Chapter 5

Summary and Discussion

Infectious diseases, caused by pathogenic microorganisms, continue to pose major health challenges in developed as well as developing countries. A proper functioning immune system is essential for defense against infectious agents. The immune system is functionally organized into two components: innate (natural) and adaptive (acquired) immunity. The innate immune system is inborn and provides immediate protection against invading microorganisms by recognizing important and conserved structural components, such as molecules of microbial cell walls. A variety of immune cells orchestrate responses of the innate immune system. These include: macrophages, neutrophils, eosinophils, basophils, dendritic cells, natural killer cells, and mast cells. Innate immune cells function cooperatively with T- and B-lymphocytes of the adaptive immune system, which execute a more specific response due to recognition of distinctive molecules (antigens) of different pathogens.

Upon detection of an infection or other indications of danger, the cells of innate immune system initiate the release of pro-inflammatory mediators, which are proteins and lipids that stimulate inflammation. Increased blood flow and attraction of immune cells during inflammation helps to limit the spread of infection. However, this process needs to be properly controlled, because excessive inflammation is harmful to the body's own tissues. Inappropriate regulation of the immune system may lead to auto-immune and auto-inflammatory diseases. Both groups of disorders result from the immune system attacking the body's own tissues, which in turn results in increased inflammation. In most auto-immune diseases, such as type 1 diabetes, coeliac disease, multiple sclerosis, systemic lupus erythematosus, Crohn's disease, and rheumatoid arthritis, defective responses of the adaptive immune system play a major role. Auto-inflammatory diseases are a relatively new category of diseases and are mainly caused by the defects in the innate immune system, causing inflammation for unknown reasons.

As reviewed in **Chapter 1**, both the innate and adaptive immune systems rely on many signaling molecules for their proper functioning. These molecules have roles in pathogen recognition (e.g., pattern recognition receptors such as TLRs, NLRs, and RLRs), signal propagation (e.g. adaptor proteins, kinases, phosphatases, and transcription factors) and control of gene expression (e.g. miRNAs). Functional defects in any of these molecules may increase susceptibility to infectious diseases or result in conditions like auto-immune and auto-inflammatory diseases. The zebrafish is a recent addition to biomedical research as a model for the study of inflammation and infectious diseases. The molecular components that orchestrate immune responses are well conserved between human and zebrafish. Infection and inflammation processes can be excellently visualized in zebrafish embryos and larvae, as these are optical transparent. The absence of functional adaptive immunity in embryos and larvae makes them highly suitable for modeling innate immune responses *in vivo*. In addition, high production rates and small size facilitate high-throughput assays using zebrafish. Several infection models in zebrafish have been well established, including *Salmonella typhimurium* and *Mycobacterium marinum* models for acute and chronic infections, respectively. In this

thesis we have made use of the zebrafish to study the importance of different regulatory pathways in innate immunity.

In **Chapter 2** we have demonstrated the importance of a negative regulator of the innate immune response, which is encoded by the protein tyrosine phosphatase non-receptor type 6 gene (*ptpn6)*. It is known that mutation of this gene in mice causes inflammatory lesions in multiple organs that become lethal at 3-5 weeks of age. Furthermore, dysfunction of human PTPN6, also known as SHP1, has been linked to auto-immune and auto-inflammatory disorders. PTPN6 is a hematopoietic phosphatase. In one day old zebrafish embryos, we found *ptpn6* to be specifically expressed in macrophages and at later stages of development we also observed expression in neutrophils and developing T-cells. We used a morpholino knockdown approach to study the role of *ptpn6* in the innate immune system of zebrafish embryos. Knockdown of *ptpn6* did not affect the early stages of development, but a late pleiotropic phenotype was observed with different morpholinos against this gene. At 5-6 days post fertilization (dpf), *ptpn6* morphant embryos showed more proliferation in the brain, contained more apoptotic cells, and they developed oedema and inflammatory lesions. An increased induction of the pro-inflammatory genes coding for interleukin 1b (*il1b*) and matrix metalloproteinase 9 (*mmp9*) was found in the *ptpn6* morphants. The induction of these genes was concomitant with the occurrence of inflammatory lesions that were highly infiltrated by leukocytes. This late phenotype was found to be independent of the presence of microbes, showing that an external trigger is not necessary for the development of inflammation in *ptpn6* morphants and the absence of this gene is sufficient to cause the inflammation-associated phenotype. The administration of anti-inflammatory glucocorticoids could not block the development of inflammation in *ptpn6* morphants. On the contrary, glucocorticoid treatment of *ptpn6* morphants intensified the development of inflammatory lesions as well as the induction of pro-inflammatory genes. These effects might be due to synergistic actions between the glucocortocoid receptor and several transcription factors which have been activated by *ptpn6* knockdown.

