Summary and Discussion
In order to combat infectious diseases, multicellular organisms have developed complex networks of cellular and humoral defence mechanisms allowing for the detection and destruction of pathogenic microorganisms. In vertebrates these complex mechanisms are traditionally categorized into the evolutionary ancient innate immune system (present in all multicellular organisms), and the relatively young adaptive (acquired) immune system that arose with the appearance of jawed fish.

In the last decade the study of the innate immune system has gained renewed scientific momentum as a result of the discovery of the essential receptor families that are required for pathogen recognition. It has been demonstrated that the innate immune system plays a pivotal role in the first line of defence against potentially pathogenic microorganisms, and is also required for activating subsequent adaptive responses. Many of the molecular mechanisms underlying the innate immune response have been elucidated. The recognition of invading pathogens is achieved by the innate immune system through various classes of receptors, commonly termed pattern recognition receptors (PRRs), such as the Toll-like receptor (TLR), NOD-like receptor (NLR) and RIG-I like (RLR) receptor families. These receptors are able to detect specific molecular structures of microorganisms termed pathogen-associated molecular patterns (PAMPs) or microbial-associated molecular patterns (MAMPs). Polymorphisms of these receptors and downstream signalling intermediates have been associated with increased susceptibility to infectious diseases and with several inflammatory disorders. Furthermore, both extracellular and intracellular pathogens have been shown to manipulate the signalling pathways of their hosts in order to evade detection or escape innate immune defences, underscoring the clinical relevance of studying the molecular basis of the innate immune system.

This thesis focuses on the use of the zebrafish as a model to study the vertebrate immune system to gain new insights into the mechanisms of innate immune defence against bacterial infections and TLR signalling. In zebrafish embryos, cells of the innate immune system develop prior to cells of the adaptive system, thus allowing specific analysis of innate immune functions in a genetically tractable vertebrate animal model. Furthermore, the transparency of zebrafish embryos enables real-time analysis of the response to infections.

At the onset of the project, little was known about TLR signalling function in the zebrafish embryo model. Previous studies had demonstrated the presence of TLRs and downstream signalling molecules in zebrafish, and bacterial and viral infections were found to induce expression of different zebrafish TLR genes. Furthermore, it was known that one-day-old zebrafish embryos already possessed leukocytes able to phagocytose bacteria. However, functional evidence to confirm the role of TLR signalling in zebrafish and the presence of an innate immune response in early embryos was lacking. Therefore, as described in Chapter 2, we initially aimed at a functional analysis of MyD88, an essential adaptor protein of vertebrate TLRs and of the interleukin-1 and -18 receptors that is activated through TLR signalling. Making use of morpholino-mediated knock-down in combination with a previously established
S. enterica serovar Typhimurium (S. typhimurium) infection system, we were able to demonstrate that loss of MyD88 led to a significantly impaired ability of zebrafish embryos to clear an otherwise non-pathogenic LPS O-antigen mutant of S. typhimurium (Ra strain). Thereby, we demonstrated for the first time that Myd88-dependent signalling events play a critical role in innate immune responses towards a bacterial infection in zebrafish embryos. These results were consistent with many infection studies in MyD88−/− adult mice, which showed increased susceptibility to a variety of pathogens. Our study therefore validated the zebrafish embryo as a useful model for analysis of vertebrate TLR signalling as well as the vertebrate innate immune system in general.

In Chapter 3 we took advantage of the clear temporal separation of innate and adaptive immunity in the zebrafish embryo to specifically analyse vertebrate innate immune responses to a systemic bacterial infection using microarray technology. The host response of embryos infected with a pathogenic S. typhimurium wild-type strain was compared to the response of embryos infected with the attenuated LPS mutant strain (Ra) used in chapter 2. The time-resolved transcriptome analysis revealed distinct temporal expression profiles correlated to the symptoms of disease progression for both strains. The main difference between the strains was observed at 24 hours post infection (hpi). At that point the transient infection caused by the Ra mutant was nearly eliminated, whereas accumulation of wild type bacteria further increased, resulting in lethality around 30 hpi.

