAT-6 (**Figure 1B**)[83]. However, the ESAT-6 virulence factor requires the response of the host to mediate this mechanism. A study in zebrafish showed that ESAT-6 induces the production of the matrix metalloproteinase 9 (Mmp9) in epithelial cells surrounding the nascent infectious lesion, which in turn facilitates macrophage infiltration and formation of granulomatous cell aggregates (**Figure 1B**)[83].

Here we have found that the induction of the macrophage chemokine Cxcl11aa is also important to sustain the granuloma aggregation and to maintain a proper macrophage trafficking via its receptor Cxcr3.2 (**Figure 1B**, **Chapters 3-4**). In fact, *cxcr3.2* mutant macrophages were recruited to a reduced extent to the infectious foci, which resulted in delayed granuloma expansion and reduced bacterial dissemination (**Chapter 3**). Sorting of macrophages from infected larvae followed by transcriptional quantification, showed that macrophages themselves are the main responsible cell type for the production of Cxcl11aa. These findings suggest a macrophage-autonomous mechanism by which mycobacterial infection induces production of the macrophage chemoattractant Cxcl11aa, to support further macrophage aggregation (**Figure 1B**, **Chapters 3-4**). Notably, Cxcl11aa induction does not require the presence of the RD1 locus while it requires active Myd88 (Myeloid differentiation factor 88)-dependent immune signalling (**Chapter 4**). Myd88 is a central adaptor that links innate pathogen recognition via most of the Toll-like receptors (Tlr) to a downstream machinery that modulates transcription of immune and inflammatory genes. Signalling via Tlr/Myd88 has been shown to be fundamental for the induction of inflammatory genes in the zebrafish-*Mm* model and to drive host protection, for example by activating bacterial clearance via autophagy[84]. Notably, Myd88-deficient larvae develop a more severe mycobacterial infection, but strikingly fail to upregulate Cxcl11aa (**Chapter 4**).

The studies presented above indicate that the ESX-1/ESAT-6/Mmp9 and the Myd88/Cxcl11aa/Cxcr3.2 mechanisms of recruitment represent two distinct, but synergistic, systems (**Figure 1B**). However, differently from the Myd88/Cxcl11aa/Cxcr3.2 axis, the ESX-1/ESAT-6/Mmp9 pathway is not likely to induce active recruitment, rather it would facilitate macrophage infiltration by generating local inflammation and by loosening the matrix resistance (**Figure 1B**). It is however also possible that local activity of Mmp9 might facilitate recruitment directly by mediating release of matrix-derived chemotactic peptides or by processing chemotactic mediators[85]. On the other hand, it must be noted that in mammals, MMP9 processing has been shown to exclusively activate neutrophil chemokines (and not macrophage chemokines) and to produce matrix debris that are solely able to activate neutrophil recruitment[85]. Therefore, the indirect macrophage recruitment model is the most likely mechanism of action of Mmp9. In contrast to the passive Mmp9-mediated recruitment, the Myd88/Cxcl11aa/Cxcr3.2 signalling can induce direct macrophage recruitment to the infection focus, since the Cxcl11aa/Cxcr3.2 signalling can mediate directional cell migration, sustain cell anteroposterior polarisation and increase basal motility (**Figure 1B**, **Chapters 3-4**).

The zebrafish model has helped to clarify that granuloma aggregation, which was historically regarded as a host-protective mechanism, can benefit the bacteria in many different ways: recruitment and coalescence of macrophages fuel the infectious focus with novel cells to be infected and to replace the dying ones, which is an advantage for a pathogen that essentially is adapted for an intracellular life (**Figure 1**)[22]. Additionally, by curtailing tissue necrosis with efferocytosis (collection and clearance of cell debris), newly recruited macrophages also moderate tissue inflammation to a level that is suitable to maintain the parasitic relationship (**Figure 1D**)[42,86]. Furthermore, departure of infected macrophages from a mature granuloma can seed new granulomas in healthy tissues of the host (**Figure 1E**, **Chapters 2-3**)[41,77]. Finally, establishment of an intra-macrophage niche permits host signalling subversion and the initiation of specific pathogen-beneficial programmes, which include the induction of angiogenesis (**Figure 1D-E**, **Chapter 5**) [41]. Taken together, it is not surprising that attenuation of macrophage trafficking can benefit the host and, in agreement with this, the knockdown of *mmp9* and the null mutation *cxcr3.2* confer resistance to the host, reducing infection burden and granuloma formation (**Chapter 3**)[83]. Host-beneficial effects from disrupting CXCL9-10-11/CXCR3 signalling and Mmp9 function have also been observed in murine animal models for TB and have been suggested by human clinical and genetic association studies. CXCR3 knockout BALB/c mice developed a limited disease upon exposure to *Mtb*[87] and a study performed on the Chinese population revealed a host-beneficial association between TB and a -135G>A proximal promoter polymorphism of CXCL10. This replacement, which is in the proximity of a putative NFκB binding site, was suggested to impair the infection-dependent inducibility of this gene[88]. Similarly, increased MMP9 secretion is associated with increased severity and mortality in TB meningitis[89,90] and increased expression of all CXCR3 ligands (CXCL9-10-11) is correlated with active TB[91].

Initial macrophage recruitment to mycobacteria: insights into a "chicken and egg"-like paradox

Intriguingly, both the Cxcl11aa/Cxcr3.2 and the ESAT-6/Mmp9 pathways that drive macrophage recruitment to mycobacterial infectious foci substantially require a pre-existing intramacrophage infection (**Figure 1B**). Infection-dependent induction of *cxcl11aa*

