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

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人生在勤，不索何獲 。

——張衡 (Zhang Heng, AD 78-139)

Explore the unknown in order to be able to innovate

in the distant future.

---Faculty of Science, Leiden University

For my family and Andy

献给我的家人和远方的朋友

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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-

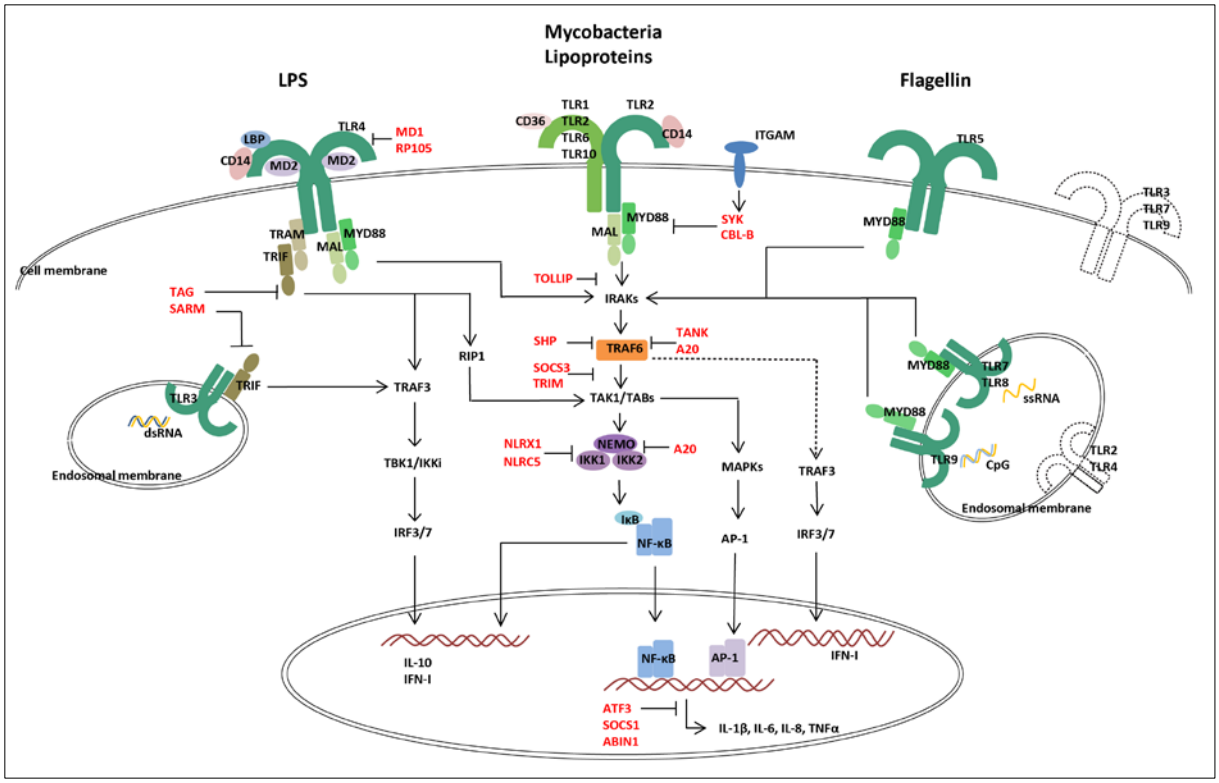

FIGURE 1 TLR signaling in mammals. The cellular distribution of TLRs can be variable. In some cases, TLR2 and TLR4 are found in endosomal membranes and TLR3, TLR7 and TLR9 are found on the cell surface. TLR signaling is activated by the recognition of ligands and microbes and is modulated by several 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

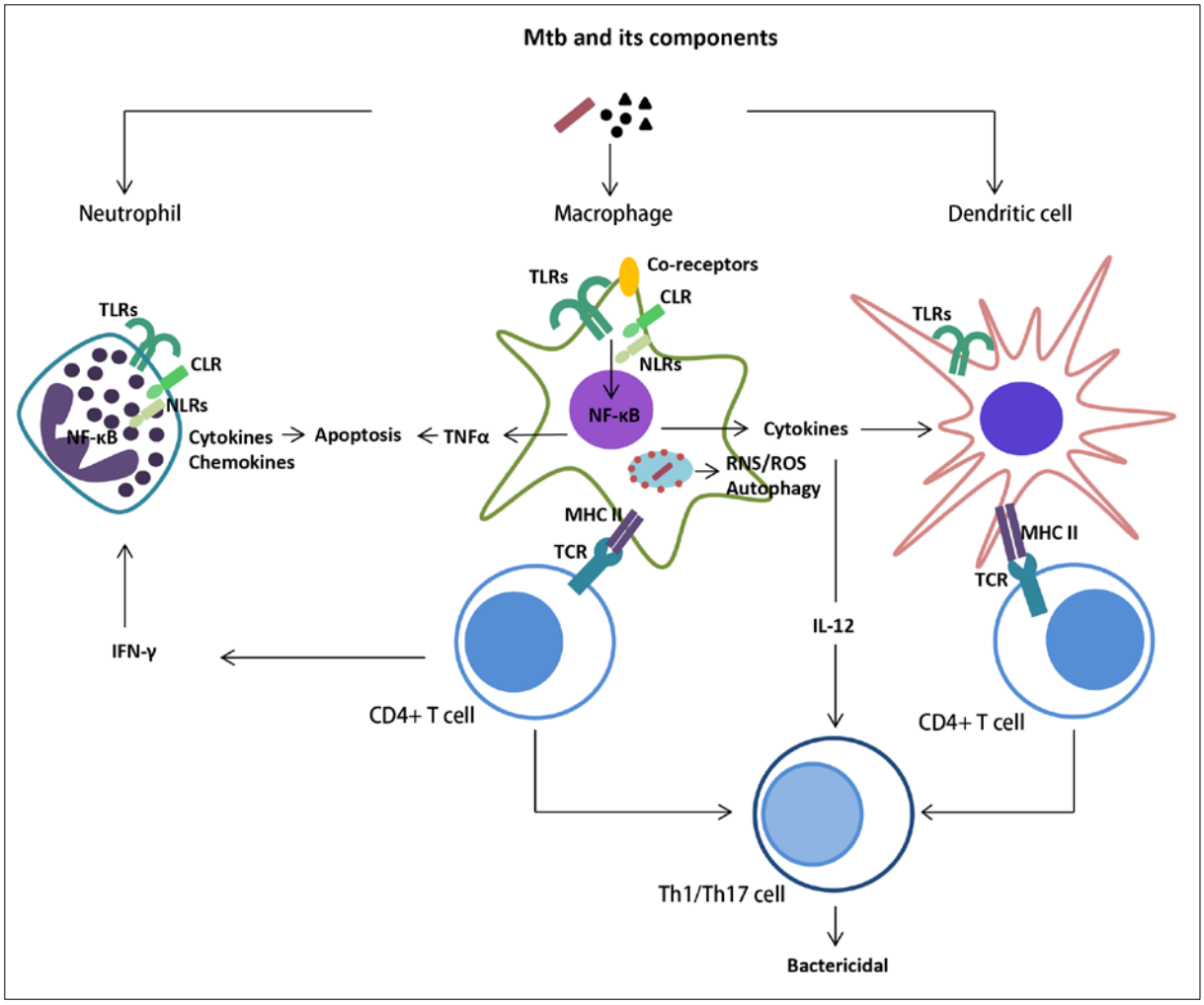

FIGURE 2 Immune cells activation in the presence of Mtb and stimulation of its signal transduction components. The strategies of host defense against Mtb infection include macrophage- and dendritic cell-mediated T cell responses, neutrophil-mediated killing of bacteria, granuloma formation, apoptosis 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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CHAPTER 2

Common and specific downstream signaling targets controlled by Tlr2 and Tlr5 innate immune signaling in zebrafish

2

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Abstract

Although the responses to many pathogen associated molecular patterns (PAMPs) in cell cultures and extracted organs are well characterized, there is little known of transcriptome responses to PAMPs in whole organisms. To characterize this in detail, we have performed RNAseq analysis of responses of zebrafish embryos to injection of PAMPs in the caudal vein at one hour after exposure. We have compared two ligands that in mammals have been shown to specifically activate the TLR2 and TLR5 receptors: Pam3CSK4 and flagellin, respectively. We identified a group of 80 common genes that respond with high stringency selection to stimulations with both PAMPs, which included several well-known immune marker genes such as *il1b* and *tnfa*. Surprisingly, we also identified sets of 48 and 42 genes that specifically respond to either Pam3CSK4 or flagellin, respectively, after a comparative filtering approach. Remarkably, in the Pam3CSK4 specific set, there was a set of transcription factors with more than 2 fold-change, as confirmed by qPCR analyses, including *cebpb*, *fosb*, *nr4a1* and *egr3*. We also showed that the regulation of the Pam3CSK4 and flagellin specifically responding sets is 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 embryo system.

Introduction

The innate immune system is referred to as the first line in host defense against invading pathogens (1, 2). Its highly developed ability to recognize microbial patterns and host-derived danger signals relies on so-called pattern recognition receptors (PRRs), especially on the Toll-like receptors (TLRs) (3-5). In humans, the TLR family is composed of 10 members, which are located at the cell surface with the exception of TLR3, 7, 8 and 9, which are localized on intracellular endosomal membranes (6-8). The TLRs are involved in the recognition of a wide variety of ligands, including pathogen associated molecular patterns (PAMPs), such as bacterial cell wall components and viral RNA, as well as damage associated molecular patterns (DAMPs) (9, 10). This leads to subsequent intracellular signal transduction, triggering the production of inflammatory cytokines and chemokines, but it can also lead to anti-inflammatory responses as has been recently shown for TLR10 acting as a heterodimer with TLR2 (11, 12).

The recognition of PAMPs and DAMPs by different TLRs is directed by structurally conserved leucine-rich repeats (LRR) motifs of the TLRs ectodomains (ECDs) (13, 14). Cell-surface TLRs can mediate binding to PAMPs by homodimerizing, like TLR4 and TLR5 that recognize lipopolysaccharide (LPS) (15) and flagellin (16), respectively. In contrast, other TLRs form hetero-dimers like TLR2 that associates with TLR1, TLR6 or

TLR10, in conjunction recognizing lipopeptides and lipoproteins (12, 17, 18). The diversity of TLR2 dimer combinations is thought to be responsible for its extensive recognition ability, ranging from the diverse components of various pathogens to the host heat-shock protein 70 (10). TLR2 plays an important role in resistance to the infection induced by *Mycobacterium tuberculosis* (*Mtb*) (19, 20). For example, McBride et al. demonstrated that TLR2 knockout mice show enhanced cell infiltration and inflammation in lungs upon low dose chronic infection with *Mtb*, and fail to stably control the bacterial burden (21). A series of components of *Mtb* can trigger the TLR2 signaling pathway upon infection, such as tri-acylated lipoprotein LprG, LpqH and PhoS1 (22, 23), and glycolipid lipo-arabinomannan (24). Pam3CysSerLys4 (Pam3CSK4) is a synthetic tri-acylated lipopeptide that mimics the triacylated lipoprotein of mycobacteria and classical gram-positive bacteria, which can be recognized by TLR2/TLR1 heterodimers and induce the production and release of pro- and anti-inflammatory cytokines (IL-6, IL-12, TNF-α and IL-10), chemokines (IL-8) and interferon (IFN-γ) (25-28). Most of these studies on the responses of the TLR2 signaling pathway have been performed in cell culture systems. A notable exception is the reported transcriptome response of mouse mononuclear phagocytes recruited to lungs challenged with Pam3CSK4 as measured by micro-arrays (29). As another example 8-day old mice were treated with Pam3CSK4 and analyzed for the expression of several inflammatory genes using qPCR (30).

The zebrafish embryonic model has much potential to study the ligand specificity of TLRs at the organism level [29]. Importantly, zebrafish offers the possibility to study the innate immune system separated from the adaptive immune system in their embryonic and larval stages (up to 3-4 weeks post fertilization) (31-33). To date, a number of TLR signaling pathway mediators have been identified and studied in zebrafish such as the adaptor proteins Myd88, Tirap (Mal), Trif and Sarm1 (34-38), and the downstream signaling intermediates Irak and Traf6 (39, 40).

