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

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CHAPTER 1

1

General introduction

Innate immunity and Toll-like receptors (TLRs)

Innate immunity is the first line of defense against infection and injury, and it plays a crucial role in maintaining homeostasis and preventing pathogen invasion of host tissues (1, 2). It eliminates a great variety of microbial invaders and is essential for the activation of the adaptive immune response (1, 2). Innate immunity consists of many components, such as humoral components including proteins from the pentraxin family and the complement system. It also includes specialized cell types such as macrophages, neutrophils, dendritic cells and natural killer cells. In addition, epithelial cells also contribute to innate immunity as physical barriers and producers of antimicrobial proteins (2). The communication between the various cell types involved in innate and adaptive immunity is orchestrated by signaling peptides, including cytokines and chemokines, as well as lipid mediators.

Recognition of pathogens and other danger signals by host innate immunity is mostly based on the specific interaction between pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) and germline-encoded pattern recognition receptors (PRRs) (3, 4). Among the most important PRRs are the Toll-like receptors (TLRs). TLRs are type I transmembrane glycoproteins, consisting of an ectodomain that contains ligand-binding motifs (leucine-rich repeats and cysteine-rich repeats), a transmembrane domain and a cytoplasmic signaling domain (Toll/IL-1 receptor (TIR) homology domain) (5). There are 10 TLRs in humans and an additional 3 in rodents. Generally, TLR1, 2, 4, 5, 6 and 10 are localized in the cytoplasmic membrane, and TLR3, 7, 8 and 9 are localized in the endosomal membrane. However, their cellular distribution varies in some tissues; for instance, TLR2 and TLR4 are also found intracellularly localized in human corneal epithelial cells (6), and TLR3, 7 and 9 are found on the cell surface of airway epithelial cells as well (7) (Fig. 1). It has been hypothesized that each TLR has a distinct function in terms of PAMP and DAMP recognition (5, 8).

TLRs recognize distinct PAMPs by forming hetero- or homo-dimers. Examples of heterodimer recognition are TLR2/TLR1 and TLR2/TLR6 that recognize tri- (9, 10) or di-acylated (11, 12) lipoprotein. TLR2 as a heterodimer also recognizes gram positive bacteria, mycobacteria and their cell wall components, such as glycolipids (13, 14) and glycoprotein (15, 16) to trigger the production of cytokines and chemokines. Typical synthetic ligands that are recognized by TLR2 heterodimers are Pam3CSK4 and Pam2CSK4. These synthetic lipopeptides can mimic the acylated amino terminus of bacterial lipoproteins, and are often used to trigger TLR2 activation in experimental settings. TLR10 has recently been shown to act as a heterodimer with TLR2 to sense microbes and fungi (17, 18). However hetero-dimerization of TLR10 with TLR2, does not lead to the activation of typical TLR-induced signaling, including NF-κB-, IL-8-, or IFN-β-driven reporters, which suggests that TLR2/TLR10 heterodimers signal through

different pathways than those used by TLR1 and TLR6 heterodimers (17, 18). This is suggested by the observation that TLR10, alone or in cooperation with TLR2, fails to activate typical TLR-induced signaling, including NF-κB-, IL-8-, or IFN-β-driven reporters (17) and that TLR10 is not involved in recognition of known TLR2 ligands (18). TLR10 has also been suggested to induce anti-inflammatory responses by hetero-dimerizing with TLR2 (6). One possible mechanism for a TLR10 anti-inflammatory function is as an inhibitory receptor when forming heterodimers with TLR2; the other one is that TLR10 mediates the specific induction of an anti-inflammatory cytokine IL-1 receptor antagonist (IL-1Ra), which is known as IL-1 inhibitor (6). TLR10 was also shown to dimerize with TLR1 in a co-immunoprecipitation study in human cells (19). TLR6 can also dimerize with TLR4 in response to endogenous ligands, promoting sterile inflammation (20). There are the following examples of TLR homodimer recognition: TLR4 recognizes bacterial lipopolysaccharide (LPS); TLR5 recognizes bacterial flagellin (21); TLR3 recognizes viral double strand RNA (dsRNA) and synthetic analogs of dsRNA, such as Poly I:C (22); TLR7 and 8 recognize viral single-strand RNA, miRNA and several anti-viral compounds (23, 24) and TLR9 recognizes unmethylated CpG DNA of bacterial and viral origin (25).

