Chapter 7

Summary and discussion

Tuberculosis is a life-threatening disease caused by infection with the bacterial pathogen *Mycobacterium tuberculosis*. Approximately one third of the world population is infected with *M. tuberculosis* and research of the World Health Organisation demonstrates that tuberculosis is second only to human immunodeficiency virus infection/acquired immunodeficiency syndrome (HIV/AIDS) as the greatest killer worldwide due to a single infectious agent. Although tuberculosis can be treated with four antimicrobial drugs, multidrug resistant strains are emerging world-wide. This highlights the need to increase our knowledge on how the host's immune system contributes to combating the mycobacterial infection. *M. tuberculosis* is a pathogen that is able to subvert the killing mechanisms of macrophages and can replicate intra-cellularly in this cell type. Central to the pathology of tuberculosis is the formation of granulomatous aggregates of infected and non-infected macrophages and other immune cells [1]. These granulomas provide a niche for the long-term survival of the pathogen inside its host. Studying this pathological hallmark of the disease requires whole organism models. Many animal models for tuberculosis are used, each having specific advantages as well as limitations [2, 3]. For example, a wealth of immunological tools is available for research in mice, but *M. tuberculosis* is not a natural pathogen of rodent species, and mice do not form the highly structured granulomas that are seen in human patients. In the recent years, the zebrafish has emerged as a new animal model for tuberculosis. Zebrafish develop tuberculosis following infection with a close relative of *M. tuberculosis*, namely *Mycobacterium marinum*. Granulomas in the zebrafish structurally resemble the human tuberculous granuloma and the transparent early life stages of the zebrafish proved to be a highly versatile model system to study the early events in granuloma formation [4-7] (**chapter 2**).

Morpholino screen identified candidate innate immune genes involved in *M. marinum* infection

To identify innate immune host genes involved in controlling mycobacterial growth during early stages of infection when granulomas are being formed we set up a morpholino-based knockdown screen. This screen identified 5 reproducible hits out of the 17 candidate genes analysed that demonstrated either an increased or decreased infection of *M. marinum* after knockdown (**chapter 3**). These hits provided a basis for further in-depth analysis to identify which step of the infection process is affected and which genes and pathways are influenced, as demonstrated subsequently in chapters 4, 5, and 6 for the hit genes *marco*, *mpeg1*, and *atf3*, respectively.

Time-resolved transcriptome analysis of *M. marinum* infection and the function of the inducible transcription factor Atf3

To increase our knowledge about the infection process of *M. marinum* we analysed RNA sequencing gene expression profiles throughout a time course of *M. marinum* infection every 2 hours until 8 hours post infection and daily from 1 until 5 days post infection,

and interrelated these to the observed characteristic host-pathogen interactions at specific time points (**chapter 4**). This analysis revealed that there is an early-, mid-, and late-phase response during *M. marinum* infection (fig. 1). The early-phase response is observed until 4 hpi during which macrophages phagocytose the *M. marinum* and travel through the vascular system. This phase was characterised by rapid induction of transcription factors and a subsequent transient peak of pro-inflammatory gene expression. This is followed by a mid-phase response between 6 hpi and 1 dpi during which infected macrophages could be seen to still travel through and occasionally leave the vascular system and the expression levels of most genes return to uninfected control levels. Such a 'silent' mid-phase is in sharp contrast with the response to an acute pathogen, like *S. typhimurium*, where expression levels of pro-inflammatory genes strongly increase over the same time frame [8]. Finally, there is a late-phase response with a progressive increase in differential gene expression between 2 dpi and 5 dpi when granuloma-like aggregates start forming containing increasing numbers of uninfected and infected macrophages with an occasional infected neutrophil. It was clear that the transition between the mid-phase and late-phase (2 dpi) is associated with strong metabolic changes. At 4 dpi many infected macrophages start dying and at 5 dpi the granuloma contains high numbers of infected and uninfected neutrophils. This period was characterised by increasing expression levels of the same genes that were induced during the early-phase response and many other inflammation-associated genes. The zebrafish infection model has previously shown that susceptibility to *M. marinum* can result from either inadequate or excessive acute inflammation balance in immune response [9]. Therefore it is not surprising that concomitant with the induction of pro-inflammatory genes and transcriptional activators, we found numerous negative regulators of innate immunity signalling pathways to be up-regulated during early- and late-phase infection. Of interest is the expression profile of complement components because they are up-regulated consistently throughout the infection, even at the mid-phase when other immune related genes show baseline levels. Similar to other expression profiles, the complement components are also increasingly induced during the late-phase. Furthermore, a striking regulatory pattern was observed of a group of apolipoprotein genes that is the exact opposite of that of pro-inflammatory genes, showing down-regulation during the early- and late-phase and up-regulation during the mid-phase. This could be an interesting group of genes to study further in more detail because they have previously been linked to mycobacterial infection by their suggested function in transferring antigenic lipids from cell to cell, thereby enabling the mycobacteria to elicit their influence beyond the cell that contains them [10, 11].