In work previously published by our group, we demonstrated that the function and regulation of the zebrafish homologs of human TLR5, *tlr5a* and *tlr5b*, are conserved with their mammalian counterparts. Both *tlr5a* and *tlr5b* are strongly up-regulated in response to *Salmonella typhimurium* infection (41). Furthermore, in the same study it was shown that knockdown of these two genes prevented or weakened the activation of genes for several inflammatory mediators like *mmp9*, *cxcl-C1c*, *il1b* and *il8* upon flagellin stimulation (41).

In this study, we aimed to study TLR2 function, in comparison with TLR5, in zebrafish using transcriptome analysis. Injection into the blood stream of the *tlr2*/*tlr1* ligand Pam3CSK4, was followed by transcriptome profiling to characterize key genes involved in the early response to this PAMPs. In addition, by comparing the transcriptome response towards treatment with flagellin, we were able to discriminate

non-specific immune responsive genes from a set of genes which are regulated by *tlr2* but not by *tlr5*.

Results

1 The immune response of zebrafish embryos to injection of PAMPs in the caudal vein

In a recent study by Stockhammer et al, it was shown that flagellin injected into the caudal vein at 27 hours post fertilization (hpf) induced several immune response marker genes as measured by qPCR (41). To further characterize the response to another well characterized PAMP, we injected Pam3CSK4 using the same method. The expression levels of cytokine genes *il1b*, *tnfa* and *il6*, the chemokine gene *il8,* and anti-inflammatory gene *il10,* were measured by qPCR at 1, 3 and 6 hour post injection (hpi) respectively (Fig. 1). The results show that there was a significant up-regulation of these genes upon Pam3CSK4 stimulation. For all these marker genes the induction was transient and followed by a gradual decrease over time. The *il1b* gene was the only marker of which up-regulation was observed at 1 and 3 hpi, with a significantly higher expression than the control group (Fig. 1A). For the *tnfa*, *il6, il8* and *il10 genes* there was a more obvious decrease of induction over time (Fig. 1B-E). These results show that Pam3CSK4 induces similar responses in zebrafish as in mammalian cells (25-28) suggesting that this response is also mediated via the *tlr2* signaling pathway.

2 The function of Tlr5 and Tlr2 in the immune response towards flagellin and Pam3CSK4

In order to study the function of *tlr2* and *tlr5* in the above described responses to Pam3CSK4 and flagellin we used morpholinos to knockdown these genes. There are two orthologous genes of human *tlr5* in zebrafish, *tlr5a* and *tlr5b* and previous studies in our group showed that they are required for activation of host defense genes upon flagellin stimulation. This was shown by simultaneous co-knockdown of *tlr5a and tlr5b* by morpholinos (41). In this study, *tlr5a* and *tlr5b* morpholinos were injected separately and, subsequently the morphants were stimulated with flagellin at 27 hpf. Embryos treated with standard control morpholino were used as a control (41). The expression of *il1b* was measured at 1hpi by qPCR. Our results revealed that abrogation of both *tlr5a* and *tlr5b* effectively prevented the *il1b* up-regulation observed in control embryos upon flagellin stimulation (Fig. 2A).

The function of *tlr2* in recognition of Pam3CSK4 was tested in the same manner. Our results showed that *tlr2* morphants did not exhibit up-regulation of *il1b* expression upon Pam3CSK4 stimulation, while the control morphants did (Fig. 2B). In contrast,

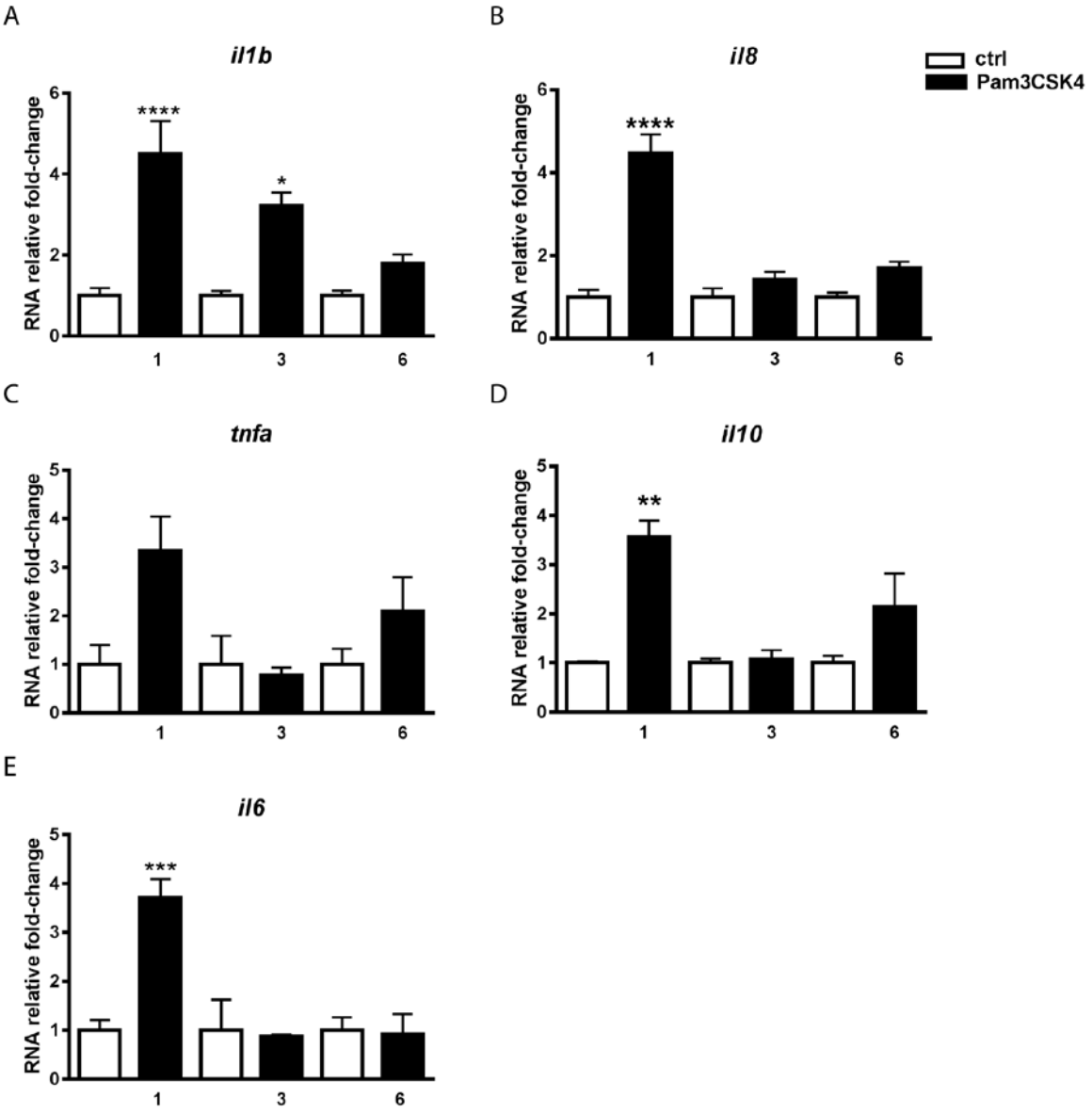


FIGURE 1 Immune genes expression at different time points upon Pam3CSK4 stimulation. Embryos were injected at 27 hpf with 1ng Pam3CSK4 and expression levels of *il1b* (A), *il8* (B), *tnfa* (C), *il10* (D) and *il6* (E) were determined at 1, 3 and 6 hours post injection by qPCR. Data (mean ± SEM) are combined from at least three biological replicates (n=15 embryos per group) and expressed relative to their corresponding mock injection (water) control, which is set at 1. Statistical significance of differences between mock and Pam3CSK4 groups was determined by ANOVA analysis and Tukey's multiple comparisons test, **p<0.05, **p<0.01, ***p<0.001*.

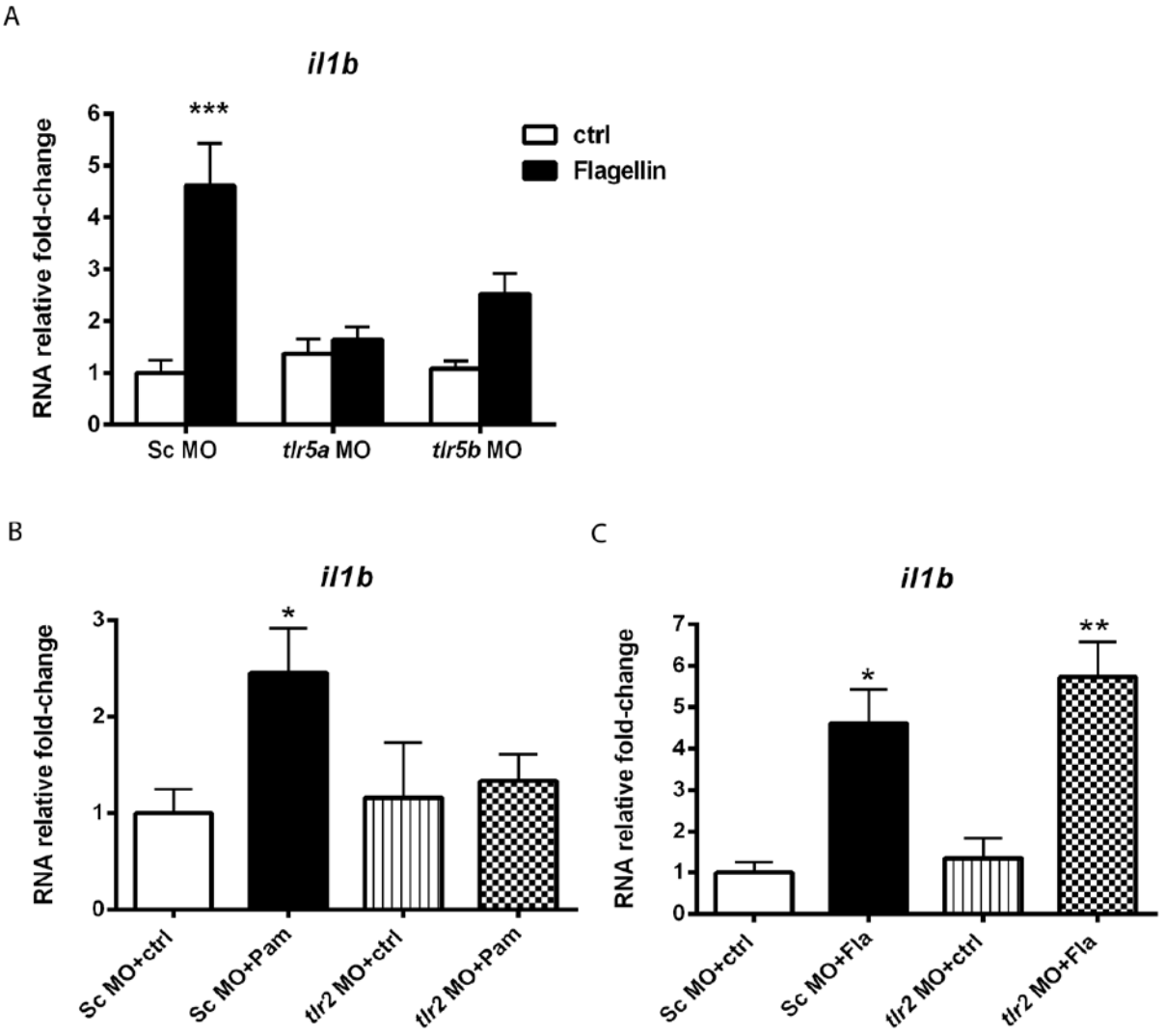


FIGURE 2 *il1b* expression in *tlr2* and *tlr5* morphants following PAMPs stimulation. Embryos were injected at the 1-2 cells stage with standard control (Sc), *tlr2, tlr5a or tlr5b* morpholino (MO) and subsequently injected with Pam3CSK4 at 27 hpf, flagellin or water as a mock control. Expression of *il1b* was determined by qPCR at 1 hpi. A, *tlr5a* and *tlr5b* knockdown effect on *il1b* RNA expression in response to flagellin. B, C *tlr2* knockdown effect on *il1b* RNA expression in response to Pam3CSK4 (B) or flagellin (C). Data (mean $\pm$ SEM) are combined from at least three biological replicates (n=10 embryos per group) and expressed relative to their corresponding water control, which is set at 1. Statistical significance was determined by ANOVA analysis and Tukey's multiple comparisons test, **p<0.05, **p<0.01, ***p<0.001*; Pam, Pam3CSK4 injecton; Fla, flagellin injection.

tlr2 morphants stimulated with flagellin, still showed a significant induction of *il1b* expression (Fig. 2C).