Besides dimerization between each other, TLRs also need other accessory molecules or PRRs to recognize microbes and ligands. For instance, TLR4 needs the assistance from LPS binding protein (LBP), CD14 protein and the MD2 protein for recognition of LPS from various gram negative bacteria (26). TLR2 recognizes lipoteichoic acids (LTA), peptidoglycan (PGN), and lipopeptides (LP) and other membrane constituents from gram positive and negative bacteria and mycobacteria. Recognition of this diverse group of compounds needs the cooperation of TLR2 with other proteins (27). CD14 is reported to enhance TLR2/1 surface recognition of tri-acylated but not di-acylated lipopeptides (28), and significantly enhances TLR2 activation by facilitating lipopeptide binding and TLR2 hetero-dimerization (29). Scavenger receptors are also linked to TLR2 function (30); for instance, CD36 is a selective and non-redundant sensor of microbial di-acylglycerides that signals via the TLR2/6 heterodimer (31). In addition to these co-receptors, integrin β3 (32), Dectin-1 (33) and CXCR4 (34) are also reported to function as co-receptors for TLR2, and assist to modify the recognition of pathogens and the subsequent signal transduction. We believe that more co-receptors of TLRs will be found, and that a better understanding of the interaction between TLRs and their co-receptors is necessary. This will help us to understand the similarities and differences of intracellular signaling induced by different TLRs and to elucidate the mechanism of pathogen-defense by the host innate immune system.

TLR signaling pathways

After TLR activation, the intracellular signaling starts with the recruitment of adaptor proteins to the TIR domain. Adaptor proteins include myeloid differentiation factor

(Myd88) (35), MyD88 adaptor-like (Mal) (also called TIR domain-containing adaptor protein, Tirap) (36, 37), TIR domain-containing adaptor protein inducing interferon-β (TRIF) (38), TIR-containing adaptor molecule (TICAM) (39), and TRIF-related adaptor molecule (TRAM) (40). With the exception of TLR3, Myd88 functions as a recruitment factor for all TLRs. For TLR2 and TLR4 the additional adaptor Mal is needed as well. For TLR3, the recruited adapter is TRIF, which can also cooperate with TRAM to recruit for TLR4 (Fig.1). After recruitment of TIR-domain containing adaptors, IL-1R-associated protein kinases (IRAKs) (1, 2 and 4) are activated (41), followed by activation of tumor necrosis factor receptor-associated factor 6 (TRAF6) (42). Through TRIF, the kinase receptor interacting protein 1 (RIP1) is activated (43). Subsequently, TRAF6 and RIP1 activate a complex of TGF-β-activated kinase 1 (TAK1)/ TAK1-binding proteins (TAB1, 2 and 3), which in turn activates an IκB kinase (IKK) complex (IKK1, 2 and IKK-γ, also named NEMO) and the mitogen-activated protein kinases (MAPK) family, including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 MAPKs. Lastly, activator protein 1 (AP-1) and NF-κB are activated (Fig.1), inducing cell survival, proliferation, activation, and production of chemokines and cytokines. In addition to activation of AP-1 and NF- κB, TLR9 can signal through MyD88 to trigger TRAF6/IRAK4/TRAF3-dependent activation of IRF7 to induce type-I interferon (IFN) expression (44) (Fig.1). TLR3 and 4 can trigger Myd88-independent signaling through TRIF and TRAF3, inducing non-canonical IKKs, TANK-binding kinase 1 (TBK1), and IKKε pathways, and finally, they activate the transcription factor IRF3 and induce type-I IFN (IFN-β) and IL-10 production (45) (Fig.1).

Negative feedback regulation of TLR signaling

The activation of TLR signaling and the production of cytokines, chemokines and type-I IFN are crucial for host defensive responses against pathogens (15, 46, 47). However, the aberrant and excessive activation of TLRs may contribute to auto-immune diseases and chronic inflammatory responses and even to an increase in infections (48-51). To avoid these excessive responses, the host needs to negatively regulate TLR activation. Such negative regulation mechanisms include dissociation of adaptor complexes, degradation of signal proteins and transcriptional regulation (52). In terms of dissociation of adaptor complexes, TRAM adaptor with GOLD domain (TAG) and sterile alpha- and TIR motif-containing protein (SARM), were reported to inhibit the TRIF-dependent pathway (53, 54). The Toll-interacting protein (TOLLIP), first discovered as a regulator of the IL-1R pathway (55) inhibits IRAK phosphorylation. TOLLIP also directly associates with TLR2 and suppresses the phosphor-rylation and kinase activity of IRAK1 and thereby facilitates termination of TLR2 signaling (56). TRAF family member-associated NF-κB activator (TANK), TNFα-induced protein 3 (TNFAIP3, known as A20), suppressor of cytokine signaling 3 (SOCS3) and the small heterodimer partner (SHP, also called NR0B2) can inhibit TRAF6 ubiquitination (57-60). NOD-like receptor (NLR) family CARD domain containing 5 (NLRC5) inhibits NF-