To analyze the function of *ptpn6* during infection we focused on the first days of embryonic development, prior to the manifestation of the spontaneous inflammatory lesions and the induction of pro-inflammatory genes. We challenged the *ptpn6* morphants at 1 dpf with *S. typhimurium* and *M. marinum* infections. A more rapid proliferation of *S. typhimurium* was observed in *ptpn6* morphants compared with control embryos. Furthermore, *ptpn6* morphants showed impaired control of the growth of an attenuated *S. typhimurium* strain, which could be more efficiently cleared by the immune system of control embryos. Infection with *M. marinum* also led to heavier bacterial loads and increased granuloma formation in *ptpn6* morphants compared with the controls. The infection-dependent induction of pro-inflammatory *il1b* and *mmp9* genes was higher in *S. typhimurium*-challenged *ptpn6* morphants than in the control embryos. Increased induction of *mmp9* was also observed in *M. marinum*-infected *ptpn6* morphants. Thus, hyperinduction of these genes proved to be disadvantageous for zebrafish embryos in combating infections.

We chose the *S. typhimurium* infection model to further investigate the specific effects of *ptpn6* knockdown on the innate immune response by microarray analysis. This analysis was performed 8 hours after challenge with *S. typhimurium* at 1 dpf, when no visible phenotypic effect of *ptpn6* knockdown was present. The total number of probes with a significant responsive to infection was approximately 2-fold larger in *ptpn6* morphants than in control embryos. Analysis of a group of 258 genes with higher up-regulation in infected *ptpn6* morphants showed significant overrepresentation of TLR, NLR, RIG-I, p53, MAPK, JAK-STAT and other immune-related KEGG pathways. More specifically, genes showing higher up-regulation in *ptpn6* morphants included cytokine/chemokine/interferon genes such as *il1b*, *il8*, *tnfa*, *tnfb*, and *ifnphi1*, matrix metalloproteinases such as *mmp9* and *mmp13*, and many transcriptional regulators of the ATF, CEBP, AP1, NFκB, and STAT families. Similar to the increased induction of pro-inflammatory genes, infected *ptpn6* morphants also showed increased induction levels of several negative regulators, such as *irak3*, *socs3a* and *socs3b,* and NFκB inhibitor genes (*nfkbiaa*, *nfkbiab*, *nfkbib*, *nfkbiz*). In contrast, the anti-inflammatory cytokine gene *il10* did not show increased induction in *ptpn6* morphants. The microarray results were confirmed by using an RT-MLPA assay that allows the simultaneous semi-quantitative PCR analysis of 34 innate immune genes. Thus, under *ptpn6* knockdown conditions, embryos responded to *S. typhimurium* infection by an enhanced gene induction profile of the innate immune response. Therefore, we could conclude that *ptpn6* functions as a key player regulating the inflammatory response to infection through a negative feedback mechanism. This function of *ptpn6* is crucial for preventing host-detrimental effects of inflammation and is essential for a successful defense mechanism against invading microbes.