Subsequently, we compared the zebrafish transcriptome data to a meta-analysis of microarray data of various human cell lines challenged by different pathogens. The comparison revealed a considerable overlap between the zebrafish host response to S. typhimurium and those genes that were commonly induced in all cell lines upon pathogen challenge. The overlap included genes for well known immune responsive transcription factors, cell surface receptors, signal transduction intermediates, adhesion factors and proteins involved in tissue remodelling, as well as various interferons, chemokines, pro-inflammatory and anti-inflammatory cytokines (Fig.1). Our results therefore further underscore the predictive value of the zebrafish embryo model.

As we are particularly interested in TLR immune signalling, we analyzed our transcriptome data using GenMapp-based visualisation of the expression data on the TLR pathway. This pathway analysis showed that various signalling intermediates at different levels in the TLR pathway were induced upon S. typhimurium infection in vivo. Interestingly, the two isoforms of zebrafish Tlr5 were induced throughout the infection time course. In the mammalian system TLR5 signalling has previously been shown to be triggered by bacterial flagellin. We found that flagellin stimulation in zebrafish embryos was sufficient for strong induction of several of the genes that were also induced by S. typhimurium infection, including the inflammatory cytokine gene il1b, the chemokine genes il8 and cxcl-Cic, the matrix metalloproteinase gene mmp9, and the putative negative regulator of TLR signalling, irak3. Using morpholinos directed against the two isoforms of Tlr5 followed by flagellin stimula-
tion, we attempted to assess Tlr5 signalling in zebrafish embryos. Our results clearly demonstrated Tlr5-dependent gene activation of \textit{il1b}, \textit{il8}, \textit{cxcl-C1c}, \textit{mmp9}, and \textit{irak3} in the zebrafish embryo. Extending the work described in Chapter 2, we additionally assessed transcriptional effects of Myd88 on downstream target genes of innate immunity signalling. Our results demonstrated a clear dependency of \textit{mmp9}, \textit{il1b} and \textit{irak3} on MyD88 for transcriptional activation upon \textit{S. typhimurium} challenge. In contrast, \textit{ifn1} and \textit{il8} did not show changes in their induction levels upon bacterial challenge, demonstrating MyD88-independent activation of these genes. Taken together, these results are the first demonstration of a conserved TLR ligand specificity and of the presence of MyD88-dependent and MyD88-independent signalling pathways in the zebrafish embryo.

The detailed transcriptome analysis of the host response to \textit{S. typhimurium} described in chapter 3 has linked a large set of zebrafish genes to the process of bacterial infection, providing a case study for future immunity research in the zebrafish embryo model. For many of these genes the specific function is still unknown, both in zebrafish and humans. Taking advantage of the rapid gene knock-down assays, the zebrafish embryo model will be of great use for the future functional characterization.

**FIGURE 1.** Schematic representation of the zebrafish embryonic host response (adapted from Jenner and Young, Nature Reviews Microbiology, 2005). Genes shown in red were up-regulated in zebrafish embryos after \textit{S. typhimurium} infection. Genes shown in black were present on the microarray platform but not induced after infection.
of these genes, thereby supporting further annotation of the human genome.

Analysis of the function of Traf6 in the innate immune response of zebrafish embryos towards *S. typhimurium* infection is described in Chapter 4. TRAF6 functions downstream of MyD88 in the vertebrate TLR pathway and also transduces signals emanating from members of the TNF receptor superfamily that are involved in both the immune response and developmental processes. The fact that Traf6 is a key player at the cross-roads of development and immunity makes the analysis of its *in vivo* molecular function a great challenge. In mice, severe developmental defects and early lethality caused by Traf6 deficiency have interfered with analyses of the immune response. In our approach, this problem was solved by titrating the effect of a Traf6 morpholino down to a level where developmental defects in zebrafish embryos were avoided. The morpholino-treated embryos were subsequently challenged by a *S. typhimurium* infection and the transcriptional response was assessed by microarray and RNA deep sequencing (RNAseq) analysis.