3 Identification of a common response gene set for Pam3CSK4 and flagellin stimulation

Since comparisons of the transcriptome response to PAMPs that activate different TLRs has not been described before in a whole organism we decided to perform RNA deep sequencing (RNAseq) of embryos treated with flagellin and Pam3CSK4 at 1 hpi.

Embryos injected with sterile water were used as control and RNA was isolated from a pool of at least fifteen embryos per condition. Triplicates of biological samples were analyzed with Illumina RNAseq and at least 7.2 million mapped reads were obtained for each library (Fig. 3A, Supplemental table IV). Although such reads numbers are insufficient to detect changes in very lowly expressed genes (see Veneman et al, 2014 (42)) approximately 10 million total reads is currently a good cost efficient number that matches the sensitivity of microarrays (43). The results of the transcriptional responses are summarized in Fig. 3A and Supplemental Fig. 1. The results show that at any given *p*-value (or false discovery rate-adjusted *p*-value), flagellin leads to a higher number of differentially expressed genes (DEGs) than Pam3CSK4. To further analyze the data, we arbitrarily used a threshold of 2-fold change and *p*-value <0.01. This p-value corresponds to FDR adjusted p-values ranging from 0.23 to 0.35 in the different experiments. These selection criteria are not very stringent so as to prevent loosing genes that are very lowly expressed and therefore with the used sequencing depth will have obtained only low numbers of reads. The entire list of responses without any selection criteria is given in Supplemental tables V and VI. Applying these settings we obtained 264 DEGs from the Pam3CSK4 stimulated group, composed of 169 up- and 95 down-regulated genes, and 306 DEGs from the flagellin injected group, composed of 180 up- and 126 down-regulated genes (Fig. 3A). Therefore, with both treatments there are more genes up-regulated than down-regulated. In the list of top induced and repressed genes there is a lack of any annotation in the data bases (supplemental table V and VI). We compared these two groups of genes and found an overlap set of only 80 genes that include many immune marker genes, such as *il1b*, *tnfb*, *irak3* and *irg1l*, and transcription factors, like *fos*, *fosl2* and *junba*, as shown by the gene ontology terms (GO terms) annotation in Fig. 3B and Supplemental table III.

The induction of *il1b*, *tnfa* and *il8* by Pam3CSK4 shown using qPCR (Fig. 1) was confirmed by the RNAseq data (Fig. 3C), but in the case of *il6* and *il10* there was no induction with flagellin.

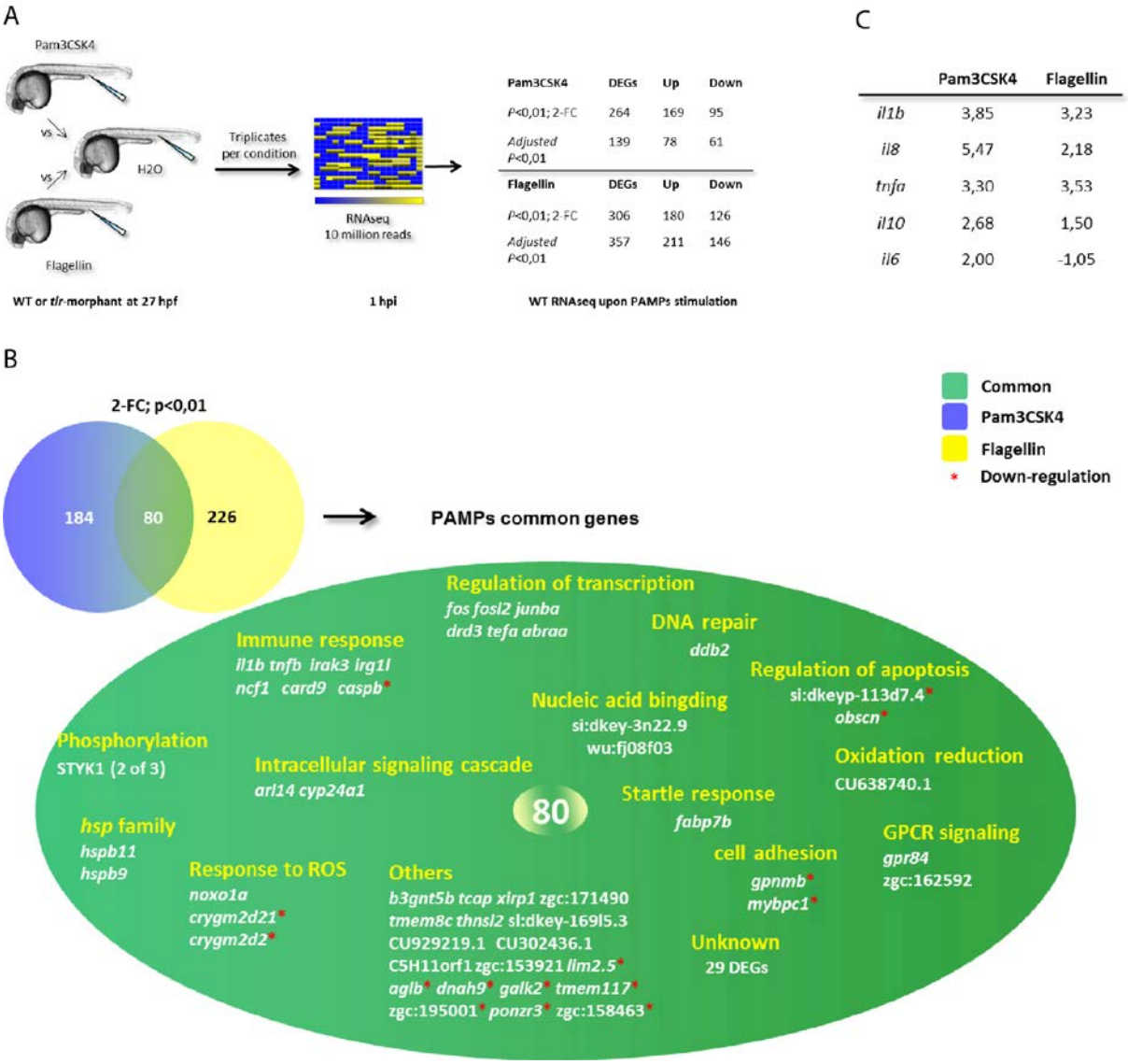
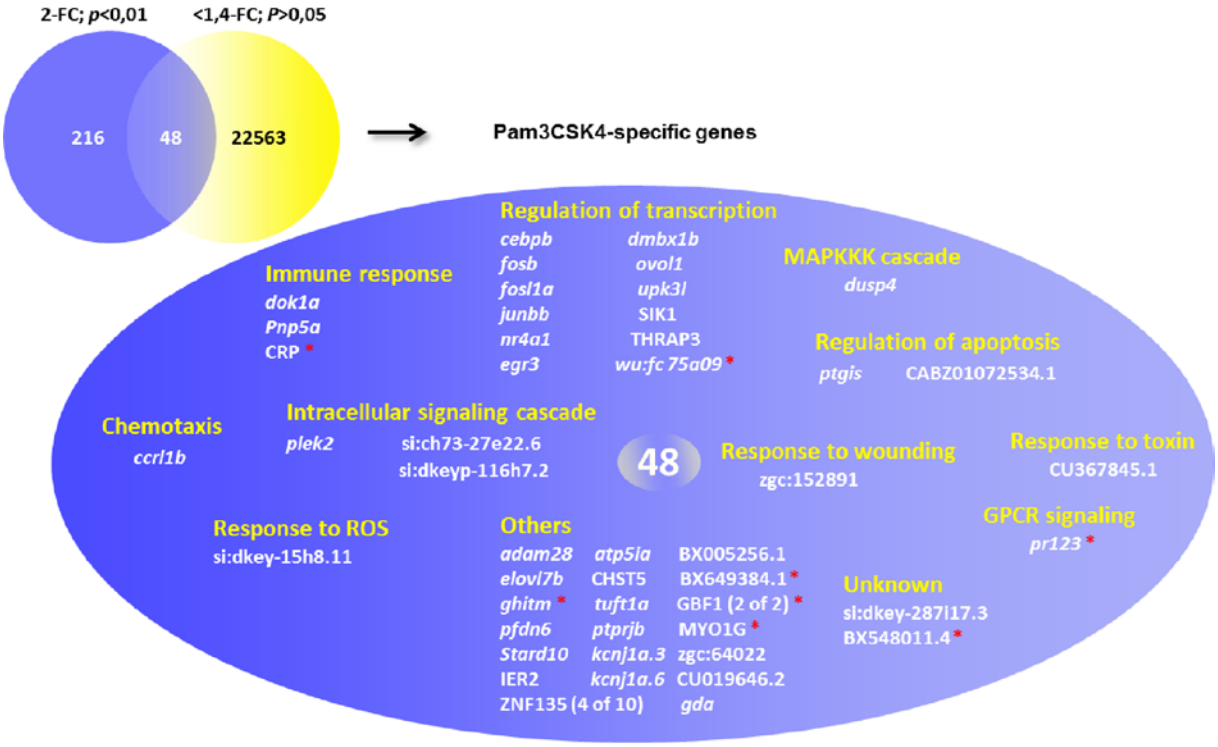


FIGURE 3 RNAseq experimental setup and comparison of gene sets responsive to Pam3CSK4 or flagellin stimulation. A, setup of the RNAseq experiment. Zebrafish embryos received a 1nl injection of 1 mg/ml Pam3CSK4 and 100 µg/ml flagellin respectively into the caudal vein at 27hpf. Control embryos were injected with water. Samples for RNAseq were taken at 1hpi. The numbers of differentially expressed genes were assessed by two criteria: 1) p<0.01, 2 fold-change or 2) adjusted p-value <0.01, without FC cut-off. B, Venn diagram showing the overlap between DEGs from Pam3CSK4 and flagellin stimulations and their GO terms annotation. C, Fold-change values of inflammatory genes in RNAseq. D, Filtering of 264 DEGs from Pam3CSK4 stimulation (p<0.01, 2 fold-change) by the flagellin non-specific set (22611 genes, p>0.05; <1.4-fold change) results in 48 Pam3CSK4 specific genes, which are grouped according to their GO terms annotation. E, Filtering of 306 DEGs from flagellin stimulation (p<0.01, 2 fold-change) by the Pam3CSK4 non-specific set (22391 genes, p>0.05; <1.4-fold change), results in 42 flagellin specific genes, which are grouped according to their GO terms annotation. DEGs, differentially expressed genes; FC, fold-change.