In **Chapter 3** we used Illumina RNA deep sequencing (RNA-Seq) technology to determine transcriptome profiles of different immune cell types and to further analyze the effects of *ptpn6* knockdown at a cellular level. Transgenic zebrafish lines with fluorescently marked macrophages (*mpeg1:egfp*), neutrophils (*mpx:egfp*), and early T-cells (*lck:egfp*) were used for this study. Zebrafish larvae were dissociated at 5 dpf and the fluorescent cells were isolated by fluorescence activated cell sorting (FACS). First, to develop a protocol for RNA-Seq analysis from FACS-sorted cells, we used the *mpeg1:egfp* macrophage marker line. From pools of approximately 250 dissociated larvae we could obtain 4000-10,000 GFP-labeled cells. Using the Ambion RNAqueous-Micro Kit for RNA isolation in combination with the Clontech SMARTer Ultra Low RNA Kit for Illumina Sequencing, we succeeded in obtaining good RNA-Seq libraries, even from samples with an estimated RNA input below 100 pg. We used the DEseq program for statistical analysis of libraries from the GFP-positive and GFP-negative cell fractions of four biological replicates with approximately 10 million paired-end reads per library. The GFP-positive cell fractions of all biological replicates showed specific enrichment of KEGG pathways and GO-terms related to the immune system and approximately 50% of significant genes were common between each pair of replicates. These results demonstrated the robustness of our assay.

Having established a reproducible procedure for RNA-Seq analysis from FACS-sorted cells, we compared the macrophage signature genes obtained from *mpeg:egfp*-positive cells with FACS-sorted neutrophils from an *mpx:egfp* transgenic line and early T-cells from an *lck:egfp* transgenic line. As expected, known macrophage markers were specifically enriched in *mpeg1:egfp*-positive cells, including *csf1r*, *cxcr3.2*, *irf8*, *marco*, *mfap4*, *mhc2dab*, and *mpeg1* itself. Neutrophils showed a higher expression of *mpx*, *lyz*, *mmp9* and *mmp13a* than macrophages. Furthermore, the expression profile of *lck:egfp*-positive cells showed very specific expression of T-cell markers including *cd2*, *cd28*, *cd4*, *ikzf1* (*ikaros*), *rag1*, and *rag2* along with *lck*. There was more overlap between the expression signatures of *mpx:egfp*-positive and *mpeg1:egfp*-positive cell fractions than there was between these myeloid cell fractions and *lck:egfp*-positive lymphoid cells. The RNA-Seq libraries gave us the opportunity to look for other specific and more abundant genes that may prove to be valuable markers for developing new transgenic lines and antibodies for distinguishing leukocyte lineages in the zebrafish model. Genes that showed higher abundance than *mpeg1* in macrophages were members of the families of chemokines, immunoglobulins, olfactomedins, granulins, cathepsins, fibrinogens, lectins, transmembrane receptors, complement factors, and MHC II class proteins. Among these, *granulin 2* (*grn2*) had a more than 10-fold higher expression level than *mpeg1* and was highly specific for macrophages. Several other genes, for example the MHC class II genes *cd74* and *mhc2dab,* were also detected almost exclusively in macrophages. In neutrophils, *lyz* and *npsn* showed higher abundance than *mpx*, and *il34* was identified as another good marker. Highly specific T-cell markers included *lck*, *rag1*, *ccr9b*, *il17r*, *p2rx1*, *rorc, foxp3a,* and several genes for T-cell specific immunoglobulins.

We also used the RNA-Seq technology to investigate the immune response-related gene expression pattern of zebrafish challenged with *M. marinum*. Following its uptake by macrophages, this pathogen causes a chronic infection in zebrafish with the formation of granuloma-like aggregates resembling the hallmark of tuberculosis in human. Infected cells carrying mCherry-labeled fluorescent *M. marinum* were isolated by FACS sorting from larvae at 4 days post infection (dpi). We compared the expression signature of the *M. marinum*-infected cells with that of uninfected *mpeg1:egfp*-positive macrophages. A set of 40 distinctive macrophage markers was detectable in the *M. marinum*-infected cells, but most of these genes were strongly down-regulated compared with uninfected macrophages. Furthermore, many genes that were expressed in uninfected macrophages, including *mpeg1*, were not detected in the infected cells. In addition to down-regulation of macrophage markers, the infected cells showed specific enrichment of genes that could be grouped into five main clusters of gene ontology terms: ribosome, oxidative phosphorylation, proteolysis, ion transport, and chromatin assembly. The induction of these gene groups may reflect activation of protein translation and defense mechanisms in the infected cells. Examples of these genes include the anti-microbial hepcidin gene (*hamp1*) and a ribosomal protein gene (*rps3*) for which an extra-ribosomal regulator function in innate immunity has been reported. It is an interesting possibility that the increased expression of chromatin assembly genes in infected cells may be associated with the *M. marinum*-induced

