The Function of Toll-like receptor 2 in Infection and Inflammation

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Chapter 5

General summary and discussion

1. Zebrafish as a model to study infectious and inflammatory diseases

The activation of the innate immune system depends on the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) from the invading pathogens. PRRs are also involved in recognition of damage-associated molecular patterns (DAMPs) from damaged tissues during infection [1, 2]. The Toll-like receptors (TLRs) family is one of the most important member of the PRRs families. The discovery of TLRs as gatekeepers to activate innate immunity was awarded by the 2011 Noble Prize in Physiology or Medicine [3]. It triggered an explosion of research into the functions of TLRs in modulating a broad spectrum of physiological and pathological processes [4, 5]. TLR2 is conserved in most vertebrates and plays an important role in modulating infectious, and inflammatory diseases by recognizing a large number of PAMPs and DAMPs (**Chapter 1**). The broad function of TLR2 makes it a promising therapeutic target (**Chapter 1**). However, the function of TLR2 is still controversial in some studies and its role in several diseases is still inconclusive [6]. Therefore, it is vital to further study how TLR2 signaling functions in the host innate immune responses.

For this purpose, we utilized the zebrafish model to study the function of Tlr2 in inflammation and infection in this thesis. Zebrafish larvae already have a functional innate immune system within 5 days post fertilization at which time the adaptive immune system is not functional yet [7, 8]. This makes zebrafish an excellent model to study the vertebrate innate immunity in the absence of adaptive immunity. Moreover, the zebrafish model is becoming more and more popular in research because of the ease of genetic manipulation, omics studies of large groups of larvae, and live imaging. The last ten years, considerable progress has been made in studying infectious and inflammatory diseases by using the zebrafish model. Van der Vaart et al. found that zebrafish embryos deficient in Myeloid differentiation factor 88 (Myd88), which is a crucial adaptor by all TLRs except for TLR3, are more susceptible to bacterial pathogen infection [9]. This finding is similar to the conclusions derived of studies of MYD88 deficient mutants in human *in vitro* cell cultures and mouse in *vivo* models [9]. In addition, Hosseini et al. reported the function of Myd88 in limiting mycobacterial growth in a tail fin infection zebrafish model [10]. Furthermore, Yang et al. demonstrated that the function of Tlr2 signaling is similar in zebrafish embryos and in mammalian cells. In both systems TLR2 regulates the expression of a similar set of immune genes after the systemic stimulation by the synthetic model lipopeptide ligand Pam3CSK4 [11]. These studies paved the way to further investigate the function of Tlr2 in the zebrafish model. **In Chapter 2**, we studied the function of tlr2 in

defense against *Mycobacterium marinum* infection in zebrafish by measuring infection phenotypes and corresponding transcriptome responses. **In Chapter 3**, we investigated how Tlr2 and Myd88 regulate leukocytes migration behavior in the absence of infection, but with tissue damage, by using a zebrafish tail wounding model. **In Chapter 4**, we studied the function of tlr2 during the infection with *Mycobacterium avium* by comparing it with *M. marinum* infection with special attention to the responsive cell migration behavior.

2. Tlr2 plays a role in defense against mycobacterial infection in zebrafish larvae

In several studies it has been reported that TLR2 polymorphisms increases susceptibility to mycobacterial infection in the human population, although there is a small number of studies that found no effect of TLR2 polymorphisms (**Chapter 1**) [12, 13]. In addition, there is still controversy about the role of TLR2 in host defense against *Mycobacterium tuberculosis* in several rodent studies (**Chapter 1**) [14-16]. **In chapter 2**, we, therefore, generated a *tlr2* zebrafish mutant to study Tlr2 function in innate immune defense during mycobacterial infection. To characterize the effect of *tlr2* mutation, we first compared the transcriptome of homozygous mutant larvae with that of heterozygote larvae in the absence of infection. We found differences in the gene expression profiles of $tlr2^{-/-}$ zebrafish larvae and its control siblings, such as differently expressed genes involved in glycolysis (Chapter 2, Figure 2 and S3). This result is consistent with a previous study in the human *in vitro* and mice *in vivo* models which showed that TLR2 plays a key role to switch the host cellular metabolism toward aerobic glycolysis after *M. tuberculosis* infection [17]. In accordance, a previous study in our lab using zebrafish also suggested that MyD88 plays a role in metabolism [18]. In addition, this study showed that Tlr2 and its adaptor MyD88 are crucial for the response of the host to the microbiome [18]. This indicates that the different gene expression profiles we found in the *tlr2* mutant are caused by a dysfunctional response to the microbiome.