One of the transcription factor genes up-regulated during the early- and late-phase of infection is *activating transcription factor 3* (*atf3*), which in mice is known to function as a transcriptional repressor of genes involved in the immune response [12, 13] and is used as an ER stress marker that is expressed in the macrophage-rich area of *M. tuberculosis*-induced granulomas [14]. Embryos in which *atf3* is knocked down have a decreased *M. marinum* infection (**chapter 4**), however, we did not observe a strong effect on the

transcriptional signature in these morphants. Instead, we found that Atf3 functions in suppressing macrophage and neutrophil migration towards local damage-induced inflammation sites. To determine whether *atf3* morphants also recruit higher numbers of macrophages and neutrophils to the forming *M. marinum* granuloma, an important process in granuloma formation, we imaged this process in a double transgenic zebrafish line with fluorescent macrophages and neutrophils. Knockdown of *atf3* resulted in an attenuated infection level as soon as 2 dpi and leukocyte numbers seemed to be increased around the infection locations at 1 and 2 dpi. This suggests that increased leukocyte recruitment during the early stage of infection might be responsible for better restriction of *M. marinum* growth in *atf3* morphants. Although further research is necessary to determine the mechanism of Atf3 function during *M. marinum* infection our results indicate that the induction of *atf3* during *M. marinum* infection antagonises an effective innate immune control of this pathogen.

Marco functions in phagocytosis of *M. marinum* and controls the hosts pro-inflammatory signalling response to *M. marinum* infection

The first line of defence against *M. tuberculosis* is phagocytosis by macrophages and it has been shown in mice that the Scavenger receptor MARCO plays an assisting role in this process by binding to the cell wall component glycolipid trehalose 6,69-dimycolate (TDM) of this pathogen [15]. Furthermore, recent studies on single nucleotide polymorphisms (SNPs) in humans have identified MARCO to be associated with increased susceptibility to tuberculosis [16, 17]. We used the zebrafish tuberculosis model to study the function of Marco during *M. marinum* infection. Using two independent morpholinos we demonstrated that Marco is a key player in the rapid phagocytosis of *M. marinum* following intravenous injection of the pathogen (**chapter 5**, fig. 1). This response was specific because knockdown of a second scavenger receptor, *cd36*, had no effect on phagocytosis. The presence of multiple other receptors that can initiate phagocytosis of mycobacteria [18-20] indicates how remarkable it is that knockdown of a single receptor leads to a significant delay in phagocytosis.

After TDM binding to MARCO in mice, it was demonstrated that TLR2 signalling pathways were activated in response to the mycobacteria. This response also required the co-receptor CD14 which is suggested to enhance the proximity of mycobacteria to the receptor complex [21-23]. We showed that Marco in zebrafish functions as an essential player in the establishment of an initial transient pro-inflammatory response to mycobacteria at 4 hours after infection (**chapter 5**, fig. 1); however, it is likely that a parallel Marco-independent signalling route exists because knockdown of *marco* did not block the *M. marinum*-induced pro-inflammatory response completely. In addition, the accessory molecule CD14 does not exist in the zebrafish genome indicating that Marco is capable of functioning independently of the co-receptor CD14 in mediating a pro-inflammatory response to *M. marinum.* Further research is therefore necessary to identify the exact signal transduction pathway associated with Marco in zebrafish.

