Toll-like receptor signaling in the innate immune system of zebrafish larvae

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CHAPTER 5

Summary and general discussion

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Scientists started to address the issue of innate immune specificity about thirty years ago, when the interleukin 1 receptor was cloned in 1988 (1) and Charles Janeway (2) proposed the concept of pattern recognition receptors (PRRs) in 1989. Toll-like receptors are representative members of the group of PRRs. Human TLR4 was first identified as the signaling receptor for lipopolysaccharide (LPS) in 1989, initiating intensive research on the other TLR family members in mammals (3). TLRs are highly conserved across different vertebrate species. After recognition of specific pathogen associated molecular patterns (PAMPs) from microbes or damage associated molecular patterns (DAMPs) from damaged tissue, TLRs mediate the activation of downstream NF-κB, MAPK and AP-1 signaling pathways involved in host defense against invading pathogens and maintaining homeostasis.

Zebrafish have a 3-4 weeks separation stage between development of innate and adaptive immunity after fertilization (4, 5), which gives the possibility to study the host innate immune response to infection in the absence of adaptive immunity responses. In the beginning of our study, there was little known about the function of TLR signaling in zebrafish larvae. In this thesis I studied the functions of the zebrafish orthologs of the human TLR5 and TLR2 genes that were shown to be responsible for recognition of bacterial flagellin and a broad spectrum of bacterial cell wall components, respectively. One of the focal points of this thesis is the difference at the transcriptomic level of the downstream pathway of the TLR5 and TLR2 receptors and the roles of TLR signaling in host innate immune responses to infection by *Mycobacterium marinum*, a close relative to *Mycobacterium tuberculosis* and a natural pathogen of zebrafish. The new possibilities for analysis of transcriptomes using RNA deep sequencing make it highly attractive to analyze the responses of an entire test animal model at the system biology level. Furthermore, we used genetic knockdown and knockout tools to further analyze the function of TLR5 and TLR2 and downstream signaling partners in innate immunity, infectious disease and insulin resistance.

In **chapter 2**, we demonstrated that Tlr2 signaling functions similarly in zebrafish embryos as in mammalian cells through regulating immune gene expression after TLR2 ligand systemic stimulation. For this study we used the compound Pam3CSK4 which is a synthetic lipopeptide which can mimic the acylated amino terminus of bacterial lipoproteins, and is often used to trigger TLR2 activation in mammalian cells. Further comparative analysis of transcriptomic profiles of Tlr2 and Tlr5 signaling upon Pam3CSK4 and flagellin stimulation, respectively, were conducted using RNA deep sequencing. The results show that there is a relatively limited overlap between the transcriptome responses towards Pam3CSK4 and flagellin. The overlap includes well known downstream immune mediators that were previously shown to be induced by flagellin such as *il1b*, *tnfa*, *irak3, mmp9, cxcl-c1c* and *il8*. In contrast, *il6* and *il10*, that are associated with an anti-inflammatory response, were induced much stronger by Pam3CSK4 than by flagellin. A relatively much larger group of genes showed a

differential response to flagellin or Pam3CSK4, including a group of genes of which the transcription is specific for activation by one of the two treatments. In the Pam3CSK4 specific set, we identified a group of transcription factors with more than 2 fold-change in expression, as confirmed by qPCR analyses, including *cebpb*, *fosb*, *nr4a1* and egr3. We also showed that the regulation of the gene sets that were specifically responding to Pam3CSK4 and flagellin were inhibited by knockdown of *tlr2* or *tlr5*, respectively. Our studies show that Pam3CSK4 and flagellin can stimulate the Tlr2 and Tlr5 signaling pathways leading to common and specific responses in the zebrafish larval system.