D


2-FC; p<0,01
<1,4-FC; P>0,05
216
48
22563
Pam3CSK4-specific genes
Regulation of transcription
cebpb
fosb
fosl1a
junbb
nr4a1
egr3
dmbx1b
ovol1
upk3l
SIK1
THRAP3
wu:fc75a09 *
MAPKKK cascade
dusp4
Immune response
dok1a
Pnp5a
CRP *
Regulation of apoptosis
ptgis
CABZ01072534.1
Chemotaxis
ccrl1b
Intracellular signaling cascade
plek2
si:ch73-27e22.6
si:dkeyp-116h7.2
48
Response to wounding
zgc:152891
Response to toxin
CU367845.1
GPCR signaling
pr123 *
Response to ROS
si:dkey-15h8.11
Others
adam28
elovl7b
ghitm *
pfdn6
Stard10
IER2
ZNF135 (4 of 10)
atp5la
CHST5
tuft1a
ptprjb
kcnj1a.3
kcnj1a.6
gda
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BX649384.1 *
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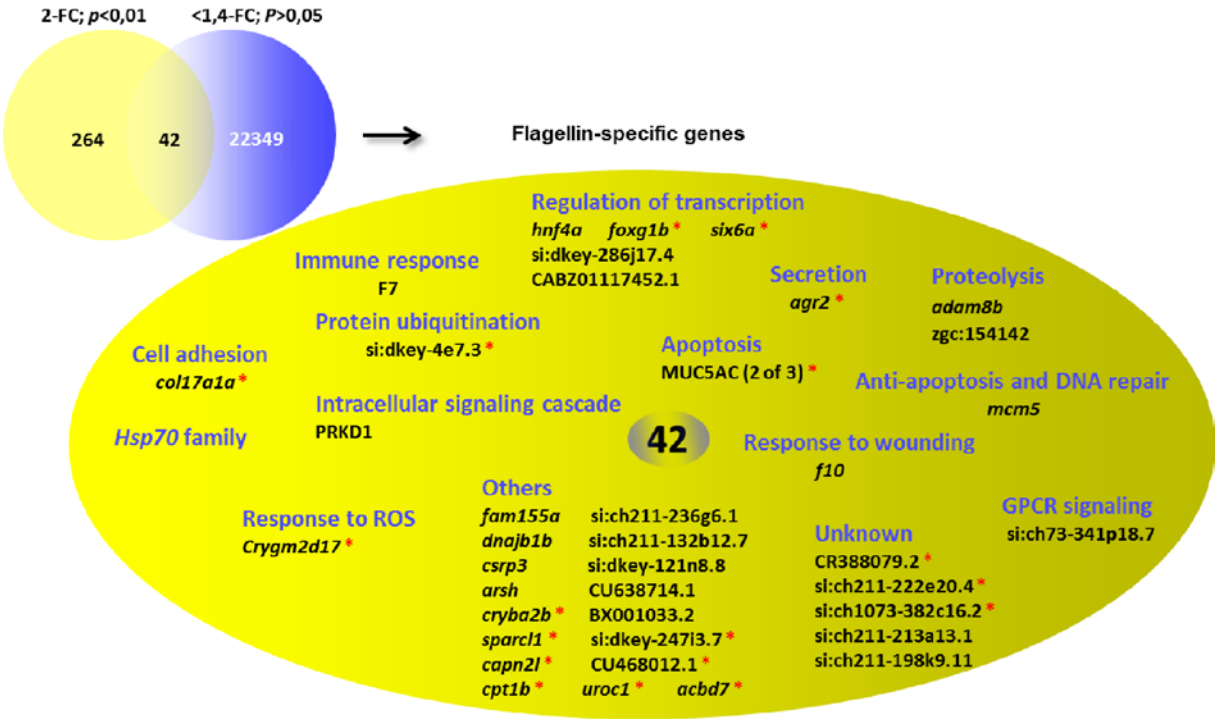
BX548011.4 *

E


2-FC; p<0,01
<1,4-FC; P>0,05
264
42
22349
Flagellin-specific genes
Regulation of transcription
hnf4a
foxg1b *
six6a *
si:dkey-286j17.4
CABZ01117452.1
Immune response
F7
Secretion
agr2 *
Proteolysis
adam8b
zgc:154142
Protein ubiquitination
si:dkey-4e7.3 *
Cell adhesion
col17a1a *
Apoptosis
MUC5AC (2 of 3) *
Anti-apoptosis and DNA repair
mcm5
Intracellular signaling cascade
PRKD1
Hsp70 family
42
Response to wounding
f10
Others
fam155a
dnajb1b
csrp3
arsh
cryba2b *
sparcl1 *
capn2l *
cpt1b *
si:ch211-236g6.1
si:ch211-132b12.7
si:dkey-121n8.8
CU638714.1
BX001033.2
si:dkey-247i3.7 *
CU468012.1 *
uroc1 *
acbd7 *
Response to ROS
Crygm2d17 *
GPCR signaling
si:ch73-341p18.7
Unknown
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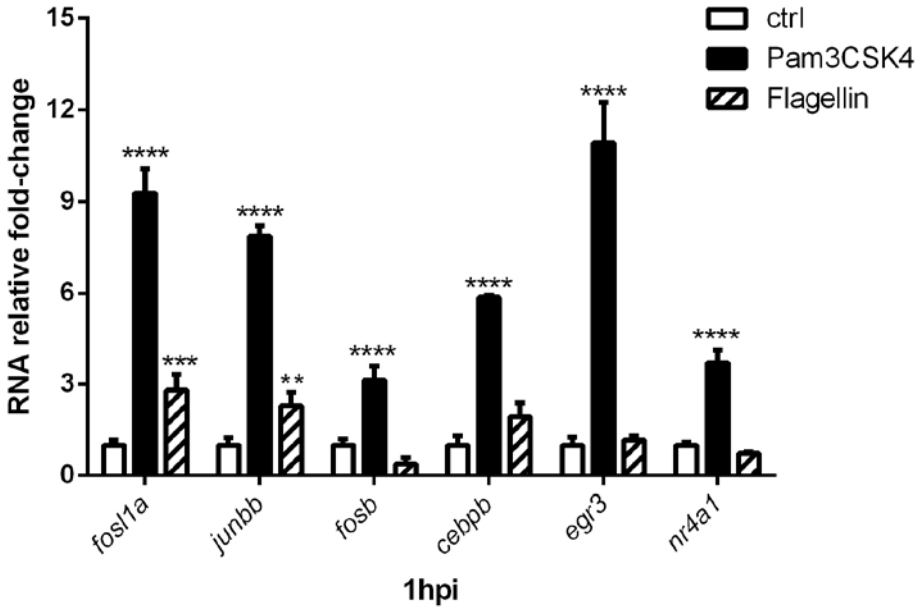


FIGURE 4 qPCR analysis of transcription factors genes responsive to PAMPs stimulation. Expression levels of *fosl1a, fosb, junbb, cebpeb, egr3* and *nr4a1* following Pam3CSK4 and flagellin stimulation are determined by qPCR. Data (mean ± SD) are combined from at least tree biological replicates (n=15 embryos per group) and expressed relative to their corresponding water control, which is set at 1. Statistical significance was determined by two-way ANOVA analysis and Tukey's multiple comparisons test, **p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*.

4 Identification of gene sets that are specifically regulated by Pam3CSK4 and flagellin

This study unexpectedly revealed that there is a relatively large group of genes that are only induced or repressed by either Pam3CSK4 or flagellin (Fig. 3B). To further test whether this specifically induced group is completely unaffected by the other PAMP treatment we used a rigorous filtering approach as shown in Fig. 3D and E. For this approach, the DEGs from the Pam3CSK4 (264 genes) and flagellin (306 genes) stimulation groups were compared with the group of genes that were not affected by flagellin (22611 genes) and Pam3CSK4 (22391 genes), respectively, with a cut-off setting at <1.4-fold change and $p>0.05$. By taking the overlap of these sets we thereby exclude genes that were inducible by the other ligand even at very low stringency. This resulted in a set of 48 genes (Fig. 3D) for which the response is specific to Pam3CSK4 and a set of 42 genes (Fig. 3E) for which the response is specific to flagellin. GO analysis indicated that these two groups contain different categories (Fig. 3D, E). Most notably, genes with high fold-change (>2) from the Pam3CSK4 specific group include many transcription factors involved in the TLR signaling pathway, such as *junbb*, *cebpb*, *fosb*,

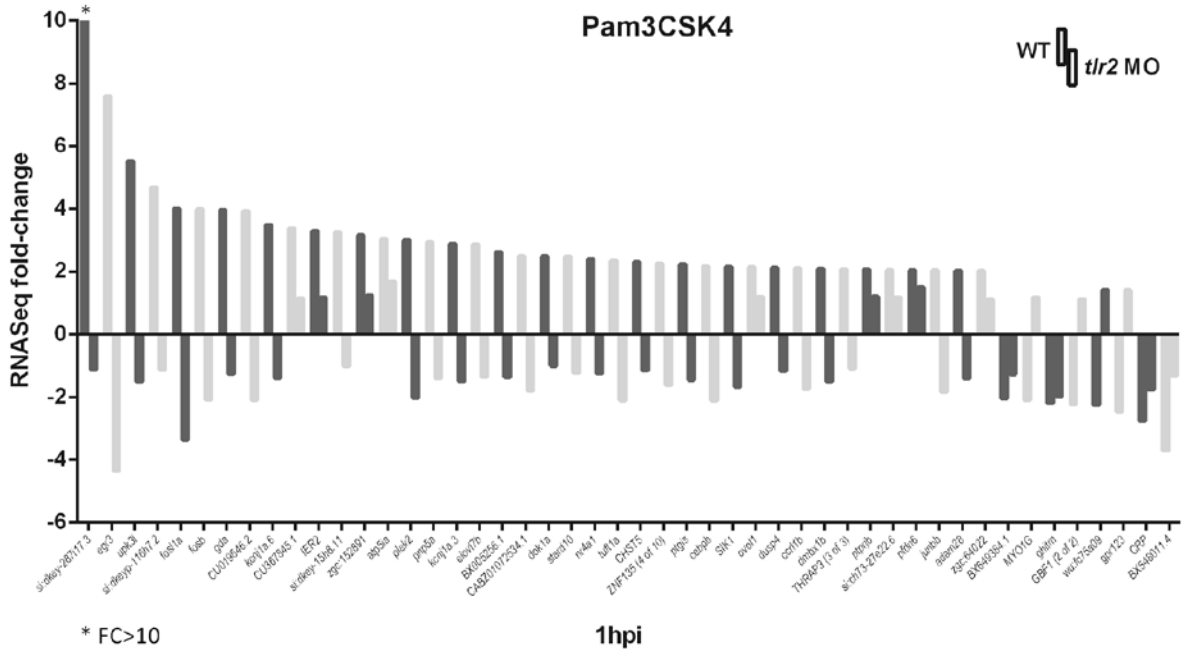
fosl1a, *egr3* and *nr4a1*. In the flagellin specific group of genes this is not the case and an obvious enriched category could not be identified.

To confirm the result of deep sequencing, qPCR was performed to verify the responses of genes from the Pam3CSK4 specific gene set, namely *junbb, cebpb, fosb, fosl1a, egr3* and *nr4a1* (Fig. 4). As expected, the expression level of all these transcription factors confirmed the deep sequencing result. Moreover, these genes did not exhibit an apparent differential expression in zebrafish upon flagellin stimulation except *fosl1a* and *junbb* (Fig. 4). Even though the expression of these both genes showed significant induction upon flagellin stimulation, the induction level was still far lower than that upon Pam3CSK4 stimulation.

