Summary

The main objective of the work enclosed in this thesis has been to analyse the functions exerted by chemokines in shaping the host-pathogen interface at play between the cells of the innate immune system and mycobacterial pathogens. Chemokines are small chemotactic cytokines, which are host signalling molecules that occupy a central position in immunity and inflammation. There is a large body of evidence that several chemokine mediators are activated in response to mycobacterial infections. These chemokines can have host protective functions, but may also contribute to disease pathology, as exemplified by the work in this thesis.

Human tuberculosis (TB) is a global health concern caused by *Mycobacterium tuberculosis*. This human-adapted mycobacterial pathogen has been parasitising the mankind since prehistorical eras, provoking debilitation, illness and death worldwide. In the 21st century and more than 130 years after the discovery of its infectious agent, we are still far from eradicating TB, since it is estimated that about one third of the global population is chronically infected by *M. tuberculosis* and may therefore develop an active disease. A major complication of modern days' TB control is the emergency of drug-resistant strains that do not respond to the conventional therapeutic regimen, which consists of prolonged treatment with a cocktail of antibiotics disrupting different processes essential for bacterial growth, such as cell wall synthesis or DNA and protein synthesis. The problem of multi-drug resistances emphasises the need of novel strategies to counteract the disease. Establishment of *M. tuberculosis* infections is intimately connected with the capability of the pathogen to manipulate the host signalling machinery. Therefore, it is an attractive hypothesis that drugs for TB treatment might be identified that act on the host rather than on the pathogen itself. Identifying the host targets for development of such drugs would not only largely expand the therapeutic opportunities, but also provide a more difficult system for the pathogen to develop new drug resistances.

The chemokine signalling system, by exerting a crucial function in immunity, may be involved in relevant aspects of TB. On one hand this signalling might function in host defence; on the other hand it might also be exploited by mycobacteria, similarly to other immune-related pathways that are subverted by these pathogens. Chemokines act via a class of G-protein coupled receptors, which are currently the most successful drug targets in practice for a number of diseases. Therefore, the chemokine axes may represent easily druggable therapeutic targets that could be used to develop novel host-directed therapies to combat TB.

As we further elaborate on in **Chapter 1** and **Chapter 2**, the zebrafish (*Danio rerio*) has proved to be an excellent animal model to study both the biology of innate immune cells (macrophages and neutrophils) and the molecular and cellular basis of infectious diseases. This is especially true for the embryonic and larval stages of zebrafish, which are optically transparent as well as chemically and genetically tractable. The zebrafish is naturally susceptible to *Mycobacterium marinum*, a bacterium that is closely related to *M. tuberculosis*. The zebrafish-*M. marinum* model is characterised by a disease that faithfully recapitulates the main molecular, cellular and histopathological aspects of human TB. The

striking similarity of fish TB with the human disease is not only interesting from an evolutionary point of view, but also scientifically attractive for the study of TB. This is particularly significant if we consider that *M. tuberculosis* does not fully reproduce the human disease in the murine model and there are severe restrictions to work with this human pathogen. In recent years, it additionally became clear that several human chemotactic signalling axes maintain remarkable molecular and functional conservation in zebrafish, suggesting that their role in limiting or driving susceptibility to mycobacterial infection might have homologies in the two infectious systems. Therefore, the embryonic/larval zebrafish platform represented for us an insightful system to model and challenge mycobacterial disease, and led to the discovery of new virulence mechanisms that involve chemokine signalling.

In **Chapter 3** we found that zebrafish conserves a Cxcr3.2/Cxcl11aa-11af signalling axis that exerts important functions in macrophages, as its orthologous CXCR3/CXCL9-10-11 axis does in mammals. Homozygote *cxcr3.2* mutants displayed deficiency in macrophage recruitment to recombinant Cxcl11aa-11af and to bacterial infections (including *M. marinum),* which promptly induce the Cxcr3.2 ligands. Strikingly, *cxcr3.2* mutation conferred resistance against mycobacterial infection, since this mutation, by attenuating macrophage trafficking, reduced mycobacterial dissemination and delayed the expansion of the so-called granulomas. Granulomas are infectious and inflammatory lesions consisting of immune cells (especially macrophages) that collect the pathogen and the necrotising tissue debris. Tuberculous granulomas are the disease-causing hallmark of mycobacterial infection and represent the natural niche of pathogenic mycobacteria. Within the granuloma, mycobacteria essentially persist intracellularly in the macrophages and extracellularly in the necrotic centre that results from the death of infected macrophages. Therefore, the Cxcr3.2 receptor, by increasing the engagement of macrophages with mycobacteria, represents a pro-granuloma determinant. The small CXCR3 inhibitor NBI74330 could phenocopy the deficient macrophage mobilisation to mycobacteria in zebrafish, therefore suggesting that CXCR3 antagonistic therapy might be used to curtail TB infection.