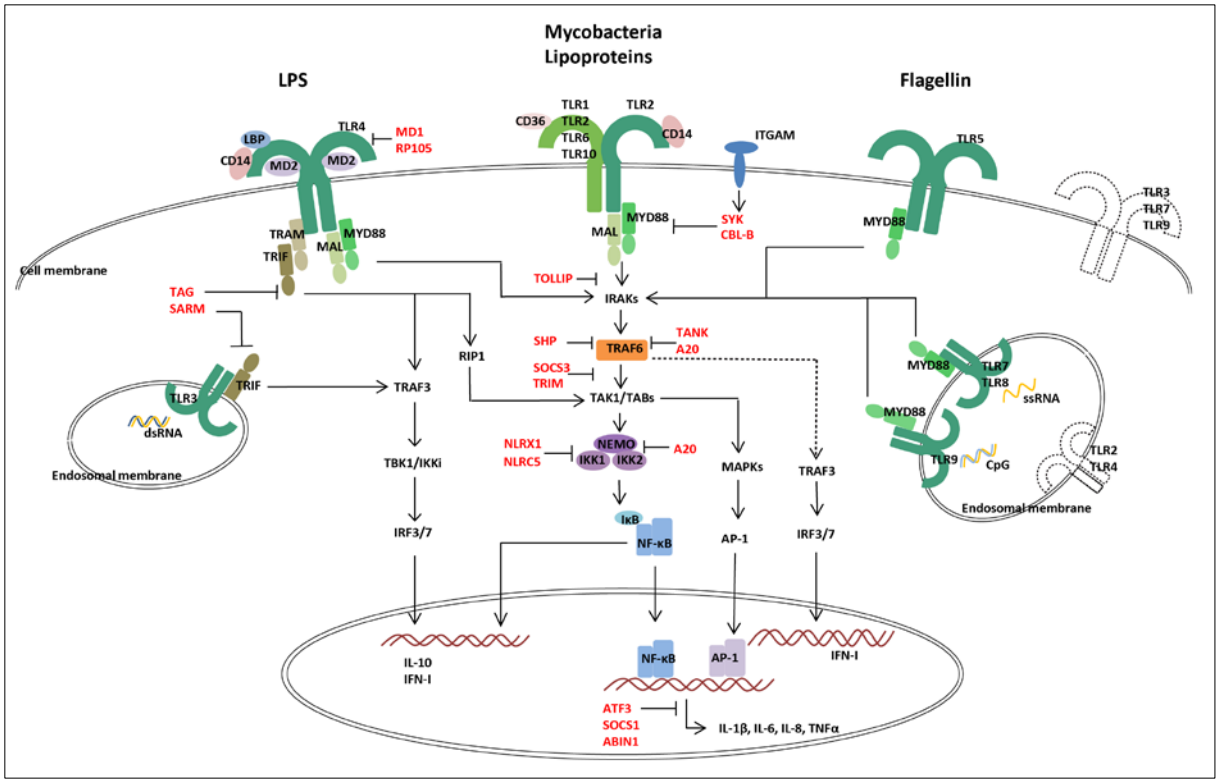

FIGURE 1 TLR signaling in mammals. The cellular distribution of TLRs can be variable. In some cases, TLR2 and TLR4 are found in endosomal membranes and TLR3, TLR7 and TLR9 are found on the cell surface. TLR signaling is activated by the recognition of ligands and microbes and is modulated by several negative regulators. The inhibitors or the negative regulators of the TLR signaling are indicated in red color. Figure adapted from McClure and Massari (61).

κB-dependent responses by interacting with IKKα and IKKβ and thereby blocks their phosphorylation (62). A20 also blocks IKKs activation through stimulation of polyubiquitin binding to NEMO (IKKγ) (63). NLR family member X1 (NLRX1) negatively regulates TLR-induced NF-κB signaling by targeting both TRAF6 and IKK complex (64) (Fig.1).

With regard to degradation of signaling proteins, SOCS proteins promote deterioration of TIRAP (MAL) or TRAF proteins (65). Moreover, the integrin CD11b is activated by TLR-triggered phosphatidylinositol 3-OH kinase (PI(3)K) and the effector L-lysine cyclodeaminase (RapL) and feeds back to inhibit TLR signaling by activating the tyrosine kinases Src and Syk. Syk interacts with and induces tyrosine phosphorylation of MyD88 and TRIF, which leads to degradation of these adaptor molecules by the E3 ubiquitin ligase Cbl-b (66) (Fig.1). Pellino-3, a member of the E3 ubiquitin ligases family, was also reported to act as a negative regulator of TLR2 and TLR4 signaling (67). Other inhibitors that promote degradation of TLR signal proteins

involve PDZ and LIM domain protein 2 (PDLIM2) (68), tripartite-motif containing proteins (TRIM) (69, 70) and peptidyl-prolyl isomerase (Pin1) (71).