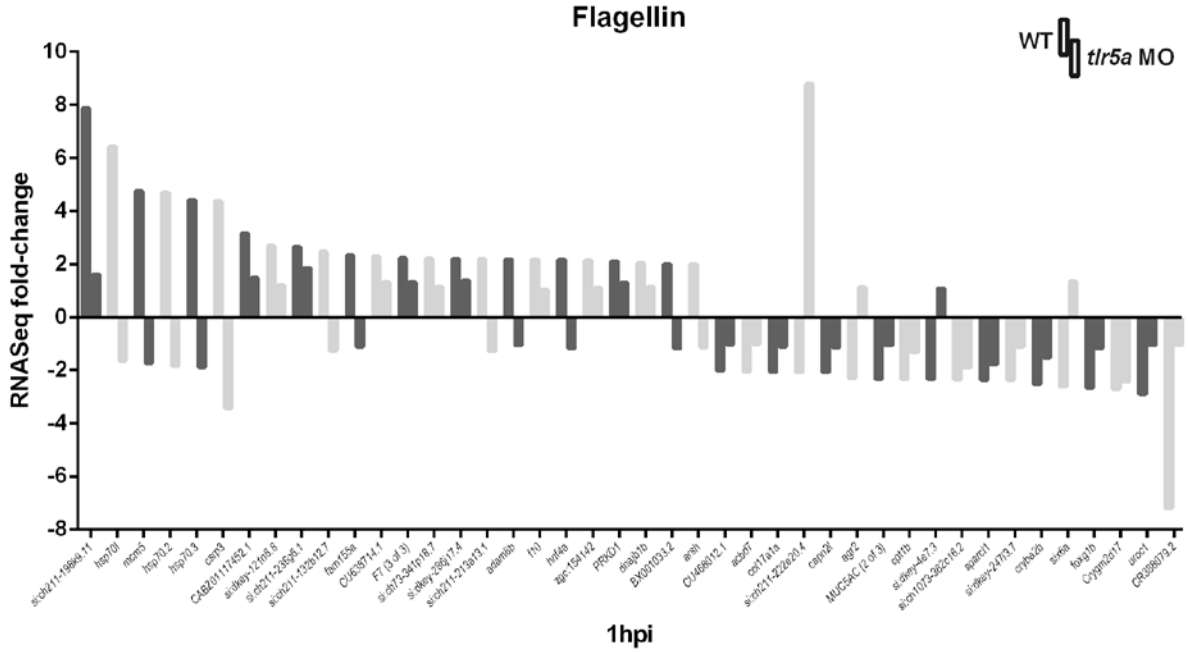
5 Function of the tlr2 and tlr5a genes in the transcriptome responses to Pam3CSK4 and flagellin

To confirm that the transcriptome responses upon PAMPs stimulation described above (Fig. 3B and Fig. 4) are *tlr2* or *tlr5* specific, we performed RNAseq analyses of the Pam3CSK4 and flagellin responses under *tlr2* and *tlr5a* knockdown conditions, again using biological triplicates of each group. Setting a threshold of 2 fold-change and $p<0,01$, we found that the 80 common DEGs responsive to both Pam3CSK4 and flagellin were reduced by *tlr2* and *tlr5a* knockdown (Fig. 5C, D). Furthermore, all the 48 genes (40-up and 8-down-regulated) from the Pam3CSK4 specific group showed no longer a differential expression or an anti-correlated expression after *tlr2* knockdown and, similarly all the 42 genes (24-up and 18-dow-regulated) from the flagellin specific group showed no longer a differential expression or an anti-correlated expression upon *tlr5a* abrogation (Fig. 5A, B). Overall, these results confirm the specificity of both morpholinos and show that zebrafish *tlr2* and *tlr5a* are key mediators of the transcriptomic response triggered by injection of Pam3CSK4 and flagellin respectively.

A


Pam3CSK4
WT
tlr2 MO
RNASeq fold-change
10
8
6
4
2
0
-2
-4
-6
*
* FC>10
1hpi

B


Flagellin
WT
tlr5a MO
RNASeq fold-change
10
8
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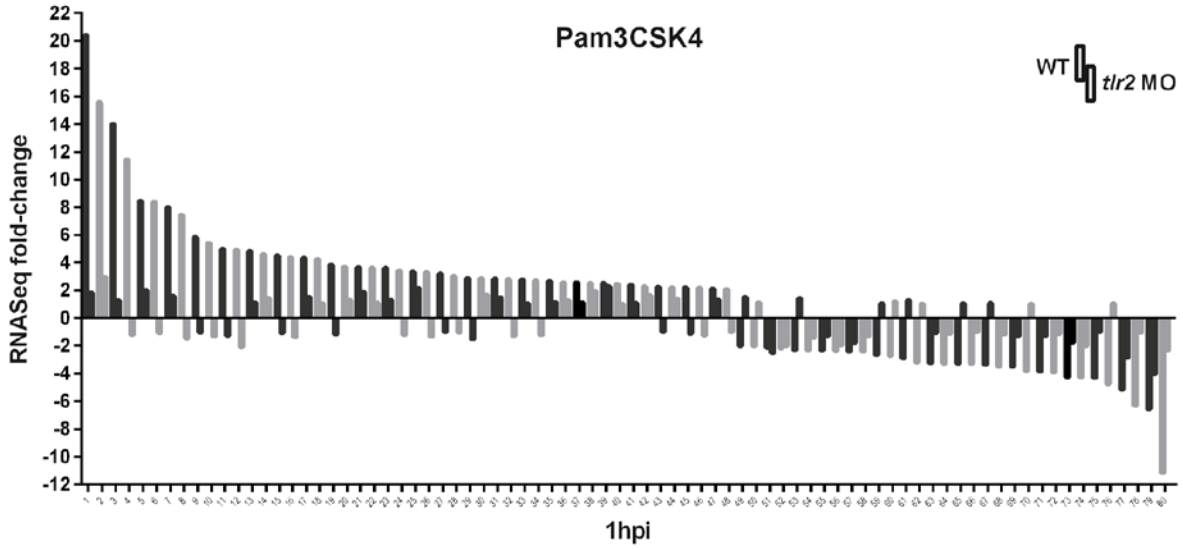
2
0
-2
-4
-6
-8
1hpi

C



D

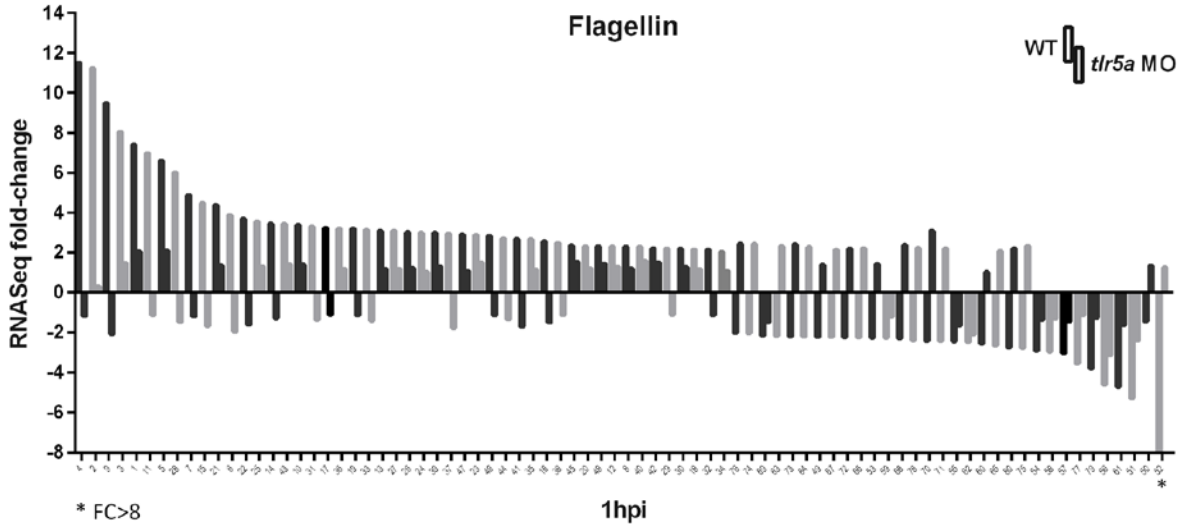


FIGURE 5 Effects on *tlr2* and *tlr5* knockdown on the expression of Pam3CSK4 and Flagellin responsive genes. A, based on RNAseq data, all the 48 Pam3CSK4 specific genes are inhibited or down-regulated in *tlr2* morphants (*tlr2* MO) upon this PAMP stimulation. B, based on RNAseq data, all the 42 flagellin specific genes are inhibited or down-regulated in *tlr5a* morphants (*tlr5a* MO) upon this PAMP stimulation. C, D, all the 80 common genes are inhibited or down-regulated in *tlr*2 and *tlr*5a morphants. FC, fold-change. Panel A, B, C, D: for the quantitative data and accession numbers of the shown genes (or numbers) we refer to Supplemental table II (panel A), III (Panel B) and IV (panels C and D).

Discussion

The signaling pathways underlying recognition of PAMPs have been studied intensively and this has led to a broad understanding of key regulators of innate immunity based on studies of cell cultures and the use of knockout rodent mutants. 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. In this manuscript we have chosen the zebrafish embryo model for such an approach and have included functional analysis of Tlr5 and Tlr2 in the response towards two well-known PAMPs, flagellin and Pam3CSK4.

The results show that there is a relatively limited overlap between the transcriptome responses towards flagellin and Pam3CSK4 (Fig. 3B). The overlap includes well known downstream immune mediators that were previously shown to be induced by flagellin (41) 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 (Fig. 4).

GO terms analysis of the genes specifically regulated by these two PAMPs show that there is an enriched category of transcription factors in the Pam3CSK4-specific group, of which most of genes are up-regulated and only one is down-regulated. Additionally, a less enriched category of immune response genes is found in this group as well, which include a down-regulated CRP (C-reactive protein). For the flagellin-specific group of genes, there are only five genes under the GO-term regulation of transcription of which two are down-regulated. Many of the genes specifically induced by Pam3CSK4 have also been shown to be strongly regulated by infection in the zebrafish embryo system (44) and therefore we would like to further study this group of genes in more detail in future research.

For functional analysis of the transcriptome response towards flagellin and Pam3CSK4 we used morpholinos that were selected on basis of their blocking effect on downstream signaling using qPCR and subsequently confirmed by RNAseq analysis. Surprisingly for *tlr5* we found that a morpholino against each of the two copies of this gene, *tlr5a* and *tlr5b* had an effect on downstream signaling, with the *tlr5a* morpholino giving a complete block of induction of *il1b* by injection with flagellin and a partial effect of the *tlr5b* morpholino. These data suggests that these two *tlr5* copies function in concert, perhaps by forming heterodimers.

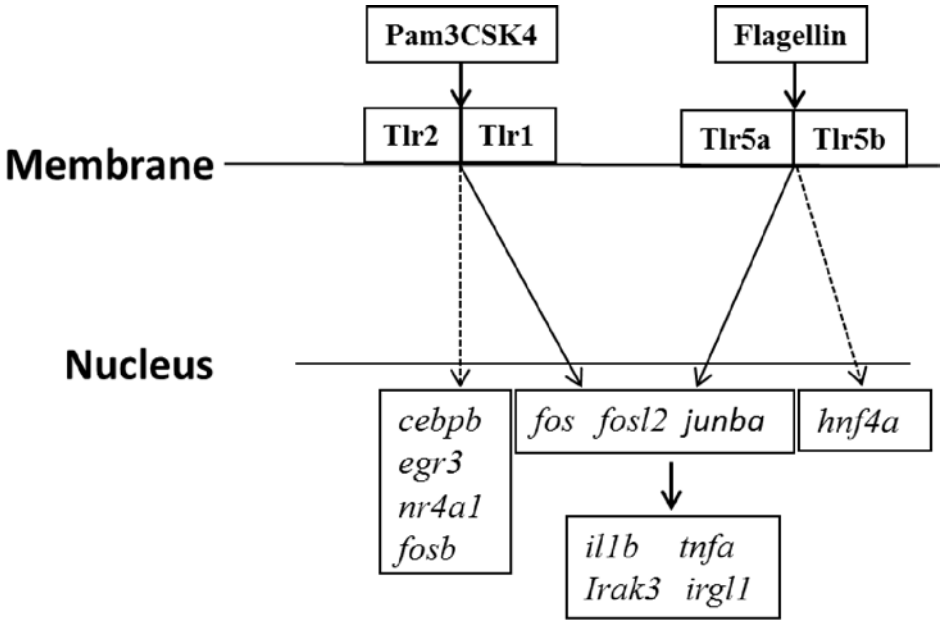


FIGURE 6 Specific and common responses to Tlr2 and Tlr5 ligands. The whole organism transcriptome response of zebrafish embryos to treatment with the Tlr2 ligand Pam3CSK4 or the Tlr5 ligand flagellin results in the induction of specific and common transcription factor genes as indicated in the figure. The common transcription factor genes (in cooperation with other non-inducible factors, e.g. of the NF-κB family), likely function upstream of the effector genes commonly induced by Pam3CSK4 and flagellin. The transcription factor genes induced by only one of the two ligands are likely to contribute to further specificity in the transcriptional responses of downstream effector genes.