To study the role of Tlr2 in defense against *M. marinum* infection in zebrafish, we injected these bacteria in *tlr2* loss-of-function mutants and their homo- and heterozygote siblings. We found that the bacterial burden was significantly higher in *tlr2* mutants and was accompanied with a higher extracellular bacterial burden and less granulomas than in $tlr2^{+/-}$ and wild-type larvae at 4 dpi (Chapter 2, Figure 3 and 4). This result is consistent with previous studies in mice that show a function of Tlr2 in zebrafish host defense [15, 16, 19]. In addition, our transcriptome analysis showed that the number of up-regulated and down-regulated genes in response to

infection was greatly diminished in infected *tlr2* mutant zebrafish compared to their heterozygotes sibling controls (Chapter 2, Figure 5-7). Moreover, we found many signaling pathways that have been demonstrated to be linked to tuberculous in humans are differentially regulated in *tlr2* mutant zebrafish larvae. For example, we found that the Tlr8 signaling pathway was strongly affected, which indicates that Tlr2 signaling is connected to the function of Tlr8 (Chapter 2, Figure S10). In addition, the vitamin D receptor pathway genes were down-regulated in *tlr2* mutant zebrafish. It has been demonstrated that vitamin D plays an important role to control tuberculosis infection [20]. Therefore, the hyper-susceptibility of *tlr2* mutants to *M. marinum* infection could be caused by aberrant vitamin D signaling. Chemokines constitute the other gene category which was affected by the *tlr2* mutation during *M. marinum* infection. In previous work, Torraca et al, demonstrated that the Cxcr3-Cxcl11 axis was involved in macrophage recruitment after *M. marinum* infection in zebrafish larvae [21]. In agreement, we found that the expression levels of *cxcl11aa* and *cxcl11ac* were significantly lower in the *tlr2* mutant after infection (Chapter 2, Figure 5). This result shows a clear connection between Tlr2 function and macrophage chemotaxis.

3. New insights of Tlr2 functions in regulating leukocyte migration from live imaging

Considering the large number of chemokines that are controlled by Tlr2 (Chapter 2), we hypothesized that Tlr2 is a key factor in the control of chemokine expression in order to regulate cell recruitment in innate immunity. To test this hypothesis, **in Chapter 3**, we first investigated the function of Tlr2 and Myd88 in modulating leukocytes migration behavior in the absence of mycobacterial infection. For this, we utilized a zebrafish tail wounding model which is widely used for anti-inflammatory drugs screening [22, 23]. We found that the number of recruited neutrophils and macrophages was decreased in *tlr2*$^{-/-}$ and *myd88*$^{-/-}$ groups compared to their wild type sibling controls (Chapter 3, Figure 2 and 3). Subsequently, live cell imaging of the tail-wound area in zebrafish was performed in *tlr2* and *myd88* mutants and their corresponding wild type siblings. Leukocyte migration in the *tlr2* and *myd88* mutants upon wounding was analyzed using quantitative analyses of cell migration tracks (Chapter 3, Figure 4 and Table 1). Our results demonstrate that the *tlr2* and the *myd88* mutations affect the migration of neutrophils that are distantly located of a wound by negatively affecting their directional persistence, but not their migration speed (Chapter 3, Figure 5 and 6). Not only the directional persistence of macrophages that are distantly located from a wound was significantly decreased

in the *tlr2* and the *myd88* mutants, but also their migration speed (Chapter 3, Figure 7 and 8). This study shows for the first time that TLR signaling is directly involved in controlling behavior of cell migration of neutrophils and macrophages during wounding, stimulating further studies also in other model systems.