For transcriptional regulation, Cyclic AMP-dependent transcription factor (ATF3) (72), B-cell CLL/lymphoma 3 (Bcl-3) (73), nuclear receptor related 1 protein (Nurr1) (74) and transcription factor aryl hydrocarbon (Ah) receptor (75) are involved in the negative regulation of TLR signaling (Fig.1). Interestingly, the Ah receptor has recently been shown to function also as a PRR (76).

Negative regulation of ligand recognition by TLRs

In short, the positive and negative regulation of TLR signaling depends on the cooperation between TLRs and other accessory molecules or PRRs to form functional multi-receptor clusters, and also on the interaction between the multi-receptor clusters of TLRs and the microbial and endogenous ligands. For instance, through elucidating the crystal structures of staphylococcal superantigen-like protein 3 (SSL3) and its complex with TLR2, Koymans et al. (77) showed that SSL3 interferes with TLR2 activation with both ligand binding and receptor dimerization to prevent the downstream signaling (77). In addition, Yoo et al (78) demonstrated that crystal structures of TLR8 in complex with two most active compounds confirmed important binding interactions that play a key role in ligand occupancy and biological activity. These studies suggest that structure-specific binding processes are the foundation of the recognition by TLRs of different microbes and PRRs. On the other hand, some additional upstream factors are also involved in the interactions between TLRs and various ligands or microbes. In *Drosophila*, Toll and other TLRs are not directly involved in ligand recognition, but the extracellular factor Spaetzle (or Spätzle [Spz]) initiates protease cascades leading to the activation of TLR signaling (79, 80). In a recent, study Hepburn et al. (81) identified a functional equivalent of Spaetzle in vertebrates, a neurotrophin named nerve growth factor β (NFG-β) which is structurally related to cystine-knot proteins. It was shown to be released by macrophages in response to S*taphylococcus aureus* infection. The activation of the high-affinity receptor of NFG-β, tropomyosin-related kinase receptor A (TRKA), enhances the intracellular killing of *S. aureus*. These NFG-β-TRKA signaling-dependent responses are also dependent on TLR signaling, suggesting an evolutionarily conserved interaction between cysteine knot proteins and Toll family receptors (81).

TLR function in *Mycobacterium tuberculosis* infection

Mycobacterium tuberculosis (Mtb) is an intracellular pathogen and the causative agent of tuberculosis (TB), It causes ill-health among millions of people each year and ranks alongside the human immunodeficiency virus (HIV) as a leading cause of death worldwide and kills about 1.5 million people annually (82). TB can be distinguished as active and latent based on the clinical symptoms. Active lung TB is accompanied with

cough with sputum and blood at times, chest pains, weakness, weight loss, fever and night sweats (83). Latent TB does not show such symptoms but is characterized by the formation of granulomas, the pathological hallmark of TB, an orderly aggregation of host immune cells around infected macrophages, not only in lung but also in many other tissues and organs. This response is initiated by the effect of mycobacterial virulence factors on host innate immune cells and progresses as the result of a concerted action of innate and adaptive immunity (84, 85).

After Mtb infects the host, macrophages and neutrophils are the first line of host defense against its invasion. Macrophages recognize the pathogen associated molecular patterns (PAMPs) of pathogens through the surface exposed PRRs, which also bind bacteria to initiate phagocytosis. Phagocytosis is a hallmark of anti-bacterial host defense, subsequent engulfment of bacteria leads to formation of the phagosome through the invagination of the cell membrane. In the cytoplasm, the phagosome undergoes fusion with endosomal or trans-Golgi-derived transport vesicles and fission of vesicles (86). These processes are also modulated by Mtb bacteria which try to influence the fusion of vesicles and the acidification of the phagolysosome (87, 88).

After recognition and phagocytosis of Mtb and their components, macrophages induce the production of various cytokines, such as TNF-α, IL-1β, IL-6 and IL-12, and chemokines, such as IL-8, which activates neutrophils releasing more IL-8, thereby attracting monocyte-derived macrophages and other immune cells like lymphocytes to the site of infection (86). These processes are concomitant with the development of granulomas (89). The granuloma development process proceeds either to localized eradication of the infection or to localized caseation and necrosis, culminating in bacterial release and leading to more infection (89).