In **Chapter 4** we further analysed the downstream signalling dependent on Cxcr3.2. Therefore, we sorted macrophages from *cxcr3.2* mutants and we profiled their transcriptome by RNA-sequencing. Intriguingly, we discovered that *cxcr3.2* mutation leads to a lysosome stress signature and to a coordinated upregulation of several lysosomal genes, including acid hydrolases and lysosomal proton pump subunits. To corroborate these results, we demonstrated that this macrophage signature goes together with an increased acidification of bacteria-containing compartments and increased microbicidal activity against mycobacteria *in vivo*. To obtain more mechanistic insight on how lysosome stress is evoked in macrophages by deficient sensing of chemotactic cues, we tracked macrophages with stained lysosomes. Surprisingly, we found that *cxcr3.2* mutation attenuated the recruitment of lysosomes to the leading edge in moving cells. The accumulation of lysosomes at the leading edge of wildtype macrophages is possibly indicating local lysosome exocytosis, which can be triggered by chemokines and is important to provide new membrane to the protruding lamellipodia. Remarkably, we show that macrophages themselves upregulate Cxcl11aa during mycobacterial parasitosis and that active Myd88-mediated immune recognition of the pathogen is essential to Cxcl11aa synthesis. We therefore hypothesise that the existence of this immune recognition/chemokine signalling/lysosome function circuit might benefit the pathogen in

two ways: induction of Cxcr3.2-dependent signalling not only would fuel the granuloma with new macrophages to be infected, it would also generate a more permissive intra-macrophage phenotype by hijacking lysosome function.

In **Chapter 5** we preliminarily characterised the function of Cxcr3.3, another zebrafish orthologue of CXCR3, and we describe the CRISPR/Cas9 genome editing pipeline that we optimised to obtain a *cxcr3.3* mutant. Besides the technical improvements of the CRISPR/Cas9 mutagenesis method in zebrafish that we propose (as further detailed in the chapter), we found that the *cxcr3.3* mutants are more susceptible to mycobacterial infection, which is diametrically opposite to the *cxcr3.2* mutant phenotype. A *cxcr3.2/cxcr3.3* double mutant line will be necessary to shed light on this controversy. However, we found that treatment with the NBI74330 inhibitor, which is predicted to equally antagonise Cxcr3.3 and Cxcr3.2, still attenuated susceptibility to infection and evoked a more *cxcr3.2*-like phenotype. This suggested that Cxcr3.3 requires Cxcr3.2 to exert its function. In support of this hypothesis we discovered that Cxcr3.3 displays significant sequence modification, the most remarkable of which is the replacement of an Arginine residue within the Aspartic acid-Arginine-Tyrosine (DRY) motif, the most conserved motif within functional G-protein coupled receptors. The Arginine residue in the DRY motif is 100% conserved in fully functional human CXCR receptors, as it serves to interact with the heterotrimeric G-proteins and activate the downstream signalling. The DRY motif is instead recurrently modified (or absent) in atypical chemokine receptors that essentially function as ligand scavengers and tight regulators of chemokine concentrations and gradients. Notably, the existence of one atypical CXCR3 isoform is recurrent in bony fish, although lost in tetrapods and tetrapodomorph lobe-finned fish (fully aquatic species directly related to tetrapods). Therefore, atypical Cxcr3.3 receptors do not exclusively exist in zebrafish and might have important conserved function in fish immunology and could additionally help to reconstruct the evolutionary history of chemokine receptors.