An important question that comes from our work is how to explain the difference in gene sets that are regulated in response to Pam3CSK4 and flagellin? (Fig. 6) Since our detection system seems sufficiently sensitive to detect even minor effects on gene transcription, a limitation in dosing is not a likely explanation for this difference, so instead we think of another two possible alternative explanations. In the first place we could speculate that there are specific downstream signaling partners for Tlr2 and Tlr5. However, such partners have not yet been indicated by previous studies, in contrast, there are evidences that all known direct binding partners are common for both Tlr5 and Tlr2 proteins: including the adaptor proteins Myd88, and Tirap (Mal) that have been implicated in signaling of both proteins (45). Furthermore, the functions of these genes in the direct recognition of TLR2 and TLR5 ligands have not been tested yet in whole animal models. Mutants for Tirap have not been described yet in zebrafish making the specific function of this gene currently difficult to investigate. Another possible explanation is that the differential response of zebrafish embryos to these two PAMPs is the result of an additive effect of the recognition by different cell types. In this case, the common group of activated downstream genes might be encoded by the response of common immune cells which have a full repertoire of Tlr receptors

whereas the specific response might be the result of a distinct transcriptional response of specialized cells that do not encode all Tlr receptors. The detailed study of these transcription factors will provide valuable information on the specific immune transcriptional signatures elicited by different pathogens. For this purpose the genetic tractability of the zebrafish system will allow the generation of new reporter lines that will contribute to the understanding of how these responses modulate the innate immune system. In addition, such reporter lines will be of general interest since Pam3CSK4 and flagellin signaling pathways are broadly used to study the Tlrs function in inflammatory microbial infection. Furthermore, this signaling pathway is also relevant for studies of atherosclerosis and autoimmune diseases processes (46, 47). Therefore the used systemic approach can be highly useful for future studies of a broad spectrum of immune-related diseases.

Material and methods

Zebrafish husbandry

Wild-type zebrafish of the AB/TL strain were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin. org). Embryos were raised in egg water (60g/ml Instant Ocean sea salts) at 28.5 °C. For the duration of bacterial injections, embryos were kept under anesthesia in egg water containing 0.02% buffered 3-aminobenzoic acid ethyl ester (Tricaine). The breeding of adult fish was approved by the local animal welfare committee (DEC) of the University of Leiden. All protocols adhered to the international guidelines specified by the EU Animal Protection Directive 2010/63/EU.

Morpholino injections

Morpholino oligonucleotides (Gene Tools) were diluted to desired concentrations in 1× Danieu's buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM Ca $(NO_3)_2$, 5.0 mM HEPES (pH 7.6)) containing 1× phenol red (Sigma-Aldrich). For knockdown experiments, *tlr2* ATG-morpholino (*tlr2* mo, Supplemental table I) was injected with the optimal concentration at 0.5 mM and 1nl volume per embryo at 0~2 cell stage. *tlr5* translation was blocked via injecting 1nl of the *tlr5a* and *tlr5b* ATG-morpholinos at a dose of 0.1 mM and 0.5 mM at 0~2 cell stage, as previously published by *Stockhammer* and coworkers (41). Control embryos were injected with the standard control morpholino (Sc mo, Supplemental table I).

Ligands injection

For the ligands injection assay, purified Pam3CSK4 (InvivoGen) and flagellin from *S. typhimurium* (Flagellin FliC VacciGrade™, Invitrogen) were respectively diluted to 1

mg/ml and 100 µg/ml in sterile water. For their administration, 1 nl of the ligands was injected into the blood stream at 27 hpf, and sterile water was used as control. Injections were perormed using a FemtoJet microinjector (Eppendorf) and a micromanipulator with pulled microcapillary pipettes.

RNA isolation, cDNA synthesis and qPCR

RNA was extracted using TRIzol Reagent (Life Technologies) and purified by column according to the manufacturer's instructions of RNeasy MinElute Cleanup Kit (Qiagen). The concentration and quality of RNA were detected by NANODROP 2000/2000c (Thermo Scientific). 1µg cDNA synthesis reactions and qPCR were performed as described in the manufacturer's instructions (iScript™ cDNA Synthesis Kit and iQ™ SYBR® Green Supermix, BioRad) and normalized against the expression of *ppial* as a housekeeping gene (48). PCR analysis was performed using the following protocol: 95°C 3 min, 40 cycles real time of 95°C 15 sec and 60°C 45 sec, and final melting curve of 81 cycles from 95°C 1 min to 55°C 10 sec. Results were analyzed using the ΔΔCt method. Primer sequences used can be found in Supplemental table I.

Deep sequencing and data analyzing

Triplicates of 10~20 embryos of AB/TL or *tlr* morphants from three injection conditions, Pam3CSK4, flagellin or water injection, were homogenized in 500 µl of Trizol reagent (Qiagen). Total RNA was extracted and column-purified according to the manufacturer's instructions of the RNeasy MinElute Cleanup Kit (Qiagen). The subsequent sample preparation and Illumina RNA sequencing were as previously described (49). RNA samples were treated with DNaseI (Life Technologies) to remove residual genomic DNA. RNA integrity was analyzed by Lab-on-a-chip analysis (Agilent, Amstelveen, The Netherlands). A total of 2µg of RNA was used to make RNAseq libraries using the Illumina TruSeq RNA Sample Preparation Kit v2 (Illumina, Inc., San Diego, CA, USA). The manufacturer's instructions were followed with the exception of two modifications. In the adapter ligation step, 1µl, instead of 2.5 µl, adaptor was used. In the library size-selection step, the library fragments were isolated with a double Ampure XP purification with a 0.7× beads to library ratio (Beckman Coulter, Woerden, The Netherlands). The resulting mRNAseq library was sequenced using an Illumina HiSeq2500 Instrument (Illumina, Inc.) according to the manufacturer's instructions with a read length of 2×50 nucleotides. Image analysis and base-calling were done using the Illumina HCS version 2.0.12. The raw data has been submitted to the GEO database (accession number GSE64570). The total number of reads for each sample is summarized in supplemental table IV and quality control was according to the sequencing company guidelines (ZF-sceens.com). The data was analyzed using the GeneTiles software (http://www. genetiles. com) (42)using a cut-off setting of 2 fold-change and a *p*-value <0.01. In brief, Genetiles used fastq files as input for the program

Bowtie2 (50) to align the reads to the zebrafish genome (obtained from Ensembl version Zv9). Subsequently, the programs SAMtools (51), DESeq and DEXSeq (52, 53) are used for data processing. The complete data processing pipeline for Genetiles, including the used parameters, is available for download at www.genetiles.com and can also be found in Veneman et al (42). Using these settings we have mapped the numbers of reads as shown in Supplemental table IV. The triplicate data sets of Pam3CSK4, flagellin and control treatments were mapped to 27104, 26583, and 26409 ENSEMBL genes, respectively. The difference between the mapped reads of the individual samples compared with the mapped reads of triplicate samples was always lower than 12 % (Supplemental table IV). GO analysis was performed using the software package DAVID available at http://david.abcc.ncifcrf.gov/home.jsp (54).

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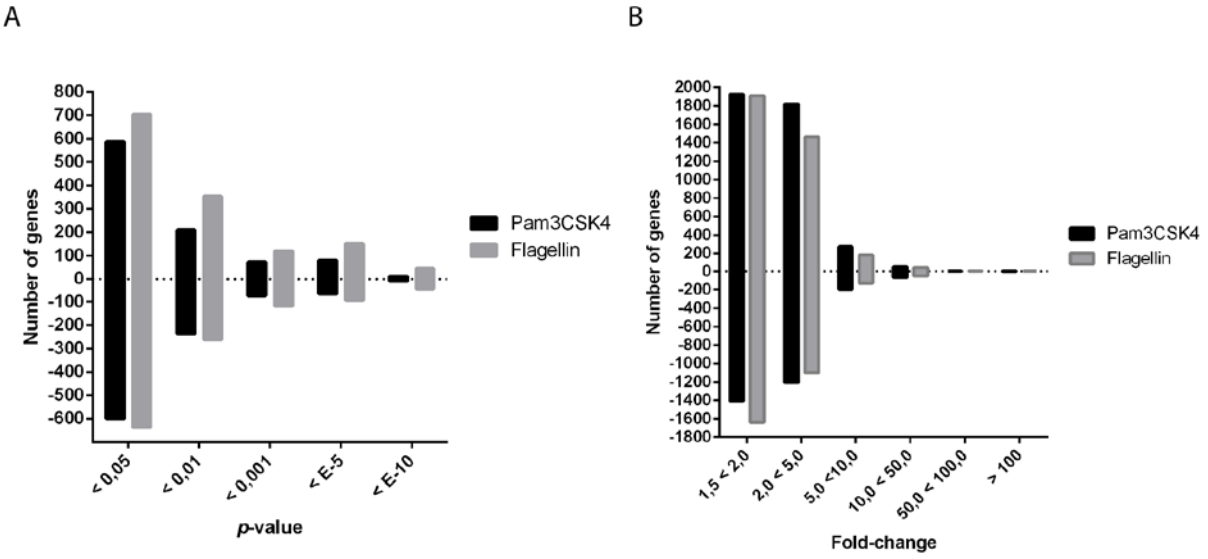
Supplementary data

Supplementary tables and supplementary material 1 can be found online at:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4514945/

Supplemental figure 1 Change trend of the number of DEGs according to different fold-change and *p-value*



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CHAPTER 3

Tlr2 function in innate immune responses to *Mycobacterium marinum* infection in zebrafish

3

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Abstract

TLR2 is known to play controversial roles in host defense against pathogens, especially *Mycobacterium tuberculosis* (Mtb). To study the function of TLR2 in mycobacterial infection, we analyzed a *tlr2* zebrafish mutant and infected larvae with *Mycobacterium marinum* (Mm), a close relative to Mtb, as a model for tuberculosis. After Mm infection, bacteria proliferated in *tlr2*$^{-/-}$ fish more than in the heterozygote *tlr2*$^{+/-}$, suggesting that Tlr2 is involved as a protective factor in host defense. We observed a reduced number of granulomas in *tlr2*$^{-/-}$ larvae when compared to the control. We also found that the *tlr2* mutant (*tlr2*$^{-/-}$) shows a reduced number of macrophages in the absence of infection. However, the macrophage migration speed and maximum migration distance from infection sites of phagocytic macrophages, are significantly higher in *tlr2*$^{-/-}$ than in *tlr2*$^{+/-}$ larvae. These results suggest that Tlr2 might be playing a role in macrophage congregation and phagocytosis. RNAseq analysis of infection experiments in mutant versus control showed that the number of upregulated and downregulated genes in the control was greatly diminished in the *tlr2* mutant, with the strongest effect on the down-regulated gene set. Detailed gene expression analysis using RNAseq and qPCR showed that Mm infection of *tlr2* mutant leads to decreased mRNA levels of genes involved in inflammation and immune responses, including *il1b*, *tnfb*, *cxcl11aa/ac*, *fosl1a* and *cebpb*. Furthermore, RNAseq analysis revealed that genes for Maf family transcription factors (Mafb/c-Maf), vitamin D receptors (Vdr) and diverse immunoglobulin domain-containing proteins (Dicps) are significantly different regulated in *tlr2* mutants with or without infection. This could result from a function in the control of induction of cytokines and chemokines as well as macrophage number, migration and phagocytosis. Moreover, our transcriptome analysis revealed Tlr2-specific pathways involved in Mm infection, which are also related to responses to Mtb infection in human macrophages. The expression analyses give further support for a function of TLR2 in host defense.

Introduction

Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis (TB), which infects nearly one-third of the world's population, and kills about 1.5 million people annually (WHO Global Tuberculosis Report 2015). TB is characterized by the formation of granulomas not only in the lung but also in other tissues and organs. This is the result of a concerted action of host innate and adaptive immunity (1, 2).