To further study Tlr2 function in host defense against mycobacterial infection, we developed a *tlr2* mutant-*M. marinum* infection model in **Chapter 3**. The Tlr2-specific genes shown in Chapter 2 also show a significant upregulation upon *M. marinum* infection. In contrast, the *tlr2* mutant showed a defect in the response of the expression level of these genes to infection. In addition, the *tlr2* mutant showed a reduced number of macrophages in the absence of infection, which might be attributed to a defect in the emergence and differentiation of hematopoietic stem and progenitor cells. The TLR2 agonist Pam3CSK4 was shown to induce a large proportion of human hematopoietic stem cells to express markers of the myelomonocytic lineage (6). Herman et al. (7) also showed that systemic exposure of mice to a TLR2 agonist leads to an expansion of bone marrow and spleen phenotypic hematopoietic stem cells and progenitors, which supports a role for TLR2 in the regulation of hematopoietic stem cells.

The *tlr2* mutant also shows a higher mycobacterial proliferation and a reduced number of granulomas in the presence of infection. However, our results suggest that the macrophage migration speed and maximum migration distance from infection sites of phagocytic macrophages, are significantly higher in the *tlr2* mutant than in the *tlr2* heterozygote control. This could indicate that the Tlr2-deficient macrophages are less active in phagocytosis which might contribute to the higher bacterial proliferation in the *tlr2* mutant. These results suggest that Tlr2 might be playing a role in macrophage congregation and phagocytosis and is involved as a protective factor in host defense against mycobacteria.

Subsequently, we performed RNA deep sequencing of *tlr2* mutant and heterozygote larvae after *M. marinum* infection, and analyzed the transcriptomic profiles. We found that *tlr2* mutation led to far less upregulation and downregulation of immune response genes after *M. marinum* infection. Many of these genes are involved in lysosome processes, chemotaxis, transcription regulation, and other immune processes, or encode diverse immunoglobulin domain-containing proteins (*dicps*). In addition we also identified genes involved in other processes, such as oxidation-reduction process, DNA repair, transcription regulation, and apoptotic process regulation. To analyze the

relationship between the genes that are differentially expressed, we constructed networks based on common expression targets. For instance, we found that the vitamin D receptor pathway genes that are normally up-regulated during infection in zebrafish larvae were down regulated in the *tlr2* mutant. Vitamin D has been shown to be an important regulatory factor during tuberculosis (8) and has been linked previously to TLR2 function in studies in cell cultures (9).

In addition, the transcriptome profiles of the *tlr2* mutant and heterozygote were analyzed in the absence of infection. We found that the largest category of genes of which the transcriptional level was significantly affected, were linked to neurological system processes. When focusing on the possible signaling pathways that could be involved we observed that there was a significant effect in the GO category of transcription factors, particularly the c-Maf factors totaling up to 546 representatives that were affected. Considering the role of Maf family transcription factors in macrophage self-renewal, these results might indicate a link to the effect of the *tlr2* mutation on the number of macrophages in the absence of infection.

Furthermore, we also conducted Gene-Set Enrichment Analysis to our deep sequencing data with *p*-value less than 0.05. We screened 61 pathways in the *tlr2* heterozygote and 67 pathways in the *tlr2* mutant related to metabolism, immunology and inflammation, respectively. Most of these pathways respond in a common way in both groups showing that the *tlr2* mutant still retains most metabolic and immunological responses to *M. marinum* infection. Subsequently, we performed Sub-Network Enrichment Analysis to identify possible key genes that are responsible for the difference in response of the *tlr2* heterozygote and mutant groups to *M. marinum* infection. In addition to 301 common pathways from both groups, there are 264 *tlr2* related specific pathways in zebrafish. These 264 pathways were compared to gene sets that were shown to be responding to *M. tuberculosis* infection (10) in human macrophages showing 56 common pathways. Of these common pathways, the pathway of TLR8 has the lowest *p*-value in both enrichments, which suggests that Tlr2 signaling is strongly connected with Tlr8 function. Furthermore, pathway analysis in the *tlr2* mutant (Chapter 3, Fig. S5 and S7) also implicated that the expression of the Tlr8 pathway is connected to vitamin D signaling. TLR8 mutations (polymorphisms) increase susceptibility to mycobacteria (11, 12). These predictions from integrated transcriptome analysis give further incentives to study the function of TLR8 signaling in our future work.