It has been shown previously in mice infection models that TLR signaling is involved in controlling infiltration of neutrophils and macrophages into injured tissues [24, 25]. Moreover, Tlr2 has been demonstrated to regulate the expression of cytokines and chemokines after the recognition of its ligands in both zebrafish and mice models (Chapter 2) [24]. Therefore, the aberrant leukocyte migration behavior, which was observed in *tlr2* and *myd88* mutant zebrafish could be caused by the insufficient level of basal transcripts for chemokines. For the tail wounding, it also could be that DAMPs released by dead cells around the wound do not lead to the secretion of chemokines in the absence of TLR2 signaling. For example, high-mobility group box 1 protein (HMGB1) is a widely studied endogenous danger signal that induces inflammatory response through its direct interaction with DAMPs recognized by TLR2 [26, 27]. Besides, reactive oxygen species (ROS) have been reported to be involved in leukocyte recruitment upon wounding in zebrafish larvae [28, 29]. And it has been demonstrated that the secretion of ROS is mediated by TLRs after tissue injury [30]. Thus, it is interesting to further study whether the generation of ROS may be altered in *tlr2* and *myd88* mutant zebrafish larvae.

4. Differences between TB and NTM infectious learned from zebrafish studies

Nontuberculous mycobacteria (NTM) diseases are defined as diseases caused by mycobacterial pathogens other than *M. tuberculosis* and *Mycobacterium leprae* [31]. Besides TB, NTM infectious diseases have recently attracted wide attention because its prevalence is increasing sharply since 2000 [32]. Although there are existing treatments for NTM infectious diseases, the treatment regimens are long and there is a high frequency of multi-drug resistant cases [33]. Thus, it is urgent to discover novel prevention and therapeutic strategies for patients infected with NTM. **In Chapter 4**, we used zebrafish larvae to establish a *M. avium* infectious model and then characterized *M. avium* infection in larvae by comparing it to *M. marinum* infection, a popular model of tuberculosis infection. It has been reported that innate immune defense against NTM infection is mainly mediated by the TLR signaling pathway [34-36]. Therefore, we first compared the transcriptome profiles of the host responses to infection specifically in relation to TLR signaling. Subsequently, we investigated the function of toll-like receptor

signaling after *M. marinum* and *M. avium* infection to compare the function of Tlr2 in infection with these two different bacteria.

We found *M. marinum* infection is more virulent than *M. avium* infection in zebrafish larvae (Chapter 4, Figure 1). Moreover, we found that *M. avium* is persisting in the macrophages with less extracellular cording compared to *M. marinum* (Chapter 4, Figure 2). *In vivo*, extracellular cording is a morphology of mycobacteria accompanied by necrotic macrophages and extracellularly replicating bacteria which prevent phagocytosis because of the size of the clusters [37, 38]. Bacterial cording is a pathogenic feature associated with hyper-virulence in *M. tuberculosis*, *M. marinum*, *M. abscessus*, *M. fortuitum*, and *M. chelonae* [37, 39-42]. Thus, the observation that *M. marinum* infected larvae show more extracellular cords may be a feature of the higher lethality and bacterial growth resulting from *M. marinum* infection. To obtain explanations for the lower virulence of the *M.avium* infection, and obtain genetic markers for further studies we performed RNAseq deep sequencing to study the whole transcriptome profile in the *M. avium* infection model comparing it to that of *M. marinum* infection model at a systemic level.

We found that *M. avium* has a distinct transcriptome response compared to *M. marinum*, especially regarding to the regulation of the following gene categories: autophagy regulators, matrix remodeling, and cytokines and chemokines (Chapter 4 Figure 3 and 4). In the category of cytokines, chemokines and their receptors, more genes were downregulated specifically in the *M. avium* infection group, such as *il1rga*, *ccr9b*, *cxcr4b*, *ccr6b*, *cxcl11.7*, *ccl36.1*, *cxcl12.b* and *ccl33.3* (Chapter 4, Figure 4). To be noted, the Cxcr4b/Cxcl12 signaling, which is related to HIV pathogenesis, tumor-sustained angiogenesis and mycobacteria-induced angiogenesis, was downregulated in the *M. avium* infection group [43-45]. Furthermore, it has been demonstrated that CXCR4/CXCL12 signaling sustains leukocytes trafficking to inflammatory sites as well as CXCL11 signaling, which mediates the recruitment of macrophages upon mycobacterial infection [46, 47]. Therefore, we hypothesize that the macrophage and neutrophil migration behaviors can be different in zebrafish larvae after infection with different NTM species.

