Chapter 1

Outline of the thesis

Zebrafish as a model for innate immunity studies

The zebrafish is an excellent model to study vertebrate innate immunity. Zebrafish embryos are fertilised externally and develop an innate immune system within the first days after fertilisation. The first innate immune cell type to develop is the macrophage, which is already functional in one-day-old zebrafish embryos. At this stage, macrophages are capable of phagocytosing pathogens and tissue debris. Neutrophils are the second innate immune cell type to develop and produce anti-microbial proteins within their characteristic granules by 2 days post fertilisation. These cells are the first responders to inflammatory stimuli and are also efficient scavengers of bacteria in infected tissues. The first immature T-cells can be detected in four day old larvae, but it takes several weeks before zebrafish also have antibody-producing B-cells and a fully functional adaptive immune system. Using the zebrafish as a host model has several advantages. Firstly, the temporal separation of innate and adaptive immunity allows us to study the isolated function of innate immune factors during infection. Secondly, various transgenic lines are available that have fluorescently labelled macrophages, neutrophils, or other immune cell types, and the behaviour of these cells can be imaged in real-time and with excellent resolution during the early life stages when zebrafish are transparent. Thirdly, the recently published sequenced genome of the zebrafish allows us to apply state-of-the-art RNA sequencing techniques to study gene expression profiles. Fourthly, many genetic tools are available to create temporary knockdowns or permanent mutations of genes of interest. All these tools combined make the zebrafish embryo an ideal model host to study the function of innate immune genes during pathogenic infections.

Mycobacterial infection studies in zebrafish

The zebrafish has become a well-accepted model to study the function of innate immunity during mycobacterial infection. Mycobacteria are the causative agents of life-threatening human diseases, including tuberculosis and leprosy. Infection with *Mycobacterium marinum*, a natural pathogen of fish and a close relative of the human pathogen *Mycobacterium tuberculosis*, leads to granuloma formation in zebrafish embryos, the key hallmark of tuberculosis. Using *M. marinum* infection in zebrafish embryos led to the discovery that innate immune factors are sufficient to initiate granuloma formation, a process that was previously thought to develop only in the presence of adaptive immunity. Furthermore, research in zebrafish has changed the view of the tuberculous granuloma, which had been historically regarded strictly as a host defence structure, but is now known to play an important role in the dissemination and expansion of mycobacterial infection (Ramakrishnan, Nat Rev Immunol 12:352, 2012). These findings illustrate the importance of using various host models to contribute to our understanding of mycobacterial pathogenesis. Zebrafish models for a wide variety of other pathogens have also been established and their use is leading to new mechanistic insights into host-pathogen interactions and is inspiring novel therapeutic strategies for infectious disease treatment (Meijer et al., Cell Microbiol 16:39, 2014).

Overview of this thesis

This thesis is focused on the innate immune defence mechanisms responsible for controlling mycobacterial growth after infection. In addition to *M. marinum,* another intracellular bacterial pathogen, *Salmonella typhimurium*, is used as a comparative model. To study the host immune responses to infection, the pathogens are delivered into the embryo by various injection techniques to create a rapid, systemic infection or a localised infection. Chapter 2 demonstrates a bacterial preparation protocol for injection, various injection techniques, and techniques for confocal imaging. We also provide representative results for the used injection methods demonstrating their versatility.

To identify candidate genes that play a role in controlling *M. marinum* infection during the early stage of infection while granulomas are formed, a morpholino knockdown screen was performed which is described in chapter 3. Morpholinos are specifically designed molecules that block correct translation of RNA of a gene of interest which leads to a dysfunctional or non-functional protein. The candidate genes used in this screen were chosen based on previous transcriptome data of zebrafish embryonic macrophages and *M. marinum* infection studies. The screen started with seventeen candidate genes and resulted in five positive hits: four leading to an increased infection and one leading to a decreased infection after knockdown. The detailed function of three of the five positive hit genes are further described in the following chapters of this thesis.

To provide a detailed description of the host's innate immune response to *M. marinum* infection, zebrafish gene expression levels were analysed by RNA sequencing at various time points during infection ranging from 2 hours until 5 days after infection and correlated with imaging data of the process of pathogenesis. These results are presented in chapter 4 where an early-, mid- and late-phase immune response to *M. marinum* is characterised. One of the transcription factors that we found to be induced during the early- and late-phase of infection is *atf3* (*a*ctivating *t*ranscription *f*actor *3*). Knockdown of *atf3* in the zebrafish embryo resulted in an increased migration of leukocytes towards local inflammation sites and a decreased bacterial burden, indicating that that the induction of *atf3* during *M. marinum* infection antagonises an effective innate immune control of this pathogen.

Directly after infection, immune cells detect the presence of pathogen-associated molecular patterns on the surface of microbes. Scavenger receptors on the cell surface of macrophages play an important role in this process through their ability to not only bind microbial ligands, but also induce phagocytosis. In chapter 5, it is demonstrated that the scavenger receptor Marco (macrophage receptor with collagenous structure) is a key player in the rapid phagocytosis of *M. marinum* and we use gene expression analysis in combination with gene knockdown studies to show that it is also essential

in the establishment of an initial transient pro-inflammatory response to *M. marinum* infection.

Once phagocytosed, *M. marinum* is capable of avoiding killing mechanisms of the host cell and can continue to grow within macrophages. This is the period when Membrane Attack Complex/Perforin (MACPF) proteins are involved in killing intracellular bacteria by their pore-forming activities. In **chapter 6**, we reveal the regulatory mechanisms and function of two macrophage specific genes, *mpeg1* and *mpeg1.2* (**m**acro**p**hage **e**xpressed **g**ene **1.2**), and illustrate that both genes encode proteins with MACPF domains. Combined results of infection experiments with *M. marinum* and *S. typhimurium* during knockdown conditions of *mpeg1* and *mpeg1.2* support their anti-bacterial function.

The findings presented in this thesis are summarised and discussed in **chapter 7**. The results complement knowledge obtained from other model organisms by providing new insights into both counteracting and supporting mechanisms underlying the innate immune response. An overview is presented of the key players in innate immune defences identified in this study. This overview highlights how an integrated study of transcriptional responses and morpholino knockdown studies can rapidly lead to functional information of a group of key genes involved in the innate immune defence system.