In addition to being involved in innate immune defense against mycobacterial infection and insulin resistance, TLR-dependent signaling is also required to maintain compartmentalization of the gut microbiome of mice (13). TLR2 showed a significantly higher expression in the small intestine in mice with conventional microbiome compared with that in germ-free mice (14). In our study with zebrafish larvae, we

found that the germ-free condition can induce the expression of Tlr2-specific genes such as *cebpb* and *fosl1a*, (Yang, Koch and Spaink, unpublished data). This induction of the transcription factors that were already indicated to be downstream of TLR2 signaling in chapter 2, was also shown to be dependent on Myd88 and Tlr2. Further studies into the function of TLR2 in the response of the gut to the microbiome are currently undertaken in our laboratory.

Tlr2 is widely expressed in various tissue and cells (15, 16) including immune cells (17), intestinal epithelial cells (18) and skin (19, 20) in teleost. We also performed studies with external stimulation with TLR2 ligand-Pam3CSK4 in zebrafish larvae that have not been included in this thesis. In these studies we used Q-PCR to analyze the expression of various genes that were predicted to be downstream of TLR2 signaling and found a dose-dependent inflammatory response (unpublished data). These results indicate that Tlr2 also functions in zebrafish skin.

To study the downstream effects of Tlr signaling in zebrafish we analyzed zebrafish TNF receptor associated factor 6 (Traf6) and inhibitor of kappa B kinase beta (Ikkβ, Ikk2) mutants in **Chapter 4**. These mutants are the result of a point mutation that leads to stop codons in the beginning of the reading frames of these genes. Both mutants did not show any visible abnormal organ phenotype during embryonic or later developmental stages, and the adults were also capable of breeding. These results are unexpected, since total deficiency of Traf6 or Ikk2 in rodents is lethal.

In the case of the *traf6* mutant, there is no significant difference with the wildtype in leukocyte development and mycobacterial infection phenotypes. We hypothesized that the *traf6* mutation does not result in a null mutant at the protein level. There could be an alternative start codon close to the mutation point that leads to reinitiation of translation of Traf6. We did find that there is another ATG, close to the predicted start codon, that if used as an alternative start codon would lead to a slightly shorter Traf6 protein. Although several of the amino acids in the resulting deletion are conserved in the mammalian counterparts, one of the predicted essential domains in the N-terminal moiety, the RING domain, would not be affected and therefore it is conceivable that a resulting protein as a result of translational reinitiation might be at least partially or perhaps fully functional. However, we do not know how likely this hypothesis is, since to our knowledge the capacity of the zebrafish translation machinery to restart translation shortly after a stop codon that pertubs a natural reading frame is currently unknown. Another explanation is that the *traf6* mutation effect has been rescued by another mutation in the genome that resulted from the saturating ENU methodology used for mutagenesis that was not bred out of the original founder line. An indication for this is that we only obtained one single homozygote after the first generation incross. Such a suppressor mutation could have led to misregulation of close paralogs of Traf6 that might take over the function of Traf6. It is also possible that the Traf6

mutation is suppressed by mutation of unrelated proteins that function in the TLR or TNF signalling pathways. This would be similar to the published suppression of mutants of Ikk2 by mutations in Tnfr1 (21). Therefore, it is needed to construct new mutants of Traf6 for further comparative studies.

We found that Ikk2 mutant larvae show a significant decrease in body size, leukocyte numbers and expression of marker genes for macrophages and neutrophils. Furthermore, the mutant also showed an increased bacterial proliferation after *M. marinum* infection. Unexpectedly, we found that in the *ikk2* mutant a significantly higher expression of *il1b* was induced by flagellin injection than in the control. This result might be attributed to the overexpression of *ikk1* in the *ikk2* mutant, which perhaps plays a compensatory role in *il1b* expression. IKK1 is necessary for activation of the non-canonical or alternative NF-κB pathway (22). Functioning as one of the catalytic subunits of the IKK complex, IKK1 shows a similar structure as IKK2 (23). This hypothesis could be further studied by the construction of double mutants of Ikk1 and Ikk2 in zebrafish.