occurs in macrophages and requires Myd88-signalling and active pathogen recognition (**Chapter 4**). Similarly, the ESAT-6 secretion by mycobacteria requires pathogen adaptation to the intramacrophage life style[83]. Therefore, these two recruitment axes seem to be predominantly exploited to maintain an appropriate macrophage supply once the lesion has been established, rather than mediate its very initial onset (**Figure 1A-B**). Another zebrafish study has suggested that an additional chemokine axis, an orthologue of the mammalian CCL2/CCR2, might be important for the initial recruitment of macrophages to the invading bacteria, via a mechanism that does not require Myd88 signalling and intramacrophage infection (**Figure 1A**)[68]. This axis was shown to rely on recognition of extracellular mycobacteria via specific mycobacterial wall lipids by the neighbouring epithelial cells. This recognition would, in turn, activate production of the macrophage chemokine Ccl2 and therefore macrophage recruitment. However, evidence from the murine model and from human disease-polymorphism association studies are contradictory on the function of CCL2/CCR2. In mice, this axis seemed to permit better containment of high doses of bacteria but did not abolish granuloma aggregation neither reduced infection susceptibility to low doses[92,93], which is in contrast with the hypothesis that the Ccl2/Ccr2 pathway would not require Myd88 signalling and classical Tlr-mediated pathogen recognition. On the contrary, these data suggest that CCL2/CCR2 may not be crucial to establish the initial parasitism (considering that CCR2-deficient mice have the same susceptibility to low-dose infection as wt), rather to contain the effects provoked by a larger and more inflammatory inoculum. Human polymorphism studies also fuel the debate on the real significance of CCL2, since some studies indicate that higher expression levels of CCL2 increase susceptibility to infection in some populations, although the same correlation could not be replicated in other populations[94,95]. A similar indication in this direction also comes from the fact that PGL (the mycobacterial lipid that has been proposed to mediate CCL2 release in Myd88-independent conditions[68]) is not essential for the virulence of *Mtb* strains and many clinical *Mtb* isolates exist that do not express PGL[96]. An alternative to the CCL2-mediated mechanism could be that the initial establishment of intramacrophage parasitosis may depend on direct chemotaxis to bacterial components or may simply not require active recruitment, since the tissues, including the human alveoli, contain resident macrophages that can readily engulf invading pathogens when these are presented in a limited number (**Figure 1A**).

Function of chemokines and cell motility in controlling macrophage intrinsic immune competence

We have found that in uninfected conditions, macrophages express basal levels of *cxcl11aa* and that the Cxcr3.2-dependent signalling can facilitate macrophage basal patrolling under physiological conditions, presumably by activating an autocrine loop (**Chapters 3-4**). This mechanism possibly generates continuous adjustments of macrophage anteroposterior polarity that leads to random walks. The capability to random patrol might be intrinsic to macrophages and might influence the ability of these cells to exert a sentinel function into tissues. In this perspective, the establishment of initial parasitism could be impacted by Cxcl11aa/Cxcr3.2 signalling, with mechanisms that not necessarily require Myd88-dependent upregulation of *cxcl11aa*. Our own evidence is that macrophages use the Cxcl11aa/Cxcr3.2 signalling pathway to maintain their capability to random patrol and that in the absence of this signalling axis, macrophages upregulate lysosomal genes and become more microbicidal (**Chapters 3-4**). Thus, our data indicate that Cxcl11aa/Cxcr3.2 signalling directly correlates chemotaxis/motility to intrinsic immune competence,

although the molecular mechanism that triggers this phenotype still remains to be elucidated. A known connection between motility and lysosome function is that chemokine signals lead to the fusion of lysosomes to the plasma membrane to sustain cell movement[97]. Therefore, one hypothesis to explain why lysosomal genes are upregulated in a situation of deficient motility is that the cell is alerted and promotes lysosomal biogenesis, in the attempt to restore normal motility dynamics. There is evidence that, if the flux of lysosomes to the plasma membrane is compromised, this leads to the CLEAR (Coordinated Lysosomal Expression and Regulation) response and production of more lysosomes[98,99,100]. In addition, macrophage motility seems to be severely affected by excessive phagocytosis and by the inability to digest phagocytosed debris[101]. In lysosomal storage disorders (LSD), macrophages are amply vacuolated and the presence of these large intracellular compartments severely perturbs their motility[101]. Activation of the lysosome pathway can rescue the LSD disease by facilitating elimination of the undigested inclusions[98,99,102], which in turn would restore motility too. Therefore, the reduction of motility below certain levels might function as an alarm signal that suggests a post-phagocytosis phenotype and therefore increased need of digestive lysosomal contents.

Several genetic diseases associated with LSD are associated with impaired lysosomal function and increased susceptibility to infections[103,104,105]. Also in zebrafish the knockdown of the orthologues of three genes linked with LSD in human (*glucocerebrosidase 1*, *hexosaminidase A*, and *arylsulfatase A*) resulted in LSD and hypersusceptibility to *Mm* infection[101]. In this respect, our study indicates an important complementary aspect: if the content of functional lysosomes is increased without leading to the LSD phenotype, this benefits the host, by boosting the intrinsic capability of macrophages to counteract infection, with beneficial rather than detrimental effects. This hypothesis is in line with the proposed idea that genetic conditions that in homozygosis associate with LSD and increased susceptibility to TB, might provide resistance to TB when carried in heterozygosis.

To discuss the hypothetical links between LSD-associated genetic conditions and susceptibility to TB, the Ashkenazi Jewish (AJ) population (and their descendants) represents an interesting case, since 6 independent mutated alleles for four LSD diseases (2 mutations leading to Tay-Sachs disease, 2 mutations leading to Gaucher disease, 1 mutation leading to Niemann-Pick disease and 1 mutation leading to mucolipidosis type IV) are fixed in this population with anomalously increased frequencies[106,107]. The selection of these four genetic disorders is unlikely derived from stochastic genetic drifting events and might be explained if these disorders provide a selective advantage to the heterozygote carriers. Notably, among the AJ population, the frequency of TB-associated deaths is reduced, when compared to ethnically-separated populations residing in similar areas[108,109.110,111]. Additionally, dividing the AJ population based on their geographical origin, it was shown that the AJ groups with ancestry in areas of higher incidence of TB have also increased prevalence of the Tay-Sachs allele, compared with the AJ groups that were originally from areas with reduced TB prevalence[112,110,111].

The heterozygote advantage hypothesis for the Tay-Sachs mutations in TB has been debated[106,113,114], since the proportion of the LSD-allele carriers does not occur at such high frequency (3 to 6% of AJ population[107,112]) to provide a substantial population protection, differently, for example, from the well-known associations of sickle-cell anaemia and other haemoglobinopathies with malarial endemic areas (3 to 40% of endemic populations[115]).