Autophagy as an alternative strategy of host defense against Mtb should also be mentioned. Autophagy targets ubiquitinylated organelles/proteins to the autophagosome, which fuses with lysosomes to generate an auto-phagolysosome (86). It has been shown that an essential autophagy control factor, called Atg5, plays an important role in defense against Mtb infection (90, 91). However, recent studies in mice indicate that conventional autophagy pathways are not essential for the control of tuberculosis and have linked the function of Atg5 to prevention of pathological inflammation during Mtb infection (92, 93). Therefore further studies are needed to investigate if other non-canonical autophagy pathways exist that control TB.

In Mtb infection, TLR2 is a key innate immune receptor, dimerizing with TLR1 and gathering other accessory molecules like CD14, to recognize the specific cell wall components of Mtb, such as lipoproteins LpqH, LprA, LprG and PhoS1, Glycolipids lipoarabinomannan (LAM), lipomannan (LM), phosphatidylinositol mannoside (PIM) and trehalose dimycolate (TDM) (94-96). Therefore it is thought that the activation of

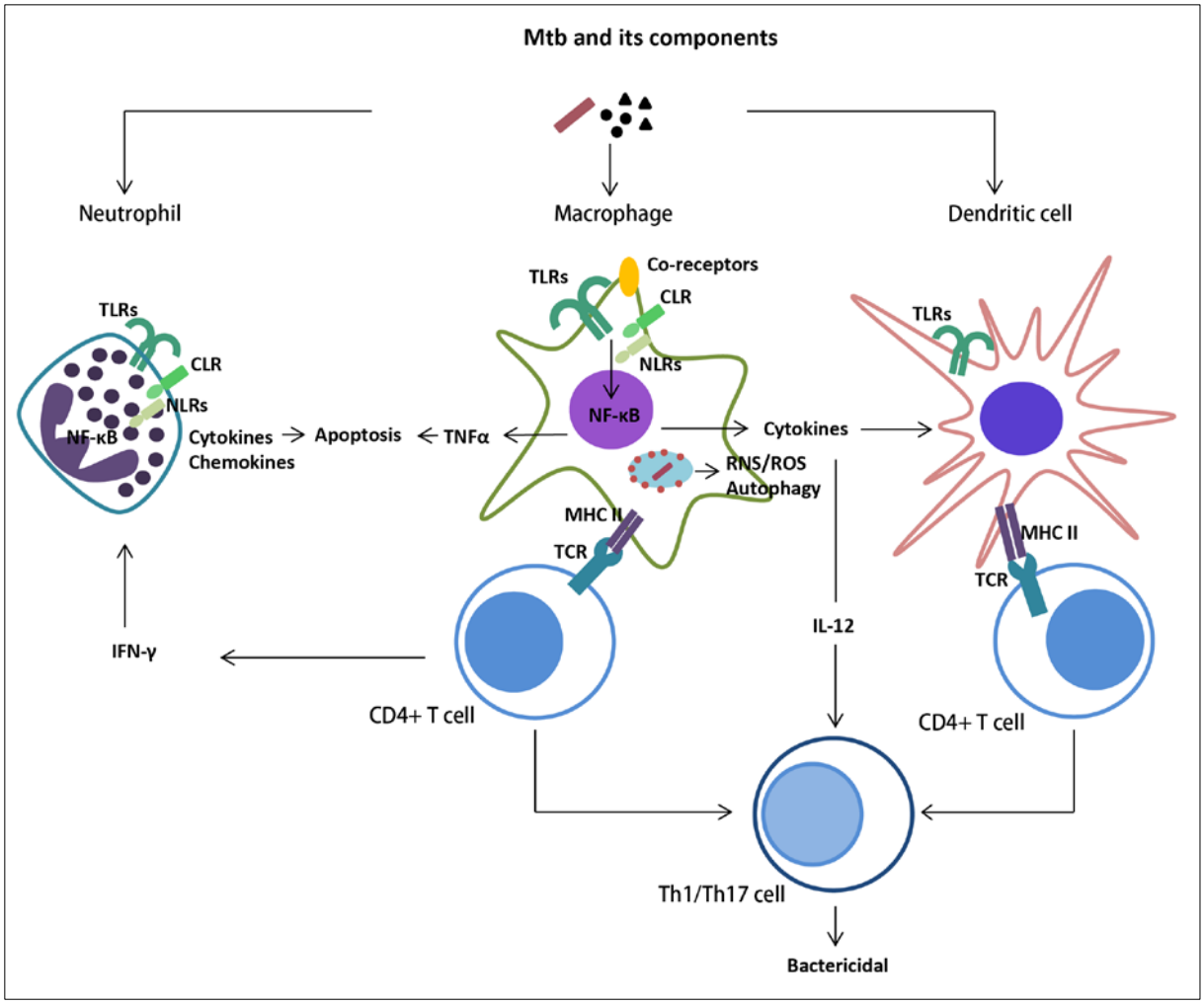

FIGURE 2 Immune cells activation in the presence of Mtb and stimulation of its signal transduction components. The strategies of host defense against Mtb infection include macrophage- and dendritic cell-mediated T cell responses, neutrophil-mediated killing of bacteria, granuloma formation, apoptosis mediated by neutrophils and macrophages and macrophage-mediated autophagy. Figure adapted from Hamza et al. (97).