IKK2 has been shown to play a role in insulin signaling in mammals. For instance, mice with Ikk2 inhibition or deficiency reverse obesity- and diet-induced insulin resistance (24-26). In our study, we found that the insulin resistance induced by hyperinsulinemia in zebrafish was modulated in the Ikk2 mutant. The mechanism underlying these results might be related to the degradation of ribosomal protein S6 kinase (S6K) in the absence of IKK2 activation (27). S6K shows a negative feedback regulation to insulin receptor substrate 1 (IRS1) under conditions of nutrient satiation (28) (Fig. 1). The activation of IKK2 inhibits the degradation of S6K after phosphorylated by c-Jun N-terminal kinase 1 (JNK1), allowing S6K activation by mTOR (mechanistic target of rapamycin), which stabilizes S6K (27) (Fig. 1). Furthermore, IKK2 can also phosphorylate IRS1 on serine residues, leading to attenuation of tyrosine kinase- mediated signaling from the insulin receptor, interference of normal insulin action, and subsequent insulin resistance (32). Similar to IKK2, activation of JNK1 also results in inhibitory serine phosphorylation of IRS-1 (31). Both IKK2 and JNK1 can be activated by the initiation of TLR signaling, which indicates that TLR activation is indirectly involved in insulin resistance. Additionally, proinflammatory cytokines, such as TNFα, play important roles in diet- or obesity-induced insulin resistance. In adipocytes of mammals, free fatty acids can induce TNFα production through TLR2 signals, combined with increased TLR2 expression, which is associated with the development of high fat-induced insulin resistance (33). Free fatty acids also can activate myeloid proinflammatory cells via TLR2 and TLR4 and JNK signaling pathways, thereby promoting inflammation and subsequent cellular insulin resistance (34). Moreover, a loss of function mutation in TLR4 in mice prevents diet-induced obesity and insulin resistance (35).