morphological changes that macrophages undergo during granuloma formation. In addition to the five main clusters of enriched genes, we found that *M. marinum*-infected cells also showed increased expression of lipid metabolism and transport genes, lectin genes, and genes with immunosuppressive functions. Some of these genes have also been implicated in human tuberculosis. Based on these results, we concluded that *M. marinum* infection has a major impact on the gene expression signature of macrophages, resulting in a general down-regulation of macrophage markers and an up-regulation of genes proposed to be involved in both host defense and immunosuppression.

Next, we decided to use the RNA-Seq profiling technology to gain further insight into the molecular processes responsible for increased granuloma formation in *ptpn6* morphants infected by *M. marinum*. Furthermore, by RNA-Seq analysis of different leukocyte populations we aimed at better understanding of the underlying causes of the inflammatory conditions that *ptpn6* morphants develop around 5 dpf. Notably, matrix metalloproteinase genes, including *mmp2*, *mmp9*, and *mmp13a*, and the *mmp* inhibitor gene *timp2b* were significantly up-regulated in *M. marinum*-infected cells from *ptpn6* morphants compared with infected cells from control embryos. Along with the *mmp* genes, *serum amyloid A* (*saa*) was up-regulated. Since this gene has previously been implicated in regulating *mmp* genes expression, we propose that increased levels of *saa* play a role in triggering increased *mmp* gene expression during *M. marinum* infection of *ptpn6* morphants. Some genes with possible functions in anti-microbial defense were also up-regulated, including *hamp1*, *lepb*, *gcga*, and *steap4.* These genes are connected with responses to nutrients and therefore may form links between the innate immune system and metabolic responses. However, the up-regulation of these possible anti-microbial genes was not sufficient to prevent the strong increase in bacterial burden of *M. marinum* infection in *ptpn6* morphants..

In contrast to what was observed during *S. typhimurium* infection of *ptpn6* morphants (chapter 2), there was no general up-regulation of pro-inflammatory genes in *M. marinum* infected *ptpn6* morphants. Therefore, we concluded that the increased bacterial burden in *ptpn6* morphants during *M. marinum* infection is mainly due to a specific hyperinduction of *mmp* genes, while in *S. typhimurium* infection a general hyperinduction of pro-inflammatory genes is the cause of the increased bacterial burden. A model summarizing our conclusions about the effect of *ptpn6* deficiency on the molecular processes involved in *S. typhimurium* and *M. marinum* infections is presented in Figure 1.