Innate immune responses play a critical role in defense against TB infection in the host, and for a major part these processes are mediated by Toll-like receptors (TLRs), a conserved family of pattern recognition receptors. TLR2 is one of the most widely reported members of the TLRs family to be involved in defense against Mtb by virtue

of its recognition of cell wall-associated components associated with this pathogen (3-6). Following mycobacterial infection in human cell cultures, TLR2 dimerizes with TLR1 or TLR6, and recognizes mycobacterial components such as cell wall glycolipids LAM and LM (7), 38-kDa and 19kD mycobacterial glycoprotein (LpqH) (8-10), phosphatidylinositol mannoside (PIM) (11), and triacylated (TLR2/TLR1) (12, 13) or diacylated (TLR2/TLR6) lipoproteins (14, 15). Then these heterodimers recruit the Myd88 and Tirap (Mal) proteins to activate the IRAK(1 and 4)/TRAF6/IKK(α or β) cascade, which subsequently leads to the ubiquitination of IκBα and the activation of transcription factor NF-κB or AP-1 to release cytokines and chemokines (16, 17). Finally, these cytokines and chemokines attract migration of macrophages and neutrophils to the infection site leading to phagocytosis of bacteria. Tlr2 is shown to be a factor that is needed for granuloma formation in Mtb infection (18). Some studies have reported that $Tlr2^{-/-}$ mice lose control to high dose infection of Mtb or show higher susceptibility to Mtb infection compared to the wildtype (19, 20). Tlr2 also mediates the inhibition of MHC-II expression on the macrophage surface and MHC-II antigen processing by Mtb bacilli, thereby preventing presentation of Mtb antigens and decrease recognition by $CD4^+$ T cells, which may allow intracellular Mtb to evade immune surveillance and maintain chronic infection (10, 21). The interaction between TLR2 and Mtb or other pathogens not only promotes the killing of bacteria, but also seems to be a part of the bacterium's strategy to evade the immune system (22-24). For instance, LprG from Mtb was reported to inhibit human macrophage class II MHC antigen processing through TLR signaling (22). Furthermore, Tlr2 mutants of mice have an increased resistance against infection of *Candida albicans* (25) and *Yersinia pestis* (26). It has been proposed that this is the result of Tlr2-dependent induction of the anti-inflammatory cytokine IL-10 (26). Furthermore, TLR2 activation inhibits the release of IL-12 via activation of the cFos transcription factor. It also has been shown that IFN-γ or IFN-γ-induced signals (27) are inhibited after infection of murine macrophages in a Tlr2-dependent fashion (28). These effects show that TLR2 activation can yield a bias to T helper Type 2 (Th2) cells (29), and by breaking the Th1/Th2 balance can lead to less Th1 type responses and reducing the killing of intracellular pathogens. However, most of the molecular mechanisms underlying TLR2 functions remain unknown and a better understanding of the TLR2-mediated immune response and immune evasion can help in planning prevention and therapy strategies against Mtb infection.

Animal models have shown their power in studies of the mechanisms of interaction of host and TB pathogens, and discovering new anti-TB drugs. Zebrafish –adult and larvae– models have become a prevailing complement for rodent studies, for three important reasons. First, zebrafish have a 3-4 weeks separation stage between development of innate and adaptive immunity after fertilization (30, 31), which gives the possibility to study the host innate immune response to infection in the absence of

adaptive immune responses. Second, zebrafish can be infected by *Mycobacterium marinum* (Mm), a natural pathogen of cold blooded vertebrates and a close relative of Mtb, which can induce granuloma formation in zebrafish, and this is similar to human TB symptoms (32, 33). Third, the transparent larvae are ideal for imaging the early steps of the infection process in real time. Hence, zebrafish has earned its place of being a versatile tuberculosis model (34). In our previous study, we demonstrated that the mammalian TLR2 ligand Pam3CSK4, a synthetic triacylated lipopeptide that mimics the triacylated lipoprotein of mycobacteria, could also specifically activate the zebrafish Tlr2 pathway, inducing *fosl1a* and *cebpb* gene upregulation (35).

In the current study, to further explore the involvement of Tlr2 in Mm infection, we conducted infection studies using *tlr2* mutant zebrafish. We found that *tlr2* mutation promoted Mm infection, affected macrophage functions and corresponded with reduced granuloma formation compared to the wildtype. In summary, we demonstrated that Tlr2 plays an important role in protecting the host during the early stage of mycobacterial infection. In addition, we performed RNA deep sequencing (RNAseq) and determined a Tlr2-specific gene list for the response to Mm infection. In particular, this revealed that most of the downregulation of genes caused by Mm infection in the control was abrogated by *tlr2* mutation.

Results

1 tlr2 mutation prevents activation of tlr2-dependent genes in zebrafish larvae

The *tlr2*^{sa19423} mutant (*tlr2*$^{-/-}$) carries a thymine to adenine point mutation that creates a premature stop codon (Fig. 1A), which is located in the C-terminus of the leucine-rich repeat (LRR) domain. This leads to a truncated protein without the Toll/IL-1 receptor (TIR) domain, which is required for the interaction with Myd88 and Tirap (Mal) (36, 37).

To confirm whether *tlr2* mutation blocks its downstream pathway, we analyzed the gene expression profiles of zebrafish treated with the TLR2 agonist, Pam3CSK4. Pam3CSK4 was injected into the blood island of zebrafish embryos at 27 hpf. One hour after injection (hpi), we collected samples and performed qPCR to analyze the expression levels of TLR2-downstream effectors, CCAAT/enhancer-binding protein beta *(cebpb*) and FOS Like Antigen 1a (*fosl1a*), as we previously reported to be specific targets of Tlr2 signaling (35). In *tlr2*$^{+/\ sa19423}$ heterozygotes (*tlr2*$^{+/-}$), the expressional levels of transcription factors *cebpb* and *fosl1a*, and inflammation marker gene *il1b* were significantly induced by Pam3CSK4, whereas *tlr2*$^{-/-}$ showed no significant response (Fig. 1B). To confirm that these results are specific for the TLR2 pathway, we injected flagellin, a TLR5 agonist into 27 hpf embryos. Flagellin induced the non-specific

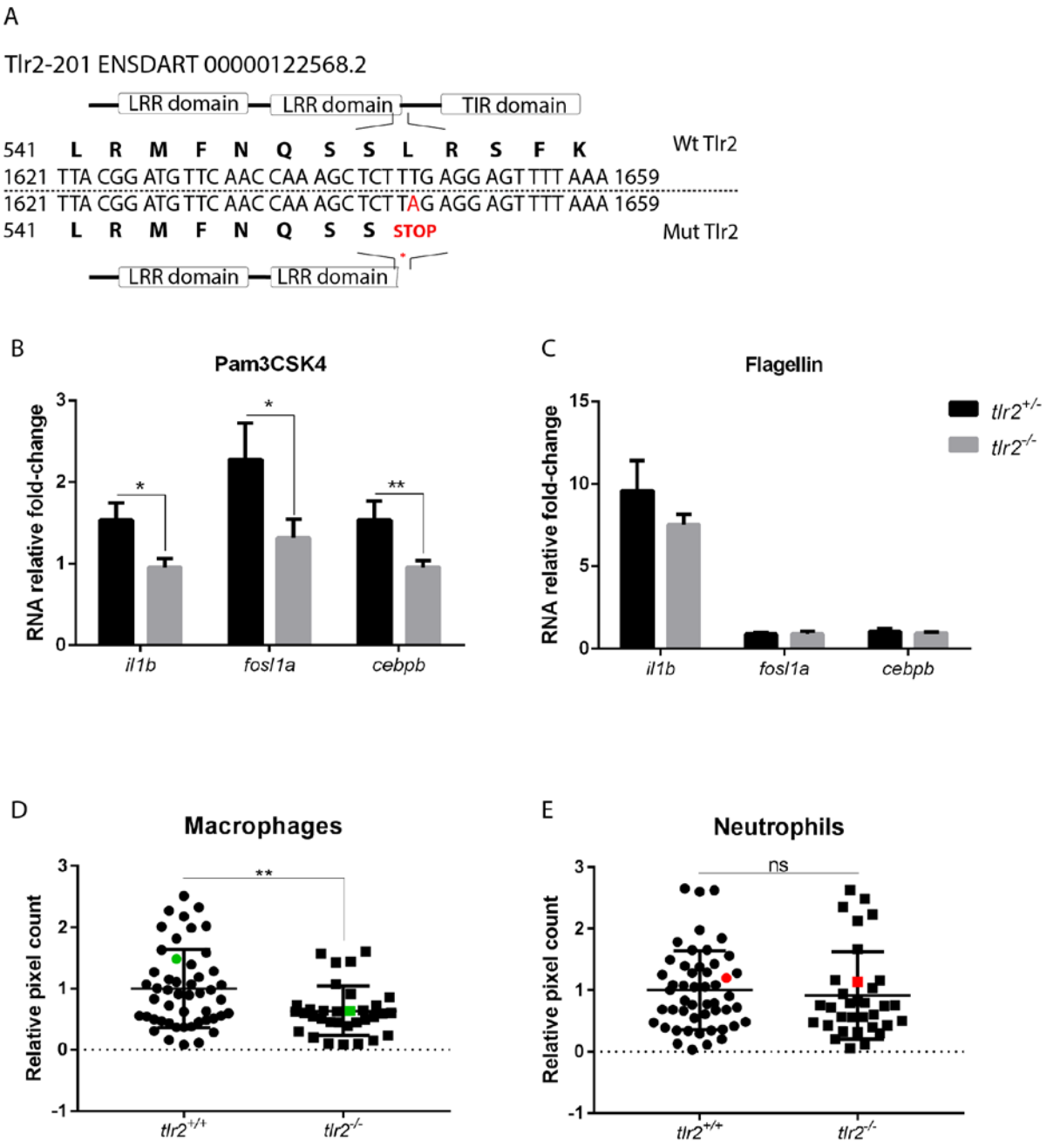

FIGURE 1 Characterization of the Tlr2 mutant. A, mutant DNA and protein sequence. A point mutation (T to A) in the C-terminal of the second LRR domain of zebrafish Tlr2 introduces a premature stop codon. The predicted truncated protein lacks the whole TIR domain. Nucleotide and amino acid positions are indicated with respect to the translation start codon. B and C, *tlr2*$^{-/-}$ and *tlr2*$^{+/-}$ embryos were injected at 27 hpf with 1 ng Pam3CSK4 or 0.1ng flagellin and expression levels of *il1b*, *fosl1a* and *cebpb* were determined at 1 hour post injection by qPCR. Data (mean ± SEM) are combined from at least three biological replicates (n=10 embryos per group) and expressed relative to their corresponding mock injection (water) control, which is set at 1. Statistical significance of differences between mock and Pam3CSK4 groups was determined by two-way ANOVA with Sidak Multiple Comparison test as a post-hoc test, *p < 0.05, **p<0,01. D, fluorescence relative pixel-count

analyses of GFP-labeled macrophages in 2dpf *tlr2*$^{+/+}$/*Tg*(*mpeg1*:*EGFP*) and *tlr2*$^{-/-}$/*Tg*(*mpeg1*:*EGFP*) embryos (at least 35 embryos per group) were performed based on stereo fluorescence images (green symbols indicate the data point for which representative examples are shown in figure S4). E, fluorescence relative pixel-count values of neutrophils derived from images of whole mount TSA immune staining (red symbols indicate the data point for which representative examples are shown in figure S4). Data (mean ± SD) are combined from two individual experiments. Statistical significance of differences was determined by T-test, **p<0.01, ns, no significant difference.

inflammation marker *il1b* expression, but not *cebpb* and *fosl1a* expression in both *tlr2*$^{+/-}$ and *tlr2*$^{-/-}$ larvae (Fig. 1C). Overall, these data show that *tlr2* mutation specifically blocks its downstream pathways.

2 Macrophage and neutrophil phenotype in tlr2 mutant

To study the effects of *tlr2* mutation in immune cells, we conducted two individual experiments of TSA staining with 2dpf *tlr2*$^{+/+/}$*Tg*(*mpeg1:EGFP*) and *tlr2*$^{-/-}$*Tg*(*mpeg1:EGFP*) larvae. TSA staining detects Mpx activity, which is specific for neutrophils in zebrafish embryos and larvae. The combination of using *mpeg1*-EGFP fish lines and TSA staining can be used to distinguish macrophages (*mpeg1:*EGFP-positive, TSA-negative) and neutrophils (*mpeg1:*EGFP-negative, TSA-positive) (38). The numbers of macrophages in *tlr2*$^{-/-}$ larvae were significantly reduced compared to those in *tlr2*$^{+/+}$ (Fig. 1D and Fig. S4) while the numbers of neutrophils was not significantly different between these groups (Fig. 1E and Fig. S4). We also conducted TSA staining in 2 dpf *tlr2*$^{+/-}$ and *tlr2*$^{-/-}$ larvae without the m*peg1:gfp* marker, and confirmed that there was no significant reduction of neutrophils induced by *tlr2* mutation Fig. S1). These results indicate that *tlr2* mutation does not affect the development of neutrophils but may influence macrophage development.