It has been demonstrated that leukocyte migration is important for bacterial clearance, containment, dissemination, and granuloma formation at the early mycobacterial infectious stages [48-51]. In previous chapters, we demonstrated that Tlr2 plays an important role tin defense against *M. marinum* infection (Chapter 2). Furthermore, we found that Tlr2 is involved

in regulating macrophage and neutrophil behavior after tail wounding (Chapter 3). Thus, Tlr2 could also participate in the regulation of migratory behavior of macrophages and neutrophils to the sites of mycobacterial infection. To assess the role of Tlr2 in the regulation of the migration of macrophages and neutrophils, we applied a tail fin infection model **in Chapter 4**, which was described before [48, 52]. *M. marinum* strain Mma20 or *M. avium* strain MAC 101 were injected into 3 days post fertilization (dpf) *tlr2*$^{+/+}$ *Tg (mpeg1:mCherry-F);TgBAC (mpx: EGFP)* and *tlr2*$^{-/-}$ *Tg (mpeg1:mCherry-F);TgBAC (mpx: EGFP)* larvae (Chapter 4, Figure 7). We conclude that macrophages play an important role in the response to both mycobacterial infections because more recruited macrophages were observed in the infected area (Chapter 4, Figure 8). The migration speed of macrophages is faster towards Mma20 infection sites (Chapter 4, Figure 8). We found that neutrophils moved faster in *tlr2* wild type larvae than in *tlr2* mutants after Mma20 injection, while *tlr2* deficiency did not affect neutrophil migration after MAC101 injection (Chapter 4, Figure 8). This altered leukocyte behavior suggests that chemokine expression profiles may be different in *tlr2* mutant zebrafish after infection by mycobacterial species.

5. Perspectives for future studies

5.1 The investigation of host and mycobacteria interactions by using zebrafish larvae

In Chapter 4, we first developed a *M. avium* infection model in zebrafish which makes it possible to directly observe the host and *M. avium* interactions *in vivo*. Through this model, we observed different phenotypes of granuloma-like clusters in zebrafish larvae after infecting with different mycobacteria (Chapter 4, Figure 2). Furthermore, the transcriptome analysis showed that the expression of the genes belonging to the category of autophagy regulator genes was significantly affected in *M. avium*-infected zebrafish larvae (Chapter 4, Figure 4). Therefore, it is interesting to compare the autophagy response and ultrastructure of granulomas resulting from infection by different species of mycobacterial clusters. For this purpose, in the near future we will apply transmission electron microscopy (TEM) and 3D block-face scanning electron microscopy (block-face SEM).

In addition, in Chapter 4, we found that Tlr2 is involved in the regulation of the migration of macrophages and neutrophils in response to infection. Interestingly, the results of cell tracking suggest that *tlr2* regulates the macrophages and neutrophils in different ways after infection by different mycobacterial species. To obtain explanations for further studies of the effect of the

tlr2 mutation on mycobacterial infection, in the future, we will investigate whether differences in expression profiles of chemokines can be observed in the early infection stage. We would also like to study whether the changes of leukocyte migration behavior in *Tlr2* mutants are due to alterations of signals from the infection site or whether they are caused cell autonomous defects in migratory abilities of the myeloid cells in the *tlr2* mutant. Thus, we will apply cell transplantation techniques to investigate the non-intrinsic and intrinsic functions of myeloid cells in the *tlr2* mutant after wounding and mycobacterial infection.