However, recent studies have elucidated how the Tay-Sachs allele could provide carrier advantage against TB. The Tay-Sachs alleles consist of genetic mutations of the gene coding for the alpha subunit of hexosaminidase A (HEXA), an important enzyme that mediates lipid degradation (especially neuronal gangliosides). Hexosaminidase A is a two-subunit enzyme and its beta-subunit is encoded by a different gene (HEXB). A second functional isoenzyme, hexosaminidase B, exists which is instead composed of two beta-subunits. In Tay-Sachs patients, the disease is caused by the homozygote mutation of the HEXA gene (and concomitant complete absence of functional hexosaminidase A isoform). Mutations of HEXB gene are very rare (Sandhoff disease), and lead to more severe dysfunctions and LSD. The main consequence of the Tay-Sachs disease is a deficient digestion of gangliosides, which accumulate into cells and provoke vacuolation. Interestingly, it has been found that Tay-Sachs heterozygotes express higher levels of hexosaminidase genes and have increased enzymatic activity of the hexosaminidase B isoenzyme than healthy individuals[109,116,117], which permits normal processing of accumulating lipids and, prevents the onset of Tay-Sachs disease. Of note, hexosaminidases are target of the CLEAR response and can be induced in lysosomal stress conditions via the CLEAR transcriptional programme[99,102]. Therefore, a possible explanation of why Tay-Sachs carriers are less susceptible to TB might be that this deficiency in heterozygosis induces lysosomal stress and the CLEAR response, in the attempt to restore normal hexosaminidase activity. However, if lysosomal stress and the CLEAR response are activated, this will lead to increased expression, not only of hexosaminidases, but also of many other lysosomal genes, and therefore to a better intrinsic microbicidal function. That dysfunction of one lysosomal component is accompanied by upregulation of many other lysosomal-related genes is a recurrent observation in LSD patients and in LSD *in vitro* models[99,102,118]. Additionally, most of LSD syndromes can be, at least partly, restored by transcriptional stimulation of CLEAR *in vitro*[99,102,119]. Therefore, these observations suggest that genetic manipulation of the lysosomal function at transcriptional level might be explored for therapeutic purposes also against bacterial infections.

It is well known that mycobacteria can counteract phagosome maturation and can mount countermeasures to persist in acidified compartments. On the other hand, it has been shown that they are susceptible to anti-bacterial autophagy and degradation via autophagolysosomes[84]. Intriguingly, among the mechanisms by which mycobacteria hijack the host macrophage, there is the promotion of lipid inclusions formation, which results in the "foamy cell" macrophage phenotype. It has been hypothesised that the fatty acids derived from lipid bodies might be an important energy source for the pathogen, or that they provide to the mycobacteria a mechanism to interfere with the eicosanoid biosynthesis and therefore with the production of pro- or anti-inflammatory components[120]. Given the fact that lipid bodies can be counteracted via induction of CLEAR activation, triggering lysosomal stress might contribute to fight this peculiar mechanism of virulence.

In mammals, the CLEAR pathway is controlled by a group of factors, including MITF (microphthalmia-associated transcription factor), TFEB, TFEC and TFE3 (transcription factor EB, EC, E3). These genes are conserved in zebrafish (**Figure 2A**), which has a single orthologue for TFEB and TFEC (Tfeb, Tfec) and 2 orthologues for MITF and TFE3 (Mitfa, Mitfb, Tfe3a, Tfe3b)[121]. Additionally, there is evidence that the genetic control of the CLEAR response is similar in humans and in zebrafish[122]. Our transcriptome data suggest that the increased lysosomal function of *cxcr3.2* mutant macrophages derives from

activation of the CLEAR response. While expression of Mitf, Tfeb and Tfe3 genes did not significantly differ between *cxcr3.2* mutants and wt, Tfec was approximately 1.8 fold downregulated in *cxcr3.2* mutants (**Chapter 4**).

TFEB is currently the most studied inducer of the lysosomal stress response[123]. However, while TFEB and TFE3 are ubiquitously expressed[123], TFEC expression is restricted to myeloid cells[124], indicating a specific function of this gene in innate immune cells. TFEC function has not been characterised to the same extent as that of the other members of this transcription factor family and its function needs further elucidation. It is known that in physiological conditions TFEC mutation does not generate any apparent phenotype in mice[125]. Interestingly, TFEC could be induced in macrophages by stimulation with T-helper 2 cytokines (IL4 and IL13) or lipopolysaccharide treatment[125]. However, even after its upregulation, TFEC did not evoke major transcriptional changes in murine macrophages. It should be noted that TFEC diverges significantly, in terms of structure, from the other TFEs (**Figure 2**). In the study that initially identified TFEC, it was found that this factor can act a negative regulator of the other members of the TFE family, by formation of non-functional heterodimers and by DNA binding competition[126]. Both TFEB and TFE3 possess an important MITF/TFEB N-terminal homology domain and a conserved bridging sequence between the N-terminal domain and the basic helix-loop-helix (bHLH) DNA-binding domain (**Figure 2B**). This linking sequence was shown to be important to activate transcription of the canonical TFE target genes, although not required for the DNA binding *per se*[126,127]. Notably, the MITF/TFEB N-terminal homology domain of TFEC is truncated when compared to those of TFEB and TFE3 (**Figure 2B**), and the region immediately upstream of the bHLH domain is also specifically divergent in TFEC. On the other hand, TFEC maintains highly conserved bHLH and basic Leucine zipper (bZip) domains, which are required to bind the DNA and to form homo/heterodimers within the TFE family members. The alterations in the N-terminal and in the bridging region involved in transcription regulation justify why induction of TFEC alone or its knockout, did not provoke large transcriptional consequences, as this factor, that antagonises TFEB and TFE3, would display transcriptional effects only in situations where TFEB and TFE3 are activated, such as during the CLEAR response. Structurally, zebrafish Tfec resembles closely the human TFEC and displays similar aberration in the MITF/TFEB N-terminal homology domain and in the region that supports target transcription in TFEB and TFE3 (**Figure 2B**). Therefore, this suggests a conserved function of Tfec and indicates that the zebrafish model could contribute substantially to understand the importance of this factor.

Reduced expression of *tfec* in *cxcr3.2* mutant macrophages indicates that *cxcr3.2* mutation might lead to the CLEAR response by suppressing a negative regulator of Tfeb/Tfe3. In future work, this hypothesis could be tested by overexpressing *tfec* in the *cxcr3.2* mutant background, which is predicted to prevent the induction of the CLEAR programme and thereby revert the increased microbicidal capacity of *cxcr3.2* mutant macrophages.