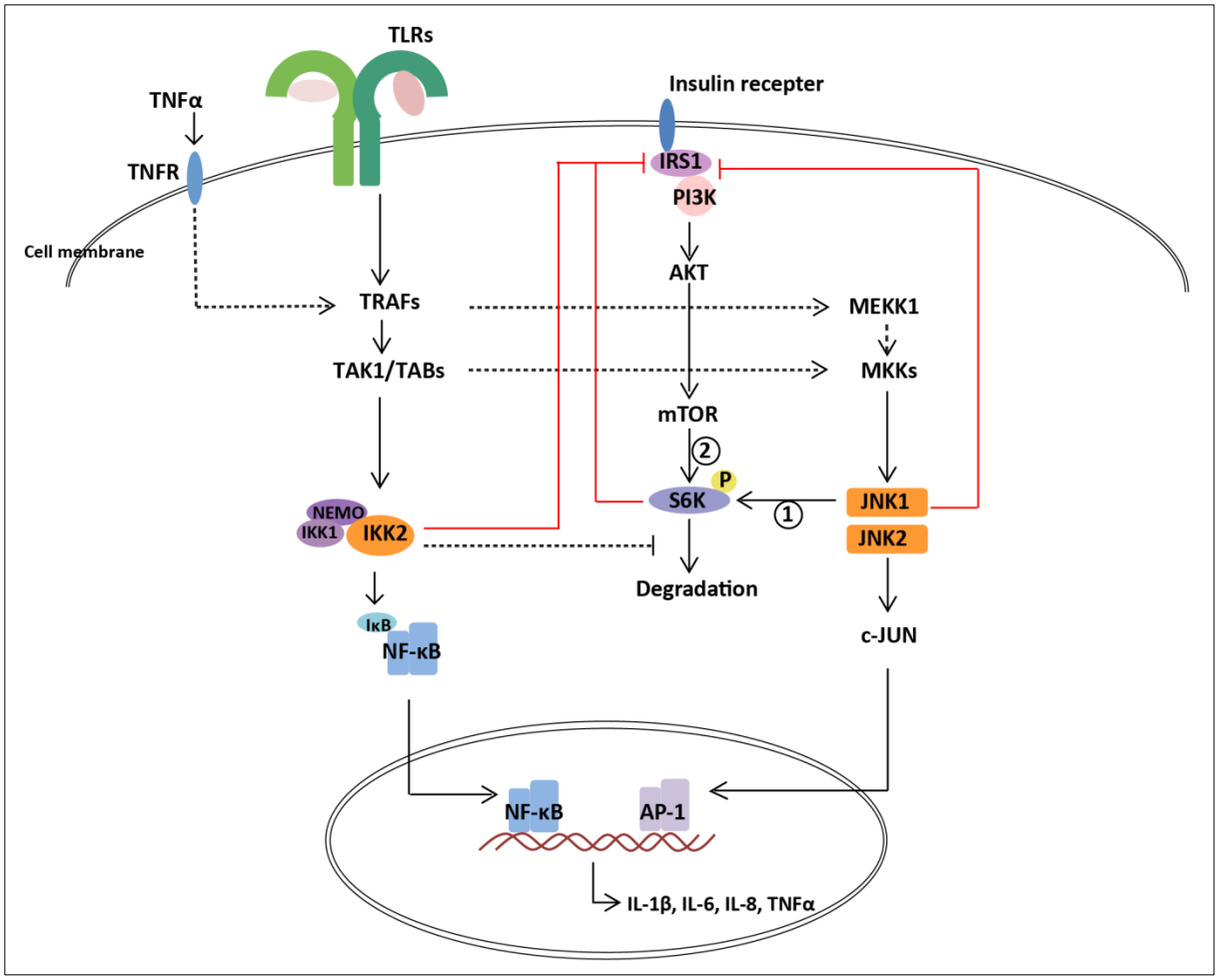

FIGURE 1: The interaction between TLR and insulin signaling in mammals. IKK2 and JNK1 modulate insulin signaling through targeting IRS1 and S6K. S6K negatively regulates IRS1 through phosphorylating Serine 307 and Serine 636/ Serine 639 sites on IRS1, which are involved in obesity- and/or diet-induced insulin resistance (29, 30). S6K activation requires phosphorylation at two stages. The first phosphorylation is mediated by JNK1 and the second phosphorylation is mediated by mTOR. Figure adapted from Patel et al. (31) and Zhang et al. (27).

However, the conclusion that IKK2 deficiency reverses insulin resistance has also been challenged by the study of Rohl et al (36), who showed that Ikk2 expression in mice skeletal muscle is not essential for obesity-induced insulin resistance in mice (36). Considering these conflicting results, additional studies of the function of IKK2 in insulin resistance are necessary. We expect that our zebrafish Ikk2 mutant will be of great use for such studies.

In conclusion, TLR signaling plays pivotal roles in regulating host innate immunity and maintaining homeostasis. Our studies describe the systemic transcriptome profiles

of different TLR signaling pathways through RNA deep sequencing, and showed common and specific downstream signaling that have not been previously described (**Chapter 2**). We show the roles of Tlr2 signaling in host defense against infection at the transcriptome- and cellular- level by studying *M. marinum* infection in a *tlr2* mutant (**Chapter 3**). Our *tlr2* mutant shows a high suitability for studies of other microbial infection agents, the responses of the host to the gut microbiome and metabolic diseases such as diabetes type II. In the study of **Chapter 4**, the *ikk2* mutant shows its functions not only in innate immune protection against infection but also in modulation metabolism such as insulin resistance induced by hyperinsulinemia. These studies provide new possibilities to further study the connection of innate immunity and the control of metabolic diseases.

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