5.2 Automated processing of zebrafish live imaging and mathematic modeling

In Chapter 3 and Chapter 4, we performed a large number of cell tracking experiments to quantify cell migration behaviors. Cell migration is an important physiological parameter for many pathological processes, including inflammatory responses [23], immune defense [48], and metastasis of malignant tumor cells [53]. Single-cell tracking using confocal real-time imaging is one of the most popular methods to analyze cell migration [54]. With the development of confocal laser scanning microscopy, it is easier to acquire massive live imaging data. However, there are many bioinformatic steps needed to be done following the acquisition of imaging. These involve the processing of large data sets, segmenting cell migration trajectories, visualization and quantification of the trajectories, and importantly, interpretation of the biological significance from the large data sets. Currently, manual data analysis is still required to assist automated data analysis. Furthermore, the availability of user friendly software programs still lags behind the requirements of researchers in the field.

Some recent reviews have summarized in detail the available commercial and free software or plug-ins for live imaging processing in cell migration studies [54, 55]. The TrackMate plug-in for ImageJ software (NIH, Bethesda, MD, USA), Volocity (Improvision; PerkinElmer Life and Analytical Sciences), and IMARIS (Bitplane) are widely used in zebrafish studies. However, still quite a large number of researchers choose manually tracking methods for zebrafish *in vivo* cell tracking, like ManualTrack plug-in, or MTrackJ plug-in for ImageJ software. This is because most of the software was designed for tracking movement of big particles or *in vitro* cell migration. However, the shape of cells *in vivo* is irregular, which makes the segmentation of the trajectories difficult and frequently results in over-segmentation [56]. Failure to segment the trajectories of cells correctly is the main reason for tracking errors. Examples of errors are that that, the trajectory output from the software is not from the same cell or that the tracked trajectory is broken in several parts. Moreover, the movement of cells *in vivo* is more

complicated than *in vitro*, especially during dynamic immune responses. Modelling of cell migration *in vivo* is not simply based on Brownian motion or Autoregressive motion, but needs to assume a combination of multiple complex motions. Single tracking algorithms in some commercial software programs results in tracking errors, which makes the quantification of trajectory unreliable. Therefore, it is necessary to establish new algorithm models based on *in vivo* movements of cells in a specific situation. In Chapter 3, we investigated the cell migration behavior regulated by Toll-like receptor signaling in tail-wounded zebrafish larvae through a manual tracking method. These manual tracking data provide a solid ground, which paves the way to develop improved cell tracking plug-ins for *in vivo* cell tracking studies in zebrafish larvae. Consequently, we plan to develop further optimized automatic tracking methods based on the large data sets in Chapter 3.

To study the mechanistic basis of the differences in cell migratory behaviors, mathematical models can provide new insights. Chemokine and ROS gradients can be modeled by partial differential equations (PDEs). These can be incorporated into cell chemotaxis models, such as random walk models, phase field models, or the Cellular Potts model, with varying degrees of cell resolution, to study leukocyte migration. Such models could provide quantitative insights into how chemokines and ROS gradients affect the migration behavior of the leukocytes, and how the cells change these gradients by binding or secretion of chemokines or by absorption and metabolizing ROS [57] which is known to affect the robustness of chemotaxis [58]. Using Bayesian inference on tracking data, one can infer a number of chemotaxis parameters, such as the flow rate, diffusion coefficient and production time of the chemoattractant [59].

6. Conclusion

A broad understanding of the innate immune system is important for host-targeted approaches for the treatment of diseases. In this thesis, we demonstrated that Tlr2 plays a crucial role in the host innate immune system. **In Chapter 2**, we show the roles of Tlr2 signaling in host defense against infection at the transcriptome level and cellular level by studying *M. marinum* infection in a tlr2 mutant. Moreover, the *tlr2*$^{-/-}$ mutant zebrafish strain described in Chapter 2 proved highly useful for the study of innate immune mechanisms underlying mycobacterial infection. **In Chapter 3,** we found that *tlr2* and *myd88* are involved in responses to tail wounding by regulating the behavior and speed of leukocyte migration *in vivo*. The large data sets acquired from Chapter 3 will be further used for developing new cell tracking algorithms and

mathematical modeling. **In Chapter 4**, we characterized a new *M. avium* infection model in zebrafish that can be further used to study the interaction between the host and NTM bacteria.

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