Concluding, our results indicate that the CXCR3-CXCL11 axis exerts different functions on macrophages, and that disruption of this axis reduces macrophage recruitment to infection, while enhancing the macrophage intrinsic microbicidal capability via induction of a lysosomal stress response (**Chapters 3-4**). In response to these functions, mycobacteria seem to have evolved several mechanisms for manipulating this axis to regulate macrophage trafficking during granuloma aggregation, drive macrophage-

mediated dissemination and suppress the basal bactericidal property of the host cell. Therefore, use of CXCR3 antagonists may represent a therapeutic regime that, by acting on a single target, could counteract mycobacterial infection at multiple levels. Additionally, considering the work performed in the murine model, CXCR3 blockade might be beneficial also to prime a more efficient anti-mycobacterial T-cell mediated adaptive immunity[87].

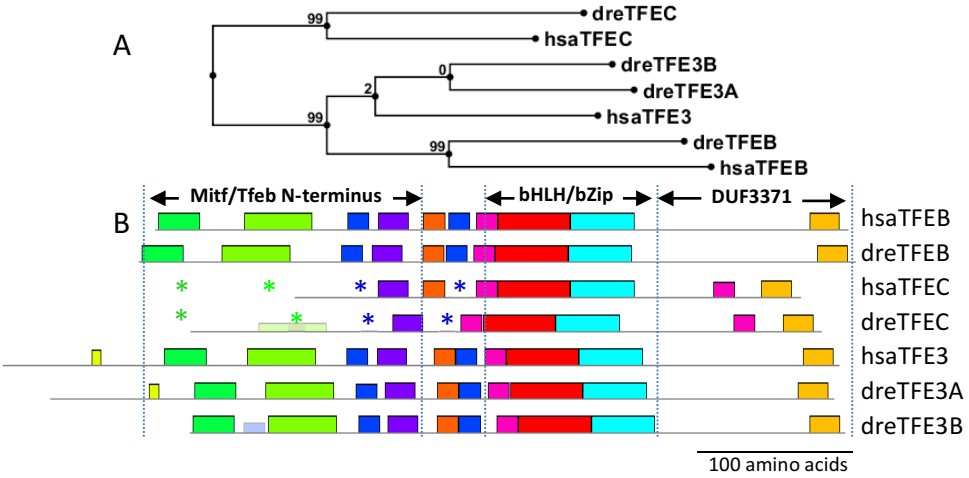

Figure 2. Conservation of Transcription factor E family members between human and zebrafish. A. Sequence alignment tree, showing that in zebrafish (dre) there are direct homologues of the three human (hsa) transcription factor E family members. There is only one homologue of TFEB and TFEC while there are two homologues of TFE3. The tree also shows that the TFEC cluster diverges from the TFEB/TFE3 cluster. **B.** Conservation of protein domains and amino acid motifs in TFE sequences. TFE proteins have essentially three domains: a conserved Mitf/Tfeb domain, a DNA binding/protein dimerisation domain composed by a basic helix-loop-helix (bHLH) followed by a basic Leucine zipper (bZip) consensus and finally a variable C-terminal domain named DUF3371 (domain of unknown function 3371). These domains encompass conserved sequence motifs, visualised here with boxes of identical colour and size. Notably, the N-terminal region of both human and zebrafish TFEC are characterised by absence (or poor amino acid conservation indicated by fainter boxes) for several motifs otherwise maintained in TFEB and TFE3 (asterisks). Like the other TFE members, TFEC proteins highly conserve instead the domain that serves to bind the DNA and to form homo- or hetero- dimers (bHLH/bZip). The N-terminal region and the area immediately upstream the bHLH/bZip are important to modulate the efficiency of transcription of target genes, which is the reason why TFEC is unable to efficiently drive gene expression and essentially is believed to act as a regulator of the other TFE members. The fact that the zebrafish Tfec has similar sequence alterations and truncation of the N-terminal as the human TFEC indicates that zebrafish Tfec may recapitulate the function of the human counterpart. The TFE tree was generated by CLC Bio workbench, from full-length protein sequence alignment. The identification of conserved sequence motifs was obtained by MEME motif discovery suite (http://meme-suite.org/).

Atypical CXCR3 receptors and emerging functions of ACKRs in inflammatory diseases

Atypical chemokine receptors (ACKRs) are 7-loop transmembrane proteins, evolutionary close to the classical chemokine receptors. However, because of a modified or missing canonical E/DRY motif and altered micro-switch elements, ACKRs are unable to interact with G-proteins and are therefore unable to induce G-protein coupled receptor (GPCR) signalling (**Chapters 1,5**)[128].

The most obvious consequence of the altered sequence in ACKRs is that these molecules are unable to directly mediate cell migration[128]. However, ACKRs are not silent molecules and can activate GPCR-independent signalling and exert important regulatory functions[129]. An important feature of the ACKR class is for example the capability to efficiently mediate ligand internalisation[130]. Because ACKRs are generally not very specific in terms of ligand binding, these receptors can intercept a wide range of chemokine ligands and mediate their transport to the lysosomes for degradation[128,130]. In some circumstances the ACKR/ligand recycling has also been seen to mediate the transcytosis of chemokines and therefore their transport across biological barriers[131]. Several ACKRs have shown to play an important function to control speed and directionality of chemotactic movements, by tightly titrating the chemokine ligand concentration and by shaping the chemokine gradients[132]. Since binding of excessive amounts of chemokine ligands to their classical receptors can induce desensitisation via receptor internalisation, the expression of ACKRs can also help to maintain appropriate signalling by avoiding complete downregulation of classical chemokine receptors[133].