In **Chapter 6** we have analysed the function of the chemokine receptor Cxcr4b and observed that Cxcr4b mutation can limit expansion of granulomas forming in the poorly vascularised tissues of the trunk. This phenotype correlated with a concomitant deficiency in triggering angiogenesis, a process that promotes the growth of granulomas in the wildtype situation. In the attempt to elucidate the mechanistic pathway that leads to granuloma vascularisation via Cxcr4b, we found that *cxcr4b* mutants had a limited induction of inflammatory mediators, especially the master regulator of inflammation, *il1b*. This pleiotropic molecule, which is also well known to exert proliferative and pro-angiogenic functions on endothelial cells, is largely expressed by infected cells in the granulomas and might represent the main connector between Cxcr4b and the induction of granuloma-associated inflammation.

In **Chapter 7** we characterised the function of the fish-specific chemokine Cxcl18b and by injection of recombinant chemokine we demonstrate that this molecule serves as a potent chemoattractant of neutrophils, while it does not affect macrophage recruitment. We additionally show that, at least partly, Cxcl18b exerts its function via the chemokine receptor Cxcr2, which was previously described as being the receptor partner of other two CXCL8/IL8-like chemokines in zebrafish, Cxcl8a and Cxcl8bb. Similarly to these CXCL8/IL8-like chemokines, Cxcl18b did not require the chemokine receptor Cxcr1, the zebrafish counterpart of mammalian CXCR1 that is redundant with CXCR2. Expression of *cxcl18b* is significantly and reliably induced by mycobacterial infection and by other

inflammatory conditions. Therefore *cxcl18b* expression may represent an important marker to longitudinally track the development of inflammation. We constructed a *cxcl18b* reporter and show that, in this line, the reporter reproduces the endogenous induction of *cxcl18b* during mycobacterial infection. Intriguingly, it seems that within the granuloma aggregate, the infected cells are not the main source of Cxcl18b. Instead, uninfected cells, that participated in the lesion but did not represent phagocytes, highly expressed the transgene. This observation suggests that further use of this reporter could permit to distinguish responses that are activated in defined subsets of non-phagocytic cells in the granuloma microenvironment, which might be important for the recruitment of neutrophils. This transgenic line could therefore be useful to study the function exerted by neutrophils in mycobacterial diseases, which remains poorly understood.

This dissertation is concluded by a general discussion in **Chapter 8**, which relates our findings with recently emerged concepts in innate immunity and mycobacterial disease. We discuss the dual function of macrophages in restricting and sustaining mycobacterial disease and the effects of macrophage-recruitment axes to establish, maintain and disseminate the infection. We elaborate on how the lysosome stress response and lysosomal storage diseases are emerging as opposing forces driving resistance or susceptibility to TB and discuss how this knowledge could be translated into novel therapeutic strategies. We try to explain our results on the atypical Cxcr3.3 receptor, of which mutation causes increased infection susceptibility, by comparing our findings to a study carried in a mice mutant of another atypical chemokine receptor, which essentially led to a comparable phenotype when infected with *M. tuberculosis*. We corroborate our findings on Cxcr4b/inflammation/angiogenesis with the complementary literature, which confirms a function of CXCR4 in induction of pathological angiogenesis and in modulating inflammation. We continue with a section discussing how the zebrafish model might help to clarify the function of chemokines in controlling neutrophilic involvement in mycobacterial diseases. Finally, we conclude with some general reflections on the pleiotropic functions and complexity behind the chemokine signalling network and on how the zebrafish model is contributing to our understanding of this and other multidimensional biological processes.

Concluding, the work described here has contributed to clarify how the chemokine network is implicated into different aspects of mycobacterial disease. We have emphasised how this intricate network can drive pathogen or host beneficial responses and we have shown that chemokine signalling not only controls recruitment of immune cells, but also their expression profile and their immune competence against the invading bacteria. More importantly, using the zebrafish model, we were able to follow longitudinally both the cellular dynamics and the molecular pathways that activate (or are activated by) the chemokine signalling *in vivo*. Finally the use of intravital imaging in this genetically tractable host model permitted to directly relate these new findings to pathological consequences at the level of the entire host during the course of the mycobacterial disease. This approach has helped us to comprehend to a deeper level the complexity behind the mycobacterial disease and how different cell types (e.g. neutrophils, macrophages, endothelium) and different host factors (e.g. chemokines, inflammatory mediators, angiogenesis factors) interact with each other and ultimately work together as a whole, in a system that eventually leads to the establishment of the disease.