the TLR2 protein contributes to host defense against Mtb infection. In *in vitro* studies, lipoproteins or other Mtb cell wall components induce TLR2-dependent cytokines, chemokines, and nitric oxide production and stimulate dendritic cell maturation (15, 98, 99) (Fig. 2). Through these actions, TLR2 mediates a nitric oxide-dependent or -independent anti-mycobacterial activity (100, 101). TLR2 activation by agonists such as one of the mycobacterial unique PE family of proteins and the lipoprotein LpqH from Mtb also mediates autophagy (102) and macrophage apoptosis (103, 104), which also contributes to bacterial elimination and inhibition of proliferation. Lancioni et al. (105) also found that LprG and LpqH can directly regulate CD4(+) T cell activation in a TLR2/1 dependent way (105). The researchers from this group subsequently showed that TLR2 engagement on CD4(+) T cells enhances effector functions and protective

responses to Mtb infection in mice (106) (Fig. 2). *in vivo* studies also showed a role for TLR2 in protection against chronic Mtb in the murine model (107, 108). In addition, IL-17 is required for early protective immunity against Mtb HN878 infection (109). Gopal et al. (109) showed that Mtb strain HN878 induces IL-17 production in an IL-1β-TLR2 dependent way. Through IL-17 receptor signaling, IL-17 mediates CXCL-13 induction, which is required for localization of T cells in lymphoid follicles and for optimal macrophage activation and Mtb control (109).

Immune evasion mediated by TLR2

In contrast to a defensive function, TLR2 can also be exploited by Mtb to evade host immunity. Previous *in vitro* studies indicate that the prolonged incubation of macrophages with the mycobacterial lipoproteins LprA, LprG and LpqH resulted in TLR2-dependent inhibition or decrease of MHC-II antigen processing and presentation (16, 98, 110). This leads to a defective recognition of infecting bacteria by CD4(+) T cells and negatively influences the CD4(+) T cell responses and control of infection. Furthermore, also polarization of T helper type 1 (Th1) cells, which show a protective role in Mtb infection (111, 112) is influenced by TB infection. The polarization of Th1 cells is promoted by the pro-inflammatory cytokines, such as IL-12, IFN-γ, TNF-α and influenced by the anti-inflammatory cytokines, such as IL-10 (113, 114). Richardson et al. (115) showed that the induction of IL-10, suppression of IL-12 and inhibition of MHC-II induced by Mtb infection in macrophages are driven by TLR2-dependent activation of ERK, which influences the Th1 polarization and thereby can contribute to immune evasion by the bacteria. In addition to these macrophage-mediated T cell responses, another possible strategy of host defense against Mtb is macrophage-mediated autophagy as described above (116). *In vivo* studies in mice show that autophagy activation can be inhibited by a microRNA, miR-125a, through targeting UV radiation resistance-associated gene (UVRAG) (117) and the expression of miR-125a is mediated by Mtb in a TLR2/Myd88 dependent way (117). These studies indicate that the exploitation of TLR2/Myd88 pathway to inhibit autophagy activation is another possible way of Mtb to evade host immunity. Similar to IL-10, another negative regulator of TLR signaling, A20, was also reported to participate in TLR2-mediated host immune evasion of Mtb in an indirect way (118). Kumar et al. (118) showed that A20 is the target of the microRNA miR-let-7, which is downregulated in Mtb-infected macrophages and enhances expression of A20, thereby attenuating inflammatory signaling and facilitating bacterial survival.

Unlike TLR2, TLR4 does not show a controversial role in host defense against Mtb. Deficiency in TLR4 compromises host immunity. For instance, Tlr4 mutant mice show impaired macrophage recruitment and pro-inflammatory responses to Mtb infection, resulting in chronic infection with impaired elimination of mycobacteria (119). The

mutants also showed an increased mortality and mycobacterial proliferation in the lung (120).

With regards to other TLRs, it is reported that TLR9 polymorphisms are associated with the host susceptibility to tuberculosis (121-123). Infected Tlr9 mutant mice display defective mycobacteria-induced interleukin IL-12p40 and interferon IFN-γ responses, indicating that Tlr9 may regulate Th1 responses in Mtb infection (124). It is worth noting that compared with Tlr2 or Tlr9 single mutants, Tlr2/9 double mutant mice displayed significantly enhanced susceptibility to Mtb infection, and a decrease in pro-inflammatory cytokines production and altered pulmonary pathology (124). These results illustrate that TLRs collaborate together in host defense against Mtb invasion.