There is increasing evidence that ACKRs also control the overall inflammatory response, by preventing exceeding chemokine-derived inflammatory signals. This condition has been recently demonstrated by using a murine knockout for the wide spectrum chemokine scavenger D6/ACKR2. The mutant animals are basically indistinguishable from wt littermates in physiological conditions. However, when challenged by wounding, chemicals, cancer or infections, these animals develop a higher inflammatory status[134,135,136,137138]. In infections with *Mtb,* the uncontrolled inflammation occurring in D6/ACKR2 knockouts is lethal and D6/ACKR2 expression is essential to prevent the inflammatory storm and to attenuate excessive infiltration of leukocytes into the lungs[137]. Similar anti-inflammatory functions have been demonstrated also for other ACKRs that scavenge inflammatory chemokines, for example DARC/ACKR1[139,140], which can sequester large amounts of circulating chemokines and dampen leukocyte activity. However, the function of DARC/ACKR1 is multifaceted, given the fact that this receptor can also sustain the transport of chemokines through physical barriers and function as a chemokine reservoir which can in some circumstances generate opposing pro-inflammatory phenotypes[141,142,143]. In fact, during inflammatory disorders of the brain, binding of chemokines to DARC can facilitate their shuttling through the blood brain barrier via transcytosis. Additionally, binding of chemokines to DARC expressed by erythrocytes can reduce exaggerated levels of freely circulating chemokines during acute inflammatory responses but also help to maintain a steadier concentration of them in the longer term, since chemokines can continue to dissociate from the receptor and become again available.

In **Chapter 5** we have identified and characterised a novel chemokine receptor, Cxcr3.3, which, based on its altered E/DRY motif (Glu/Asp-Arg-Tyr) and on altered micro-switch elements, likely represents an ACKR. Similarly to the results found in D6/ACKR2 mutants, *cxcr3.3* mutants displayed an increased infection burden, and intriguingly an opposing phenotype as mutants for the canonical chemokine receptor *cxcr3.2* (Chapter 3-5). This phenotype suggests that *cxcr3.3* may antagonise *cxcr3.2* function, for example by attenuating the inflammatory response or moderating leukocyte infiltration to the infected tissues (**Figure 1C**)[138]. Based on sequence and synteny reconstruction, we have found that atypical Cxcr3-like receptors exist in a large number of fish species, although not in tetrapods (**Chapter 5**). Due to high sequence similarity with classical Cxcr3 genes and due to its close synteny to the other *cxcr3* genes throughout fish species, it is likely that the atypical *cxcr3* products have originated from an ancestral *cxcr3* gene which encoded a functional chemokine receptor, able to bind CXCL9-10-11-like ligands. To our knowledge, in sharks (*Callorhinchus milii*) there is also only one copy of Cxcr3, which contains a normal E/DRY sequence, while in ray-fish that have diverged before the teleost-specific whole genome duplication such as the spotted gar (*Lepisosteus oculatus*), there is already existence of E/DRY-depleted Cxcr3 genes (**Figure 1**, **Chapter 5**). Therefore, the origin of atypical *cxcr3* genes may have occurred before the teleosts whole genome duplication, but after the divergence of bony fishes from cartilaginous fishes.

We currently do not know whether Cxcr3.3 acts as a scavenger of Cxcl11-like chemokines, the classical ligands of the Cxcr3.2 receptor (**Chapter 3-4**). However, since Cxcr3.3 forms a homophyletic group and is in a synteny cluster with Cxcr3.1 and Cxcr3.2 throughout fish species (**Figure 3**, **Chapter 5**), this is a plausible hypothesis. In this case, the *cxcr3.3* genes may have differentiated in the attempt to more strictly regulate the activity of the CXCR3 axis and its central role in adaptive and innate immunity. We also currently do not know whether this CXCR3 atypical axis, that appears a fish-specific system, has parallels in mammals. Intriguingly, despite the lack of atypical CXCR3 receptors, mammalian CXCL9-10-11 ligands can be still scavenged by two other ACKRs, CXCR7/ACKR3, and DARC/ACKR1[130,144,145] (**Chapter 1**).

In Actinopterygii, which includes the zebrafish, an orthologue for every human ACKR can be found, with the exception of DARC/ACKR1, which can be first found in coelacanths (*Latimeria chalumnae*), a rare group of fish regarded as living fossils that are directly related to the last fully-aquatic ancestor of tetrapods (**Figure 3**). The coelacanth does not possess an atypical Cxcr3, as its three copies of the Cxcr3 gene all contain intact E/DRY motifs. This suggests that the evolution of ACKR1 has occurred during speciation of Sarcopterygii (lobe-finned fish and tetrapods) and coincided with the loss of atypical CXCR3 isoforms. It is possible that the emergence of a novel wide spectrum chemokine scavenger (DARC/ACKR1) determined the loss of the atypical CXCR3, due to redundant function. It should also be mentioned that ACKR3/CXCR7, which is also able to scavenge CXCL11, is not redundant with DARC/ACKR1, since it is best known for its scavenging activity against CXCL12[132,144]. This receptor is the sole ACKR able to bind CXCL12 and, similarly to the classical receptor of CXCL12 (CXCR4), exerts a unique function in mammals, as demonstrated by the fact that CXCR7 mice homozygote mutants are lethal *in utero.*