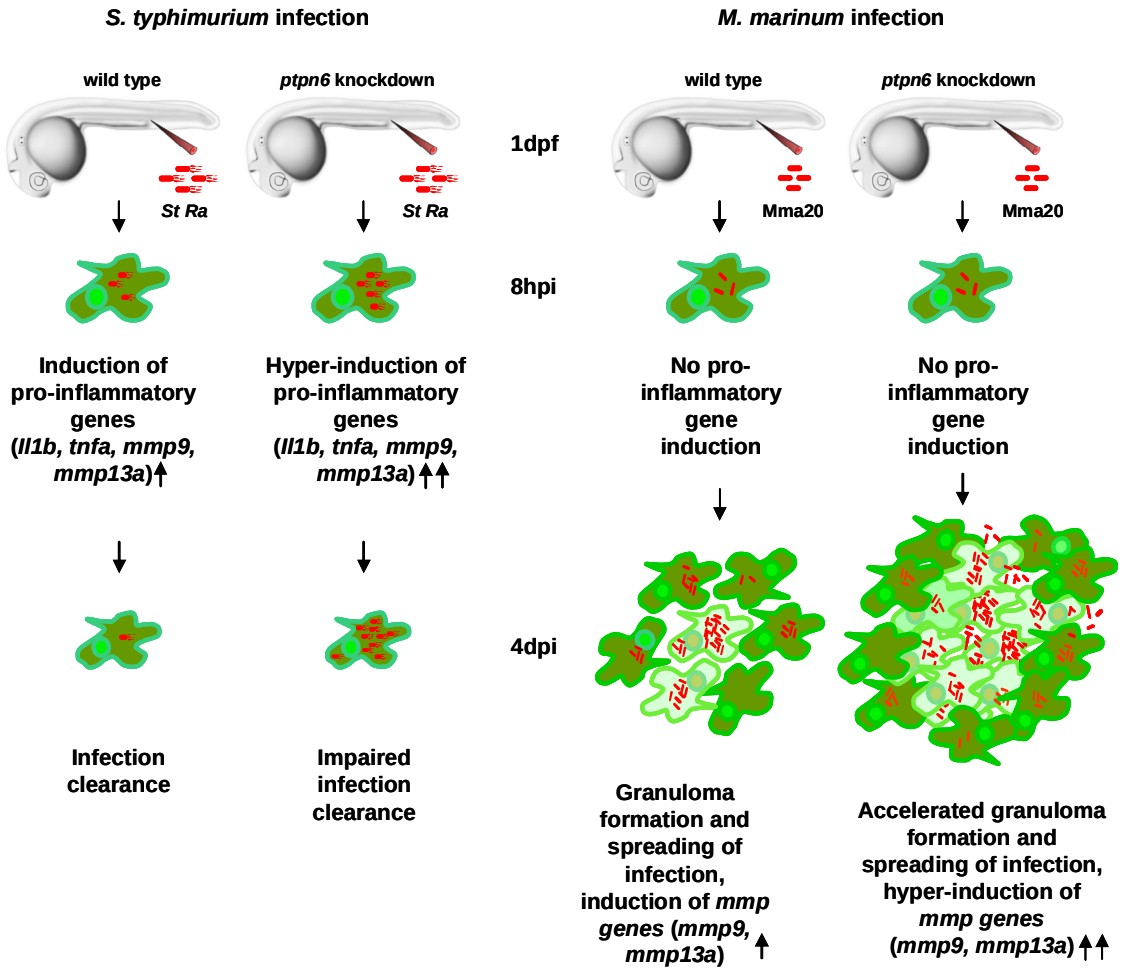

Figure 1. Schematic representation of the negative regulatory mechanisms of *ptpn6* during *S. typhimurium* and *M. marinum* infections in zebrafish embryos. Intravenous infection of wild type embryos at 1 day post fertilization (dpf) with *S. typhimurium* causes a general induction of pro-inflammatory genes (among others *il1b*, *tnfa*, *mmp9*, *mmp13a*) at 8 hours post injection (hpi), while this is not observed upon infection with *M. marinum* (Chapter 2, van der Vaart et al., 2012). In contrast, *M. marinum* infection is associated with the formation of granuloma-like aggregates of infected and uninfected macrophages. Infected macrophages in these granulomas show a down-regulation of macrophage marker genes (Chapter 3), indicated by a lighter color of green in the figure. The knockdown of *ptpn6* results in a hyperinduction of pro-inflammatory genes upon *S. typhimurium* infection. This increased inflammatory response, instead of helping the host controlling the infection, makes it more susceptible to disease progression. As a result, the host is no longer able to clear infections with the attenuated *S. typhimurium Ra* strain (Chapter 2). *M. marinum* infection with the Mma20 strain does not induce a strong pro-inflammatory response at 8 hpi in *ptpn6* knockdown embryos nor in control embryos. At later stages of infection, the morphants did not display a general hyperinduction of pro-inflammatory genes, but a specific hyperinduction of matrix metalloproteinase (*mmp*) genes was observed. This induction of *mmp* genes occurred specifically in infected and uninfected macrophages and was associated with an accelerated granuloma formation and increased bacterial burden (Chapters 2 and 3). These observations

show the importance of *ptpn6* for a balanced and productive immune response in zebrafish embryos against bacterial pathogens.