In conclusion, TLRs not only show a function in innate immune responses to Mtb infection, but also show roles in mediating immune evasion (125-129).

Study of mycobacterial infection in zebrafish

Zebrafish has become a prominent vertebrate model to study human infectious disease, especially for TB (130, 131). First, because the natural fish pathogen, *Mycobacterium marinum*, a close relative to Mtb, can induce granuloma formation in adults and larvae of zebrafish. The granulomas in adult fish are very similar to those observed during human latent TB progression and zebrafish larvae provide unique access to the earliest stages of the formation of these granulomas (132, 133). Second, the relatively small size of embryos allows infection assays to be performed at a large scale. In addition, the transparent larvae are ideal for imaging the early steps of the infection process in real time. The short generation time of 3-4 months is useful for generating transgenic lines and other genetics approaches. Last but not the least, zebrafish have a 3-4 weeks separation stage between development of innate and adaptive immunity after fertilization (134, 135), which gives the possibility to study the host innate immune response to infection in the absence of adaptive immune responses. Zebrafish have lymphatics but lack lymph nodes, so the adaptive immune cells mainly develop and function in the spleen, the kidney and the thymus (131, 136-138). Furthermore, fish do not have bone marrow and instead the anterior part of the kidney functions as a major site of hematopoiesis. Anatomically, this is therefore different from mammals, but zebrafish shares most of the primary constituents of innate and adaptive immunity with mammals (136, 139, 140). Zebrafish larvae can only activate innate immunity to provide resistance against Mtb infection. The two most important immune cell types for the innate defenses, macrophages and neutrophils, are already functional from 1 day post fertilization (dpf) (141-143).

From previous work in our laboratory several examples can be given of how zebrafish research has helped to study immune cell mechanisms that function in combatting mycobacterial infection. For instance, scavenger receptor Marco of

macrophages is required for *M. marinum* phagocytosis and the pro-inflammatory response to infection (144, 145). Additionally, the macrophage perforin Mpeg1.2, a macrophage marker, is an infection inducible factor in macrophage defense against *M. marinum* (146). As described above, autophagy could be an important mechanism in macrophage defense. DNA damage-regulated autophagy modulator (Dram1) was demonstrated to augment this process in response to infection in zebrafish (147). Moreover, the stabilization of transcription factor hypoxia inducible factor 1α (Hif-1α) and the reduction of Hif-2α can enhance reactive nitrogen species (RNS) in neutrophils and decrease mycobacterial burden, and these processes are dependent on inducible nitric oxide synthase (iNOS) signaling at early stages of infection (148). Both Dram1-mediated autophagy (147) and the production of RNS or reactive oxygen species (ROS) driven by mycobacteria (149) are dependent on the TLR signaling adaptor Myd88 and mutation of the *myd88* gene in zebrafish severely impairs host innate immunity defense against mycobacterial infection in zebrafish (150).

Overview of the TLRs in zebrafish

Various orthologs of the mammalian TLRs have been identified in zebrafish and other fish species, such as Tlr1, 2, 3, 5, 7, 8 and 9 (151, 152). In addition to these orthologs, there are fish-specific Tlrs in zebrafish, such as Tlr19, 20, 21 and 22 (151, 152). Furthermore, some Tlrs, like Tlr4, Tlr5, Tlr8 and Tlr20, have two or more copies (151, 152). Since the TLRs in fish have been recently reviewed very extensively, only a very brief summary of functional studies of Tlr4, Tlr5 and Tlr2 of zebrafish is given here.

There are two orthologs of Tlr4 in zebrafish, Tlr4a/Tlr4b, but different from the function of TLR4 in mammals, they do not recognize LPS (153, 154). This might be because of the absence of the crucial TLR4 accessory molecules Md2 and Cd14, which are essential for LPS binding in mammals. In mammals, the Md1/Rp105 complex has been shown to negatively regulate Tlr4-Md2-LPS binding (155, 156). In contrast, the Md1/Rp105 complex was found to participate in the regulation of innate immunity and viral resistance in zebrafish (157, 158). In addition, another functional component of the TLR4 complex, TLR4 interactor with leucine-rich repeats (Tril), was also identified in zebrafish (159). Chimeric Tlr4 molecules that consist of zebrafish extracellular LRR domains and mouse intracellular TIR domains demonstrated a lack of responsiveness to LPS (153). These studies indicate that Tlr4 might play a different role in mediating NF-κB activation in zebrafish that has not been described in mammals (154). On the physiological level, He et al. (160) demonstrated that Tlr4-Myd88-NF-κB signaling is both necessary and sufficient for hematopoietic stem and progenitor cell (HSPC) emergency in zebrafish and this role in HSPC emergency is evolutionarily conserved in mammals.