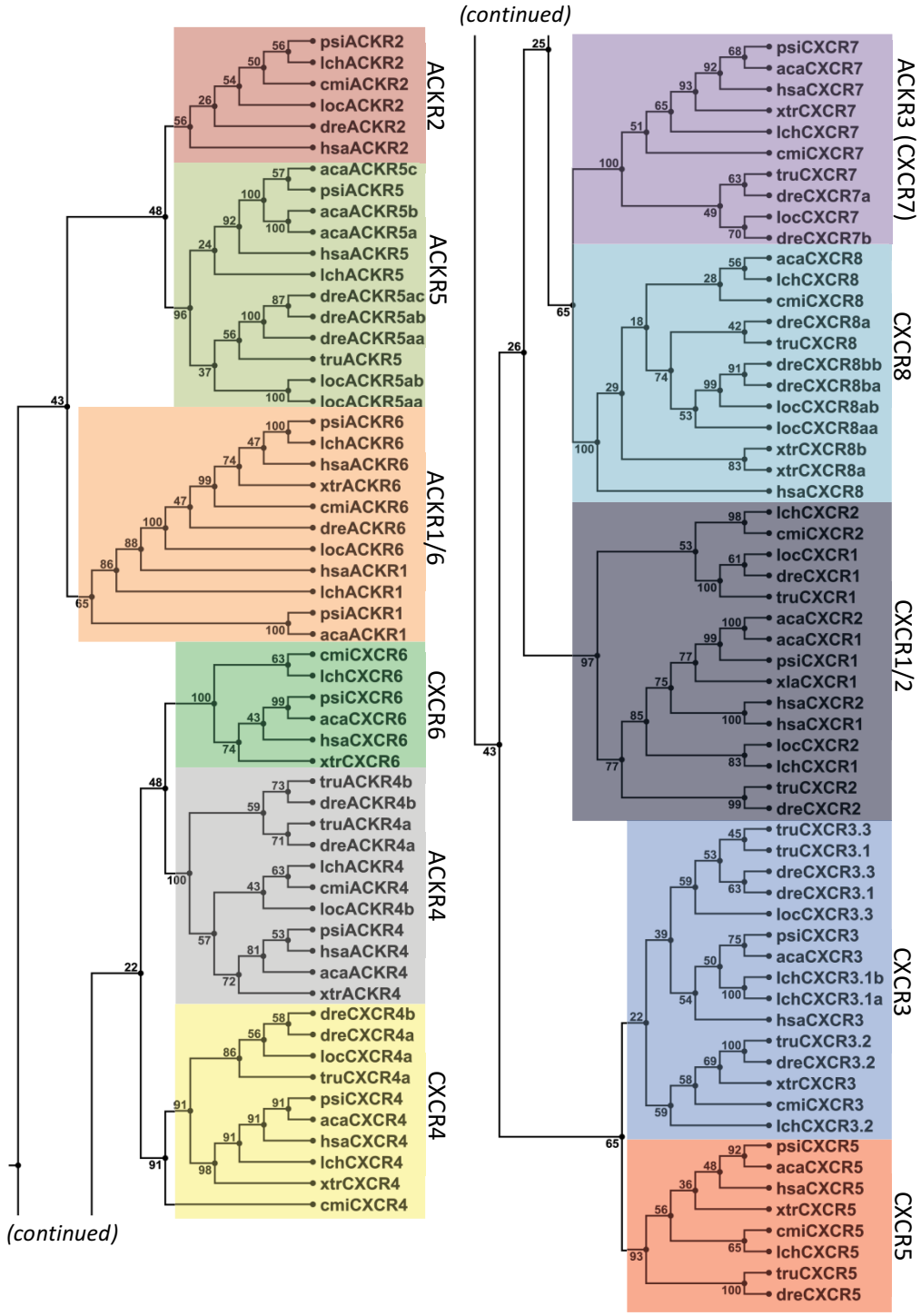

Figure 3. CXCR and ACKR phylogenetic tree *(Legend on the next page)*.

Figure 3. CXCR and ACKR phylogenetic tree *(Figure on the previous page)***.** Sequences were obtained from Ensembl, ncbi or uniprot databases. The tree was constructed from full-length sequence alignments, using CLC bio workbench. Legend: aca: *Anolis carolinensis* (anole lizard); cmi: *Callorhinchus milii* (elephant shark); dre: *Danio rerio* (zebrafish); hsa: *Homo sapiens* (human); lch: *Latimeria chalumnae* (coelacanth); loc: *Lepisosteus oculatus* (spotted gar); psi: *Pelodiscus sinensis* (Chinese softshell turtle); tru: *Takifugu rubripes* (fugu); xtr: *Xenopus tropicalis* (western clawed frog). Note: since Cxcr1/2 receptors have not been identified in *Xenopus tropicalis*, we included the CXCR1 sequence of the close species xla: *Xenopus laevis* (African clawed frog) to provide the tree with an amphibian sequence for this class of receptors.

According to alignment-based phylogenetic reconstruction, it is unlikely that ACKR1 had radiated directly from a CXCR3-like ancestor, due to large sequence differences. However, by blasting the lchDARC/ACKR1 protein to the coelacanth protein database, its closest intraspecific orthologue is in fact the lchCxcr3.1. Unfortunately, the coelacanth genome has not been assembled, which makes it impossible to support phylogenetic reconstructions with synteny studies. However, a hypothesis that could explain the large divergence of ACKR1 from the other chemokine receptors (including CXCR3) could be that stringent selective pressures have acted on this atypical receptor and mediated its diversification. This idea is supported by that fact that we have examples of large diversification of DARC/ACKR1 sequences in primates, due to selective advantages provided by DARC/ACKR1 variants against *Plasmodium* infections[146,147,148]. DARC/ACKR1 can in fact also act as a parasite receptor and facilitate entry of certain *Plasmodium* species into the erythrocytes.

Concluding, *in vivo* studies during the course of inflammatory processes have been crucial to demonstrate that ACKRs play a fundamental role, not only in the trafficking of the immune cells, but also in the regulation of the inflammatory process itself. The use of zebrafish Cxcr7/Ackr3 mutants (and the remarkable conservation of specificity for Cxcl12) has already provided fundamental insight into the function of ACKRs in homoeostatic contexts[149,150,151]. With the exception of DARC/ACKR1, all the other mammalian atypical chemokine receptors are present in zebrafish too, which is both striking and valuable in scientific terms, as it suggests high functional conservation throughout vertebrates and the possibility to use zebrafish to obtain a better understanding of the origin of ACKRs and their function in inflammatory diseases.

Function of chemokine signals as modulators of inflammation and angiogenesis in mature granulomas

As illustrated by studies in cancer treatment, targeting pathological angiogenesis has potential therapeutic applications. Inhibition of tumour-associated vessel formation has long been known to have anti-neoplastic properties, as the microvasculature contributes to the growth and dissemination of cancer cells. However, *in vivo* results suggest that, depending on the stage and nature of the tumour, in some circumstances a pro-angiogenetic therapy, rather than an anti-angiogenic one, may benefit the patient. It has been proven, for example, that improving the angiogenetic response can lead to a better delivery of chemotherapeutics and promote healing and inflammation resolution by preventing hypoxia[152]. Reasoning on TB, similar conclusions can be drawn. An anti-angiogenic treatment might help restrict bacterial dissemination and granuloma formation, while a pro-angiogenic treatment might be beneficial as adjuvant therapy to a better delivery of first line antibiotics, by promoting tissue repair, remodelling, resolution of inflammation, and by preventing granuloma caseation[153].