In addition to the immediate effects on phagocytosis and cytokine induction, knockdown of *marco* lead to a significant increase in mycobacterial burden during development of infected larvae. The delayed phagocytosis of *M. marinum* in *marco* morphants could be a reoccurring process because highly infected macrophages are known to die and be phagocytosed by newly recruited macrophages, a process in which Marco may also play a role (fig. 1). We hypothesise that the accumulated delay in reoccurring phagocytosis of *M. marinum* in combination with the dampened initial pro-inflammatory response to *M. marinum* infection leads to an inability of the embryos to control the infection sufficiently which leads to the observed higher bacterial infection in the *marco* morphants.

Mpeg1 and Mpeg1.2 have anti-bacterial functions in zebrafish

Membrane Attack Complex/Perforin (MACPF) proteins play a crucial role against phagocytosed pathogens by forming pores either directly on the surface of invading bacteria or in the plasma membranes of infected and transformed host cells, allowing the entry of cytolytic proteins. Macrophage expressed gene 1 (*MPEG1*) belongs to the superfamily of MACPF containing proteins and is conserved between human, mouse and zebrafish. In addition to *mpeg1*, the zebrafish genome also contains an *mpeg1.2* and *mpeg1*.3 gene as a result of gene duplication, although based on our expression data *mpeg1.3* seems to be a pseudogene.

The *mpeg1* and *mpeg1.2* genes are differentially regulated during infection with *M. marinum* and with *Salmonella typhimurium,* another intracellular pathogen that we used as a comparative model in our studies. At different time points after infection with either of these pathogens we observed that *mpeg1* is down-regulated and *mpeg1.2* is up-regulated (**chapter 6**). While our results of increased *M. marinum* infection during knockdown of *mpeg1* are consistent with the expected anti-bacterial function (fig. 1), we also observed an altered immune response to *M. marinum* infection indicating that Mpeg1 might have a broader effect on controlling infection. Knockdown of *mpeg1.2* did not lead to any difference in controlling *M. marinum* infection, which might be explained by the late time point (four days after infection) during which we first observed up-regulation of this gene during *M. marinum* infection. This suggests that Mpeg1.2 functions during the later stages of infection (fig. 1) when the knockdown effects of the *mpeg1.2* morpholino have diminished. To still be able to study the functionality of both Mpeg1 and Mpeg1.2 we used the more acute *S. typhimurium* infection model, in which we observed down-regulation of *mpeg1* and up-regulation of *mpeg1.2* within eight hours after infection. We showed this process to be partially dependent on the presence of functional Mpeg1, and to require the Toll-like receptor adaptor molecule MyD88 and transcription factor NFκB. Knockdown of both *mpeg1* and *mpeg1.2* increased *S. typhimurium* bacterial burdens, but unexpectedly *mpeg1* morphants showed prolonged survival. We hypothesised that the survival advantage of *mpeg1* morphants might be explained by a difference in expression of pro-inflammatory markers, but this was not the case and therefore the underlying cause of this phenotype currently remains

unknown. The combined results of the *M. marinum* and *S. typhimurium* infection models provide *in vivo* support for the anti-bacterial function of the MPEG1 family and indicate that the intricate cross-regulation of the two *mpeg1* copies aids the zebrafish host in combatting infection of various pathogens.