Studies of Tlr5 structure and function has determined its high conservation amongst vertebrates (161, 162). In zebrafish, Tlr5 possesses two orthologs-Tlr5a and Tlr5b, which are both involved in the recognition of flagellin as is the case with the single TLR5 protein of mammals. In zebrafish, the Tlr5 genes are required for the activation of host specific defense genes upon Salmonella infection (162). Flagellin-Tlr5 signaling was also shown to be crucial for a mucosal immune responses in the zebrafish intestine induced by a live attenuated *Vibrio anguillarum* vaccine (163). Concomitant with the upregulation of other PRRs, such as Tlr2, Tlr4, Nod1, Nod2 and Marco, and adapters, such as Myd88, Irak4, Irf7 and Traf1/2b/3, it was shown that Tlr5 is significantly upregulated during infections by various bacterial species in zebrafish (163, 164). To elucidate the structural basis and mechanistic implications of TLR5-flagellin recognition, Yoon et al. (161) analyzed the crystal structure of zebrafish Tlr5 in complex with Salmonella flagellin. Recently, Amelia et al. (164) described the capacity of distinct flagellar serotypes to differentially activate Tlr5 in zebrafish. Tlr5 signaling could be potentially affected by single-nucleotide polymorphisms present within coding sequences for the conserved C-terminus of various flagellin variants, which directly contact with Tlr5 (164). These studies trigger the interests to further study the binding mechanism of Tlr5 with various flagellins.

As in mammals, zebrafish Tlr2 also mediates pathological inflammatory injury. For example, the outer membrane protein of pathogenic leptospires, LipL32, can induce leukocyte infiltration and kidney injury in Tlr2 dependent manner in zebrafish (165). Despite its important role in infectious disease in mammals, further studies of TLR2 in zebrafish were lacking, and therefore this is an important subject of this thesis. These studies show that zebrafish are a valuable addition to mammalian models for understanding the function of TLR2 in infectious disease.

Outline of this thesis:

In **Chapter 2** we applied RNA deep sequencing (RNAseq) analysis to comparatively study the systemic transcriptome responses in whole zebrafish embryos upon injection with TLR2 agonist- Pam3CSK4 and TLR5 agonist-flagellin. We describe the genes whose transcription shows an early response to these PAMPs. In addition, we describe a set of genes that show a specific immune response via the function of *tlr2* but not by *tlr5*, such as *cebpb* and *fosb*.

In **Chapter 3**, we analyzed a *tlr2* null mutant zebrafish line. We applied the *M. marinum* infection model in the zebrafish *tlr2* mutant larvae to study Tlr2 function in the innate immune system during infection. The *tlr2* mutant showed a reduced macrophage number in the absence of infection, but higher migration speed and maximum migration distance from infection sites of phagocytic macrophages. The *tlr2* mutant also showed a higher bacterial proliferation. RNAseq and qPCR results revealed

that *M. marinum* infection of the *tlr2* mutant leads to decreased responses to infection at the mRNA level of genes involved in inflammation and immunity, especially the Tlr2 specific genes described in Chapter 2. Moreover, our transcriptome analysis revealed Tlr2-specific pathways involved in *M. marinum* infection, which are also related to responses to *M. tuberculosis* infection in human macrophages.

The zebrafish model can also assist with the analysis of the mechanisms of TLR downstream signaling as we show in **Chapter 4**. In this chapter we show that TLR downstream signaling pathways are also important for research of metabolic syndromes such as diabetes type II. We analyzed zebrafish Traf6 and Ikk2 mutants with a stop codon in the reading frame of the proteins (*traf6*$^{sa244/sa244}$ and *ikk2*$^{m10/m10}$). There were no consistent indications for effects on leukocytes phenotype and bacterial burden upon *M. marinum* infection in *traf6*$^{sa244/sa244}$. In contrast, *ikk2*$^{m10/m10}$ larvae showed a significant decrease in body size, leukocyte numbers and expression of marker genes for macrophages and neutrophils. Upon *M. marinum* infection, *ikk2*$^{m10/m10}$ larvae also showed a higher bacterial burden, which indicates that Ikk2 plays a role in host defense against infection. In addition, in the *ikk2* mutant the insulin resistance induced by hyperinsulinemia was modulated. Considering the phenotype of the *ikk2* mutant in both infection and insulin resistance this mutant provides new possibilities to further study the connection of innate immunity and metabolic diseases.

Finally, **Chapter 5** contains a general discussion and summary of the work described in this thesis.

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