Studies on TB patients corroborate the hypotheses that the function of angiogenesis in either limiting or sustaining development of the disease is not univocal and that in different situations, an anti- or pro-angiogenetic therapy might be advisable to counteract the diseases. In general, vascular endothelial growth factor (VEGF), an important angiogenesis promoter, has been shown to increase in the circulation of individuals with active TB at a greater extent, compared to both uninfected subjects and infected patients that do not display active TB[154,155]. A study also correlated increased levels of VEGF with the lack of lung cavitation in TB patients, suggesting that high levels of VEGF are at least protective against induction of central necrotisation of the granuloma and the formation of caseating lesions. This seems to indicate that, in an active TB state, a pro-angiogenic host-targeted therapy might be beneficial[156]. On the other hand, there is also evidence that in TB patients treated with anti-VEGF compounds, the vascular integrity was normalised, which led to beneficial effects by promoting small molecule delivery[157]. Evidence for a host beneficial effect of anti-VEGF treatment also comes from the *Mm* zebrafish infection model, where it was shown that innate immune cells, most likely macrophages, were required for the induction of granuloma angiogenesis (**Figure 1D-E**)[41]. In this model, inhibiting VEGF proved to be beneficial by reducing vascular leakage, and by reducing the oxygen availability for mycobacteria. A VEGF receptor inhibitor, Pazopanib, which is currently in clinical trials, was tested in the zebrafish-*Mm* model where it reduced bacterial burden, vascular leakiness and bacterial dissemination[41]. Additionally, this treatment increased also the effectiveness of rifampicin, a first line anti-tubercular antibiotic[41].

In **Chapter 6** we found that under knockout condition of the chemokine receptor Cxcr4b, the induction of angiogenesis to the granuloma aggregates was largely suppressed, which also reduced bacterial burden (**Chapter 6**). However, macrophage and neutrophil recruitment to mycobacteria and macrophage basal motility are unaffected in *cxcr4b* mutants (**Chapter 6**)[158], suggesting that no major mechanistic alterations are evoked in the initiation of granuloma formation. There are several hypotheses that could explain the *cxcr4b* phenotype in the regulation of angiogenesis. One possibility is that *cxcr4b* is important to complete myeloid differentiation in the haematopoietic tissues, since the Cxcr4b/Cxcl12a signalling axis is important to establish the definitive haematopoietic niches[159]. However, the number of macrophages was not largely altered in *cxcr4b* mutants in the developmental window used in our study and these cells did not display difference in basal microbicidal capability (**Chapter 6**), which indicates both quantitative and qualitative normal macrophage competence. Another hypothesis relates to the fact that Cxcr4b, like CXCR4 in mammals, is an M2-type macrophage marker that is upregulated in macrophages engaged in inflammation resolution[160]. It is possible that absence of *cxcr4b* may lead to incomplete polarisation, since M2 macrophages are also the cells that in cancer promote angiogenesis via induction of VEGF[161]. However, our study seems to indicate that *vegfaa* is induced at comparable levels in *cxcr4b* mutants and wt (**Chapter 6**), suggesting that aberration of Vegfaa signalling is not the main phenotype cause.

A more mechanistic explanation of the *cxcr4b* mutant phenotype in granuloma-angiogenesis could be that macrophages are also required to promote infiltration of endothelial cells and vessels sprouting[162] and that this mechanism would require a Cxcr4b/Cxcl12a crosstalk between the endothelium and the macrophages. Finally, although more highly expressed by macrophages, *cxcr4b* is expressed to some extent by endothelial cells too (**Chapter 6**), which would suggest that this signalling may be required for the directed recruitment of the endothelium, with a mechanism that not necessarily requires a

macrophage function[163]. Despite these considerations, our study seems to indicate that *cxcr4b* deficiency in granuloma-associated angiogenesis is related to the modulatory function that *cxcr4b* exerts on the inflammatory mediators (**Figure 1D-E**, **Chapter 6**). In TB patients and in other inflammatory disorders, the presence of inflammatory mediators such as CXCL8/IL8, IL1β and TNFα has also been correlated with the level of angiogenic activity, suggesting that, synergistically to VEGF, these mediators play a role in inflammation-associated angiogenesis[164,165,166,167,168]. Notably, the level of *il1b*, a key factor to propagate inflammation that also promotes proliferation and migration of endothelial cells[165,166,169,170], was induced to a lower extent in *cxcr4b* mutants (**Chapter 6**), which suggests that Cxcr4b can control the induction of the local inflammation. Since *il1b* is largely induced in infected cells (**Chapter 6**), it is likely that this mechanism is macrophage dependent (**Figure 1D-E**). Studies *in vitro* have shown that mammalian CXCR4 is an important co-receptor to mediate activation of Tlr signalling, for example by contributing to Lipopolysaccharide recognition[171,172]. Therefore, deficiency of *cxcr4b* might reduce the capability of immune cells to upregulate inflammatory genes via Tlr/My88/Nfκb pathway. Therefore, we propose a model where Cxcr4b deficiency, by limiting the induction of inflammation, especially via suppression of *il1b*, can reduce the levels of pro-angiogenetic signals and restrict vascularisation and bacterial growth (**Figure 1D-E**).

Overall, angiogenesis represents a promising target for granuloma-suppressive therapy, although more research is needed to determine under which circumstances its inhibition or promotion is more desirable in TB patients. In cases when anti-angiogenesis therapy can be applied, it should be considered that its direct blockade via VEGF-signalling inhibitors could have several contraindications, since this leads to risks of vascular disruption also in healthy tissues[173]. Therefore, in this respect an indirect antagonism via curtailing inflammation or CXCR4 signalling (which seems only implicated into inflammation-dependent vascularisation) might represent a better alternative. Additionally, since CXCR4 represents an important co-receptor for HIV entry into macrophages, use of CXCR4 antagonists might benefit especially patients that are both TB and HIV positive[174].

Chemotaxis of neutrophils to the granulomas and its protective function

Neutrophils can be recruited to mycobacterial infection and play an important protective role. However, when bacteria are systemically injected, macrophages are the main cell type that phagocytoses *Mm* and neutrophils are not actively engaged with mycobacteria[175]. It might be pointed that systemic injections in zebrafish are mostly started at 1 day post fertilisation, when neutrophils are not fully functional. However, the predominant macrophage phagocytosis can be reproduced also when bacteria are injected at later times, such as at 2 or 5 dpf[175]. In part, the poor phagocytosis of *Mm* by neutrophils could be explained by the fact that zebrafish neutrophils do not phagocytose well bacteria dispersed in solution and scavenge efficiently only pathogens that are associated to surfaces[176] (**Chapter 2**). In agreement with this hypothesis, *Mm* can be readily phagocytosed by both neutrophils and macrophages when infections are performed in the fin tissue[177]. It is likely that increased phagocytosis of bacteria by neutrophils in this tissue can be due to the fact that in the thin tissue, most bacteria are presented in association to surfaces[176].