This approach allowed the identification of 146 genes (confirmed by RNAseq) that were dependent on Traf6 in the context of a bacterial infection. Among those were genes with well known functions in innate immunity, such as *il1b*, *mmp9*, *mmp13*, *hamp2*, *cxcl12* and *tnfb*. Using GenMapp analysis to focus on the TLR pathway, we could show that an infection by *S. typhimurium* was no longer able to trigger Tlr5 expression after Traf6 knock-down. In addition, several genes with no known Traf6 association were also identified, such as *gnrh2* (gonadotropin releasing hormone 2), *stc1* (stanniocalcin 1), *dgat1b* (diacylglycerol O-acyltransferase homolog 1b) and *dram1* (DNA-damage regulated autophagy modulator 1). Expression trend analysis of the Traf6-dependent genes identified nine clusters with characteristic transcription response profiles, demonstrating the dynamic role of Traf6 as both a positive and negative regulator.

In our study we have also identified Traf6 targets of which no annotation could be derived either for zebrafish, mouse or human orthologs. Domain searches were equally unsuccessful in unearthing a possible function. Since the expression levels of some of these genes were strongly affected by *S. typhimurium* induction at early time points (2 hpi), as well as strongly dependent on Traf6 function, the further study of these genes in the vertebrate immune system will be of great interest. In addition, the data derived by RNAseq represent a wealth of disease-induced transcript information that will be of great value for future studies.

As discussed above, Traf6 does not exclusively mediate signalling processes of the immune system, but also has a function during development. In Chapter 5 we therefore attempted to gain insight into the function of Traf6 during early zebrafish embryogenesis using a morpholino based knock-down approach. Transcriptome analysis was performed at the stage of 30% epiboly (4.7 hours post fertilization), which is the earliest phase of gastrulation where the outer cell layer of the blastula spreads to envelop the embryonic yolk sac. Using different morpholinos directed against *traf6* mRNA, and by comparison to two control morpholinos, we were able to
identify a large gene set of 868 genes that appear to be specifically controlled by Traf6 during embryogenesis. Gene Ontology analysis showed that a Traf6 knock-down led to the induction of several genes involved in the regulation of programmed cell death. Genes such as bcl2-antagonist of cell death (bad) and bcl2-associated X protein (bax), known to promote apoptosis, were induced, whereas genes promoting cell survival such as birc2 showed decreased expression levels. Our results are in line with an anti-apoptotic effect of TRAF6 that has previously been demonstrated in mouse cell lines.

Comparison of the transcriptome data to the infection data from Chapter 4 showed only a limited overlap of 14 genes that were Traf6-dependent in early development and during infection, illustrating the diverse functions of Traf6 in development and immunity. As part of the analysis we also identified genes that showed altered expression even in response to the control morpholinos, and thus were regulated due to morpholino treatment in general. Interestingly, several of these genes could be linked to the immune system. The response of the immune system to morpholinos is consistent with the ability of the vertebrate innate immune system to sense microbial and viral DNA or RNA. However, a response to such treatments in early embryogenesis has never previously been shown. We could demonstrate that a group of four Toll-like receptors, namely tlr3, tlr4a, tlr4b and tlr9, were down-regulated in response to general morpholino treatments. From the traf6 knock-down results we were able to conclude that the general response of tlr3 to morpholino injections was not dependent on Traf6. In contrast, the response of tlr4a, tlr4b and tlr9 appeared to be dependent on Traf6.

Taken together, we could show that Traf6 knock-down led to the alteration of a large set of genes during early zebrafish development, indicating a crucial role of Traf6 also in zebrafish development. However, the specific function of Traf6 in development seems to be distinct from its function in immunity, as only a small group of genes were differentially regulated due to Traf6 knock-down under both conditions. In addition, we revealed differential regulation of various TLR genes as a general response to morpholino treatments, suggesting that immune signaling pathways might already be functional in these early stages.

In conclusion, the studies described in this thesis have strongly contributed to the validation of the zebrafish embryo model for analysis of the vertebrate innate immune system. In addition to the characterization of the embryonic host transcriptome response to bacterial infection, we have demonstrated functions for key signaling molecules in the innate immune response, including Tlr5, MyD88 and Traf6. In recent years many zebrafish infection models for human pathogens have been published and their use has already led to new insights into host-pathogen interactions, most notably in the field of tuberculosis research. Our data will now enable in depth functional follow-up studies that will give unprecedented new insights into the mechanisms of innate immune defence systems. Such insights can subsequently be validated in mammalian systems. This, in combination with future applications of ze-
brafish embryo infection models in high-throughput compound screens, holds much promise for the discovery of novel anti-microbial and anti-inflammatory drugs.