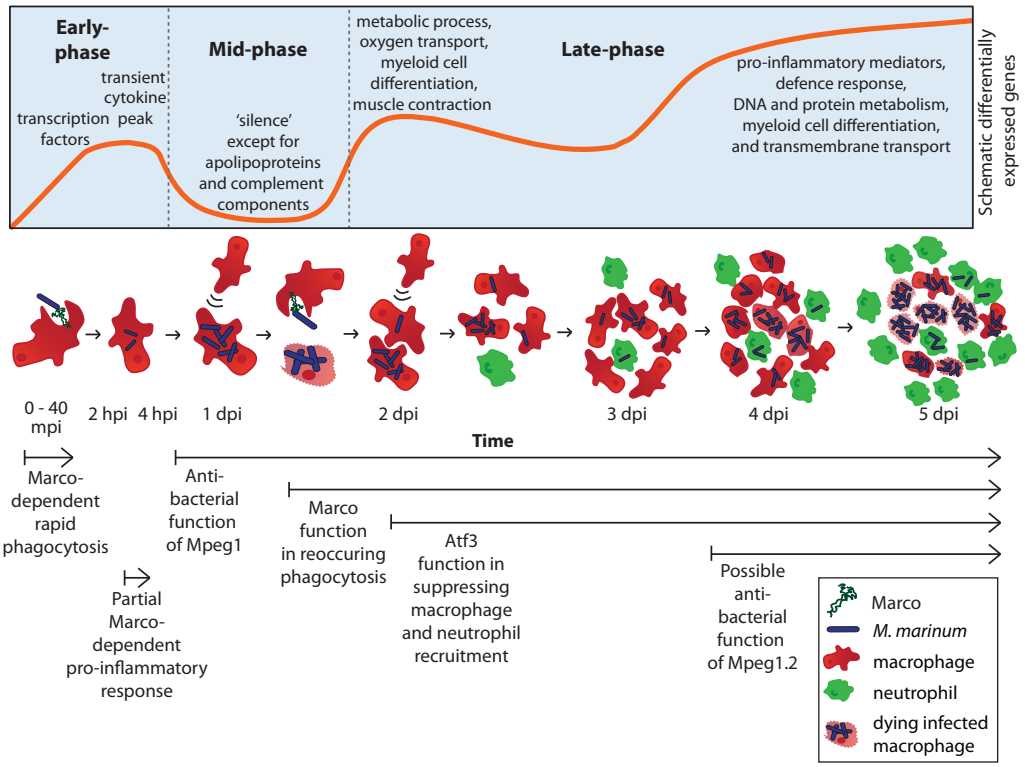

Figure 1: Schematic representation of the host immune response to *M. marinum* infection and function of Marco, Mpeg1 and Atf3 during *M. marinum* infection in zebrafish embryos. A schematic overview representing observed gene expression profile responses (orange line represents total genes up- and down-regulated during infection) and observed host-pathogen interactions. Functional properties of Marco, Mpeg1 and Atf3 are indicated below the time scale of infection.

Conclusion

The work presented in this thesis provides a great amount of information on mycobacterial infection processes which will be of great value for future studies. Providing a better framework for studying innate immune defence in the zebrafish – *M. marinum* model, we have combined a detailed time-resolved description of the host transcriptome response with confocal imaging of the precise events that take place during the process of pathogenesis. Furthermore, a successful morpholino knockdown screen revealed functions for scavenger receptor Marco in phagocytosis and initiation of the innate immune response, anti-bacterial functions for perforins of the Mpeg1

family, and a regulatory role for transcription factor Atf3 in leukocyte recruitment. Thereby, as summarised in figure 1, we have provided new insights into mechanisms involved in processes that either counteract or support the innate immune control of *M. marinum* infection.

References

1. Guirado, E. & Schlesinger, L.S. Modeling the Mycobacterium tuberculosis Granuloma - the Critical Battlefield in Host Immunity and Disease. *Front Immunol* **4**, 98 (2013).

2. Apt, A. & Kramnik, I. Man and mouse TB: contradictions and solutions. *Tuberculosis (Edinb)* **89**, 195-198 (2009).

3. Baldwin, S.L. *et al.* Evaluation of new vaccines in the mouse and guinea pig model of tuberculosis. *Infection and immunity* **66**, 2951-2959 (1998).