Interestingly, the *mmp9* and *mmp13a* genes were also up-regulated in macrophages of uninfected *ptpn6* morphants. These macrophages also showed up-regulation of actin, myosin and parvalbumin genes, of several immune-related proteases (e.g., *cpa5*, *npsn*, *cpn1*, *mmp9* and *mmp13a*), and of typical neutrophil markers like *lyz* and *mpx*. In contrast, several typical macrophage-expressed genes (e.g. *cxcr4b*, *tnfa*, *tnfrsf1a*, *mbp*, *grn2*, *mhc2dab*, *cd74*, *cd74a,* and the chemokine gene *si:ch211-149o7.4*) were down-regulated in macrophages of *ptpn6* morphants. These data suggest a complex role of *ptpn6* in regulating the activation status of the macrophages. The effects of *ptpn6* knockdown on different cell populations were very specific. The up-regulation of *mmp9, mmp13a* and several other protease genes was observed in macrophages but not in neutrophils, T-cells, or in the GFP-negative background of *ptpn6* morphants. Up-regulation of *mmp2* was detected in neutrophils in GFP-negative background cells. An up-regulation of genes involved in p53 signaling (*tp53*, *mdm2*, *ccng1*) was only observed in neutrophils. Furthermore, neutrophils showed specific down-regulation of apolipoprotein and protease genes under *ptpn6* knockdown conditions, while down-regulation of ribosomal protein genes was specific for T-cells. There was no effect on p53 signaling genes in the GFP-negative background cells of *ptpn6* morphants and no indication of a general toxicity effect in the RNA-Seq data. Based on these results, we suggest that enhanced *mmp* genes secretion by macrophages might be a major cause of the inflammation-associated phenotype that *ptpn6* morphants develop around 5 dpf.

In **Chapter 4** we studied the function of microRNAs (miRNAs) as another potential level of regulation of innate immune responses during infection of zebrafish embryos. MiRNAs are evolutionary conserved, genome-encoded small RNAs (~22 nucleotides) involved in post-transcriptional gene repression. We focused on the role of microRNA-146a and microRNA-146b (miR-146a/b), which are two closely related members of the miRNA-146 family that is well conserved between human and zebrafish. Dysregulation of miR-146a/b has been frequently linked to inflammatory diseases and malignant tumors, and therefore modulating its function might have a therapeutic potential. Previous studies in human cells and mouse models suggested that miR-146a/b function in a negative feedback pathway of TLR and cytokine signaling by targeting *IRAK1* and *TRAF6* mRNAs for down-regulation. The *IRAK1* and *TRAF6* homologs of both zebrafish and human contain putative target sites for miR-146a/b in their 3'UTRs, suggesting that miR-146 feedback control of TLR signaling is evolutionary conserved (Ordas et al., 2010). Using quantitative PCR (qPCR) analysis we found that the expression of miR-146a/b in zebrafish is inducible by infection with *S. typhimurium* and *M. marinum*, which was in line with previous microarray data from our group. Furthermore, we showed that infection-inducible expression of miR146a/b was partially dependent on the TLR pathway genes *traf6* and *myd88*. The induction of miR-146a/b during *S. typhimurium* infection was significantly reduced in *traf6* knockdown

embryos and in *myd88* knockout mutant embryos compared with the induction levels in control embryos. This demonstrated the requirement of a Myd88/Traf6 pathway for full-scale induction of miR-146a/b during infection in zebrafish. However, it is likely that a parallel Myd88/Traf6-independent induction route also exists, as neither *traf6* knockdown nor *myd88* knockout was sufficient to block the infection-induced expression of miR-146a/b completely.