When injections are performed locally in the hindbrain (at developmental stages where fully-competent neutrophils are already present), not only does *Mm* appear to essentially

reside into macrophages, it also seems to not induce neutrophil recruitment. Injection of other pathogens in this ventricle (such as *Pseudomonas aeruginosa*[175]) is able to induce comparable macrophage and neutrophil recruitment. In contrast with this phenotype, injections of *Mm* in the fin tissue seem to initially induce a comparable level of both macrophages and neutrophils[177]. It is, however, possible that injection of the pathogen in this tissue inevitably induces a significant wound response and that neutrophils may be recruited via wound-induced signals (e.g. H_2O_2)[178]. Furthermore, in the hindbrain it is possible to induce recruitment of neutrophils to *Mm* by providing additional signals, such as co-injection with *P. aeruginosa*[175]. In this case, the level of neutrophil infiltration is comparable to that of *P. aeruginosa* injection alone, which indicates that *Mm* does not chemoattract, but also does not chemorepel neutrophils[175]. Despite this scarce recruitment of neutrophils in the hindbrain model, *in vitro* mammalian cell models and *in vivo* zebrafish studies have demonstrated that neutrophil-chemotactic chemokines are induced as early as a few hours post-infection[67] (**Chapter 1**). In zebrafish systemic injections, a first wave of the neutrophil chemoattractants *cxcl8a* and *cxcl18b* is induced in the acute phase response and peaks at 4 hpi[67] (**Chapter 7**). *In situ* detection of *cxcl18b* also showed local induction to *Mm* at the injection point, as early as 3 hpi[66]. Additionally, our transgenic reporter *Tg(cxcl18b:eGFP)* is similarly upregulated in the tissue surrounding the infection, and notably not in infected or uninfected phagocytes (**Chapter 7**). However, systemic injection studies suggest that induction of neutrophil chemoattractants is transient and drops between 8 and 24 hours[67]. From 24 hpi, a second induction wave starts, which is characterised by a continuous increase of *cxcl8a* and *cxcl18b* mediators. In agreement with this, while neutrophils associate poorly to the initial mycobacterial infection, their presence in the granuloma increases over time (**Figure 1D-E**)[175]. Importantly, neutrophils that have engulfed mycobacteria can be more recurrently seen in mature granulomas, although it seems that the increased predisposition of neutrophils to get infected in mature granulomas is due to their "accidental" ingestion via efferocytosis of cell debris, which mostly derives from dying macrophages and can contain viable bacteria (**Figure 1E**)[175,177]. Engulfment of mycobacteria by neutrophils at this stage also leads to a better bacterial containment, since neutrophils can counteract the infection by induction of respiratory stress[175]. A recent study also showed that early after *Mm* infection, when the pathogen essentially resides in macrophages, uninfected neutrophils appear responsive to mycobacterial infection and increase their immune competence by activating a nitrosative stress response. This indicates that neutrophils do not exert exclusively a passive scavenging function within the granuloma, but that they possess (direct or indirect) mechanisms of mycobacterial sensing that contribute to their priming against the infection[179].

Concluding, the involvement of neutrophils increases (both quantitatively and qualitatively) with the increase of the inflammatory nature of the granulomatous lesion. Interestingly, the augmented competence and presence of neutrophils in the granuloma recapitulates the progressively increased expression of the neutrophil chemoattractant *cxcl18b* and c*xcl8a* (**Figure 1D-E**). It is possible that these molecules not only participate in the neutrophil recruitment to the granuloma, but they might be important also to activate specific antibacterial neutrophil responses. CXCL8 and other mammalian neutrophil chemokines are able to induce neutrophil degranulation[180,181], netosis[182] (a particular kind of neutrophil death that leads to release of traps composed of decondensed chromatine) and participate in the activation of the neutrophil respiratory burst[183,184] (production of reactive oxygen and nitrogen species) *in vitro*. The zebrafish model has already contributed relevantly to elucidate all these aspects of neutrophil biology upon bacterial and

inflammatory insults[175,178,179,185,186]. Therefore, further use of the zebrafish model may help to elucidate the mechanistic implication of chemokines into orchestrating not only neutrophil motility, but also their microbicidal/inflammatory function in TB and other inflammatory/infectious diseases[187].

Summarising conclusions

In summary, the work discussed in this thesis has contributed to elucidate novel mechanisms by which mycobacterial infection can benefit from chemotactic cues to initiate bacterial-beneficial programmes and to facilitate persistence of the infection. We have shown that Cxcr3.2 signalling is involved in the granuloma formation and can additionally support infection dissemination and dampen the basal microbicidal capability of immune cells. In contrast, the atypical Cxcr3.3 receptor seems to antagonise the pro-granuloma programme and exerts therefore a host protective function. We have additionally found that Cxcr4b-dependent signalling can affect the activation of the pro-granuloma angiogenesis, leading to a better infection control. Finally, we have characterised Cxcl18b and show that this novel neutrophilic cue is locally induced during the formation of granulomas.

We believe that our work has helped to shed light on the fact that chemokine signals occupy a very central position in the control of immunity. These signals, which are mostly regarded as a class of cytokines dedicated to cell migration, exert in fact extremely diversified functions, which include control of gene transcription, cell-autonomous immune functions and inflammation. The chemokine network displays an intricate texture, composed of receptors, their ligand partners and receptors that have evolved to act as ligand scavengers. Despite responding to general rules of signalling architecture, the affinity of some receptors to one specific ligand or more ligands is strikingly diversified. Additionally, the preferential activation of specific pathways of the downstream machinery can be also a prerogative of each chemokine receptor. Therefore, the application of the zebrafish model, which can be used for intravital imaging, has been crucial to obtain new insight on these highly dynamic signalling mediators and to comprehend new functions that the chemokine axes play in immunity.

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