4. Davis, J.M. *et al.* Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. *Immunity* **17**, 693-702 (2002).

5. Cosma, C.L., Humbert, O., Sherman, D.R. & Ramakrishnan, L. Trafficking of superinfecting Mycobacterium organisms into established granulomas occurs in mammals and is independent of the Erp and ESX-1 mycobacterial virulence loci. *J Infect Dis* **198**, 1851-1855 (2008).

6. Clay, H. *et al.* Dichotomous Role of the Macrophage in Early Mycobacterium marinum Infection of the Zebrafish. *Cell Host & Microbe* **2**, 29-39 (2007).

7. Volkman, H.E. *et al.* Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. *PLoS Biol* **2**, e367 (2004).

8. Stockhammer, O.W., Zakrzewska, A., Hegedus, Z., Spaink, H.P. & Meijer, A.H. Transcriptome profiling and functional analyses of the zebrafish embryonic innate immune response to Salmonella infection. *J Immunol* **182**, 5641-5653 (2009).

9. Tobin, D.M. *et al.* Host genotype-specific therapies can optimize the inflammatory response to mycobacterial infections. *Cell* **148**, 434-446 (2012).

10. Beatty, W.L., Ullrich, H.J. & Russell, D.G. Mycobacterial surface moieties are released from infected macrophages by a constitutive exocytic event. *Eur J Cell Biol* **80**, 31-40 (2001).

11. van den Elzen, P. *et al.* Apolipoprotein-mediated pathways of lipid antigen presentation. *Nature* **437**, 906-910 (2005).

12. Gilchrist, M. *et al.* Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature* **441**, 173-178 (2006).

13. Whitmore, M.M. *et al.* Negative regulation of TLR-signaling pathways by activating transcription factor-3. *Journal of immunology* **179**, 3622-3630 (2007).

14. Seimon, T.A. *et al.* Induction of ER stress in macrophages of tuberculosis granulomas. *PloS one* **5**, e12772 (2010).

15. Bowdish, D.M. *et al.* MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and Mycobacterium tuberculosis. *PLoS Pathog* **5**, e1000474 (2009).

16. Ma, M.J. *et al.* Genetic variants in MARCO are associated with the susceptibility to pulmonary tuberculosis in Chinese Han population. *PLoS One* **6**, e24069 (2011).

17. Bowdish, D.M. *et al.* Genetic variants of MARCO are associated with susceptibility to pulmonary tuberculosis in a Gambian population. *BMC Med Genet* **14**, 47 (2013).

18. Schlesinger, L.S. Macrophage phagocytosis of virulent but not attenuated strains of Mycobacterium tuberculosis is mediated by mannose receptors in addition to complement receptors. *J Immunol* **150**, 2920-2930 (1993).

19. Schlesinger, L.S., Bellinger-Kawahara, C.G., Payne, N.R. & Horwitz, M.A. Phagocytosis of Mycobacterium tuberculosis is mediated by human monocyte complement receptors and complement component C3. *J Immunol* **144**, 2771-2780 (1990).

20. Hirsch, C.S., Ellner, J.J., Russell, D.G. & Rich, E.A. Complement receptor-mediated uptake and tumor necrosis factor-alpha-mediated growth inhibition of Mycobacterium tuberculosis by human alveolar macrophages. *J Immunol* **152**, 743-753 (1994).

21. Brightbill, H.D. *et al.* Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science* **285**, 732-736 (1999).

22. Manukyan, M. *et al.* Binding of lipopeptide to CD14 induces physical proximity of CD14, TLR2 and TLR1. *Eur J Immunol* **35**, 911-921 (2005).

23. Nakata, T. *et al.* CD14 directly binds to triacylated lipopeptides and facilitates recognition of the lipopeptides by the receptor complex of Toll-like receptors 2 and 1 without binding to the complex. *Cellular microbiology* **8**, 1899-1909 (2006).