We took advantage of the morpholino knockdown technology for functional analysis of miR-146a/b in zebrafish embryos by. designing two different morpholinos for each of the miR-146 family members. The efficiency of each knockdown was confirmed by Taqman qPCR analysis. We did not observe any effects of the loss of miR-146a/b on the development or differentiation of leukocytes in zebrafish embryos. Hypothesizing that miR-146a/b might be involved in negative regulation of the innate immune response, we were interested to test whether miR-146a/b knockdown leads to hyperinduction of pro-inflammatory genes following *S. typhimurium* infection, similar as was observed in our knockdown analysis of the negative regulator *ptpn6*. RNA-Seq analysis was performed for this purpose. First, we checked if miR-146a/b knockdown had an effect on gene expression in the absence of infection. We found that only 78 genes were affected by miR-146a/b knockdown, among which 5 genes in p53 signaling. This might reflect a non-specific effect of the miR-146 knockdown, since morpholino effects on the p53 pathway are relatively common. However, considering that miR-146 has been frequently linked with cancer, a direct effect on the p53 pathway is also conceivable. *S. typhimurium* infection led to slightly higher numbers of up- and downregulated genes in miR-146 morphants than in control embryos, but there was no general hyperinduction of pro-inflammatory genes in miR-146 morphants. The only pro-inflammatory marker that was induced at higher levels in miR-146 morphants was the *mmp9* gene. Consistent with this observation, human *MMP9* was down-regulated following overexpression of miR-146a/b in cultured cancer cells and macrophages. Given that there are no predicted target sites for miR-146a/b in *mmp9*, miR-146-mediated regulation of this gene may occur via the Myd88/Traf6 pathway that acts upstream of *mmp9* induction. The fact that *mmp9* is the most strongly induced pro-inflammatory marker in *S. typhimurium* infection may explain why differences in induction of other Myd88/Traf6-dependent pro-inflammatory markers were not seen in our RNA-Seq analysis of miR-146a/b morphants and control embryos.

Although the overall effect of miR-146a/b knockdown observed in our RNA-Seq analysis was relatively minor, KEGG pathway analysis revealed a possible effect on apolipoprotein-mediated lipid transport in *S. typhimurium*-infected miR-146 morphants. This is an interesting observation considering that many studies have linked apolipoproteins to immunoregulation and host defense and that several inflammatory disorders are associated with uncontrolled lipid accumulation. MiR-146a has been suggested to be involved in negative regulation of oxidized low-density lipoprotein accumulation in human macrophages. In future work, zebrafish infection models may prove useful to further explore the role of miR-146 family members in lipid-mediated inflammatory responses.

Knockdown of miR-146a/b did not affect bacterial burdens during infection with *S. typhimurium*, but infection with *M. marinum* under miR-146a/b knockdown conditions resulted in accelerated granuloma formation and led to significantly increased bacterial burdenscompared to control infected embryos. Knockdown experiments with morpholinos targeting each of the miR-146 family members separately, showed that loss of miR-146b rather than loss of miR-146a caused the increased bacterial burden during *M. marinum* infection. It remains to be further investigated if accelerated granuloma formation under miR-146b knockdown conditions is linked with increased *mmp* gene expression, similar as in the *ptpn6* knockdown situation.

Conclusion: The work presented in this thesis has provided new insights into the mechanisms involved in the regulation of innate immune responses to infection in zebrafish embryos. Furthermore, cell-specific transcriptome profiling studies in this thesis have identified novel marker genes for distinguishing immune cell types, which is highly useful information to fulfill the demand for new fluorescent reporter lines and lineage-specific antibodies in the zebrafish model. Our functional studies of Ptpn6 show that the role of this protein-tyrosine phosphatase as a negative regulator is of critical importance to a properly balanced innate immune response and for the control of infections with *S. typhimurium* and *M. marinum*. We found that Ptpn6 has a specific regulatory function in responses to each of these bacterial pathogens. In *S. typhimurium* infection, *ptpn6* deficiency caused a general hyperinduction of pro-inflammatory genes, which was contra-productive as it impaired the control of infection. In *M. marinum* infection, a more specific effect of *ptpn6* deficiency on matrix metalloproteinase gene expression is likely to be a major underlying cause of accelerated granuloma formation and increased bacterial burden. We further concluded that Ptpn6 functions as a much stronger negative regulator than infection-inducible miRNAs of the miR-146 family, which may be involved in more subtle fine-tuning of the innate immune response. Knowledge about the distinct roles of Ptpn6 and miR-146 miRNAs has practical applicability in regard to their potential as therapeutic targets for inflammatory diseases and cancer. Our zebrafish knockdown models for these negative regulators may contribute to anti-inflammatory and anti-microbial drug discovery, for which high-throughput assays in zebrafish embryos are very powerful.