3 Mycobacterium marinum infection in tlr2 knockdown or mutant larvae

We injected 28 hpf *tlr2* morphants, standard control morphants, and *tlr2*$^{-/-}$ and *tlr2*$^{+/-}$ embryos with ~150 CFU Mma20 by blood island injection. The results of stereo fluorescence microscopy and pixel count analysis show that the bacterial burden in *tlr2* morphants was significantly higher than those of control larvae at 3 dpi (Fig. 2A). Considering the transient effect of morpholinos, we did not test later time points. In agreement, the bacterial burden was significantly higher in *tlr2*$^{-/-}$ than *tlr2*$^{+/-}$ larvae, with the most pronounced difference at 4dpi (Fig. 2B). Figure 2C represents the typical phenotype of infection in *tlr2*$^{+/-}$ and *tlr2*$^{-/-}$ larvae observed using confocal laser

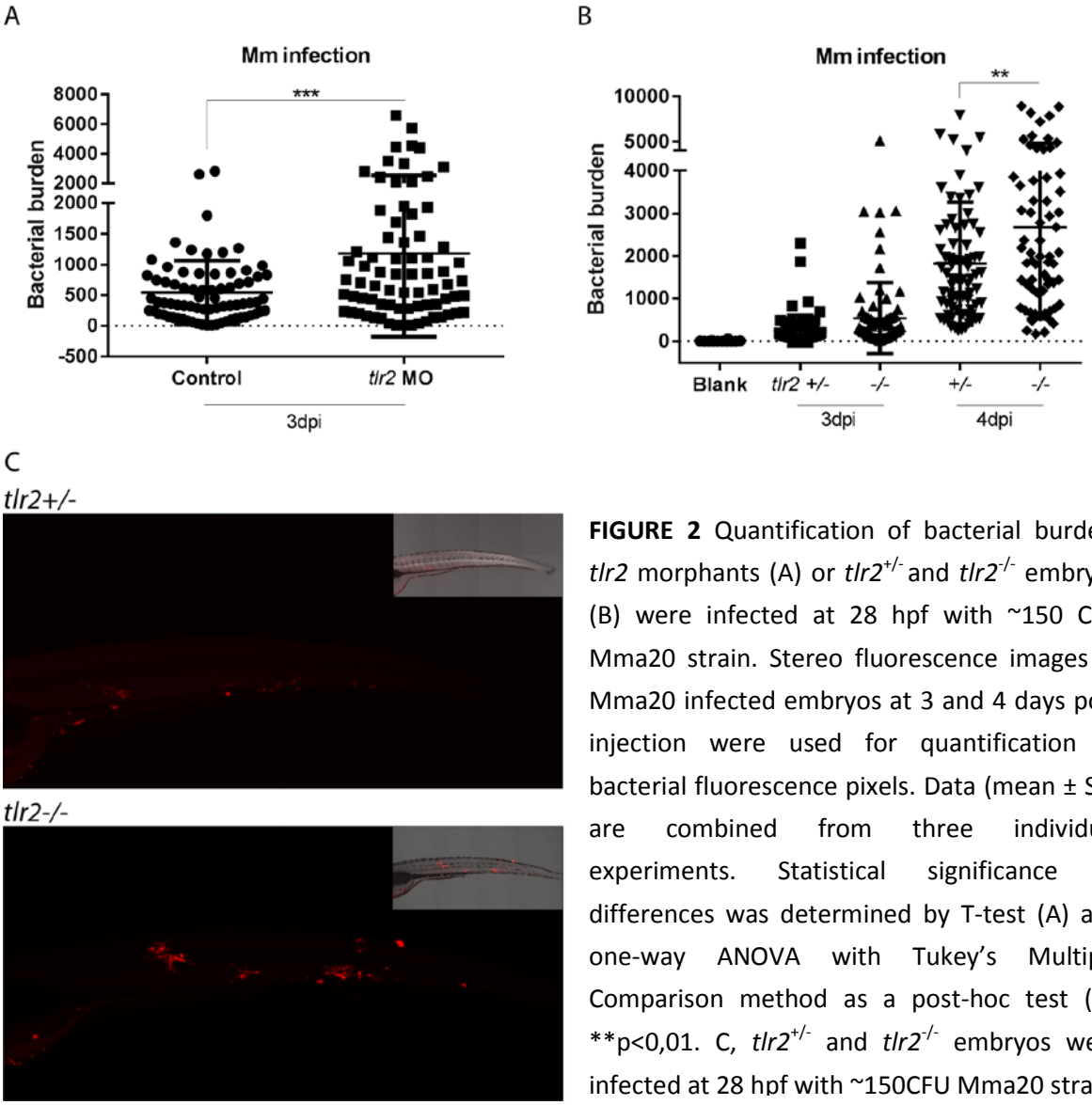

FIGURE 2 Quantification of bacterial burden. *tlr2* morphants (A) or *tlr2*$^{+/-}$ and *tlr2*$^{-/-}$ embryos (B) were infected at 28 hpf with ~150 CFU Mma20 strain. Stereo fluorescence images of Mma20 infected embryos at 3 and 4 days post injection were used for quantification of bacterial fluorescence pixels. Data (mean ± SD) are combined from three individual experiments. Statistical significance of differences was determined by T-test (A) and one-way ANOVA with Tukey's Multiple Comparison method as a post-hoc test (B). **p<0,01. C, *tlr2*$^{+/-}$ and *tlr2*$^{-/-}$ embryos were infected at 28 hpf with ~150CFU Mma20 strain.

scanning microscopy (CLSM) confirming that *tlr2* mutation promotes a higher bacterial burden during Mm infection.

Protective immunity and latent Mm infection in zebrafish are associated with the formation of mature protective granulomas (39). In mice studies, Tlr2-deficiency resulted in a defective granulomatous response (18, 20). To test whether *tlr2* mutation also suppresses the granuloma formation in zebrafish, we conducted CLSM imaging of *tlr2*$^{-/-}$*/Tg(mpeg1:EGFP)* and *tlr2*$^{+/+}$*/Tg(mpeg1:EGFP)* with Mma20 infection. We analyzed bacterial numbers and granuloma formation at several representative highly infected regions of zebrafish larvae at 4 dpi. We observed that *tlr2*$^{+/+}$ larvae showed normal granuloma formation and the majority of the bacteria was inside macrophages.(Fig. 3A-D). In contrast, in two representative locations in *tlr2*$^{-/-}$ larvae (Fig. 3E), a majority of the bacteria congested together and formed a big cluster without evidence for being

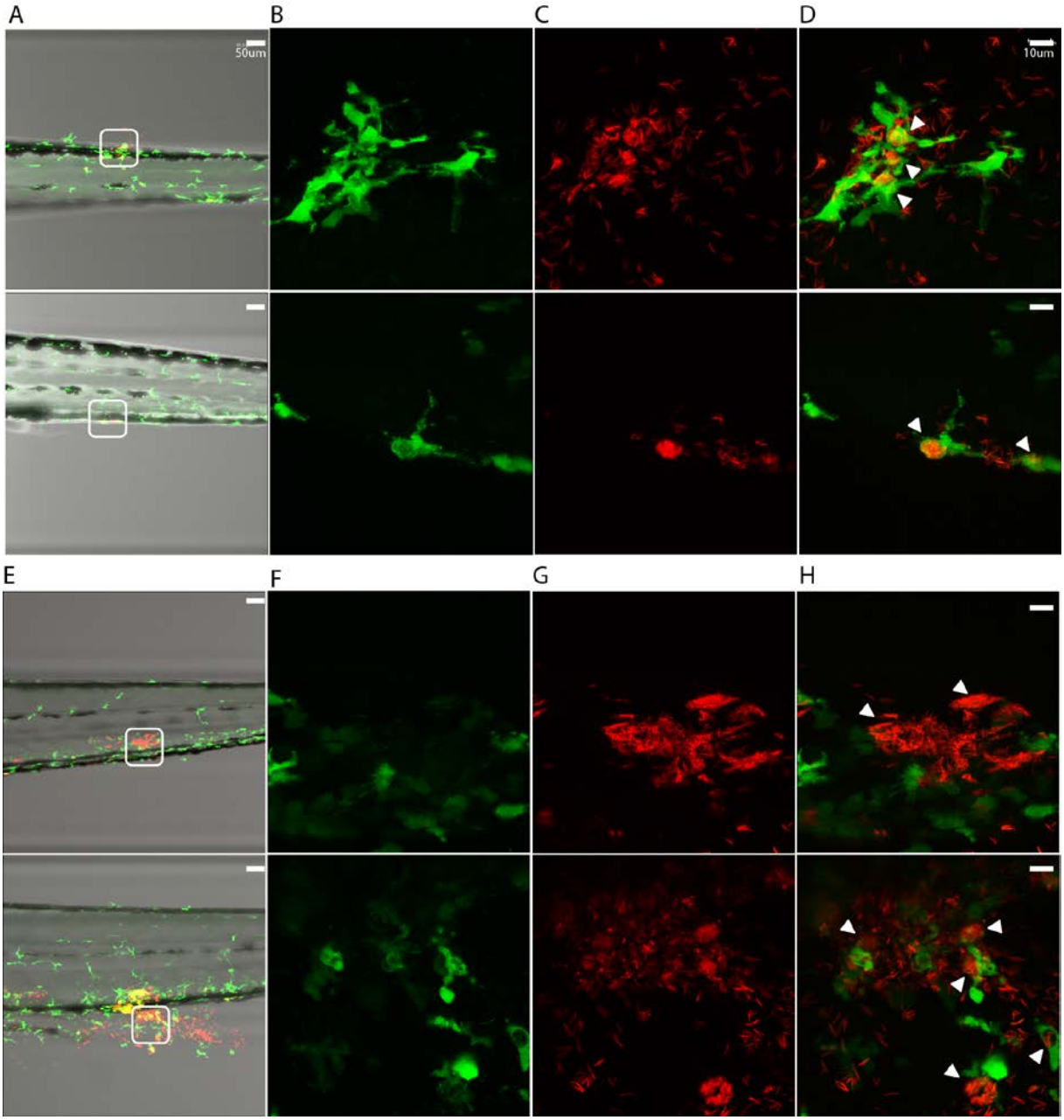

FIGURE 3 Granuloma formation and macrophage phenotypes in the Tlr2 mutant upon Mma20 infection. The *tlr2*$^{+/+}$/*Tg*(*mpeg1*:*EGFP*) (A-D) and *tlr2*$^{-/-}$/*Tg*(*mpeg1*:*EGFP*) (E-H) embryos were infected at 28 hpf with ~150CFU Mma20 strain. Confocal laser scanning imaging was conducted using a Leica SP1 microscope with 10 (A, E) and 63 (B-D and F-H) times magnification lenses. GFP-green, macrophage; mCherry-red, Mma20; white arrow head indicates the enlarged section of this picture. The size bar in bright view figure A represents 50um, in fluorescence view figure D represents 10um. (B-D and F-H) enlargements of the areas indicated in panels A and E. The positions indicated by arrowheads are the reference points. In the Tlr2 mutant they point to clusters of bacteria that are not present inside macrophages. In the control they point to macrophages that have ingested bacteria, which are observed much more frequently than in the mutant. The images are representative of images from 38 *tlr2* mutant and 42 wildtype embryos.

inside macrophages. (Fig. 3E-H). Furthermore, these results show that the *tlr2*$^{-/-}$ mutant allows higher Mm proliferation and is impaired in granuloma formation compared to the *tlr2*$^{+/+}$ control.

To further quantify the migration of macrophages after Mm infection, we performed cell tracking to measure the maximum migration distance from the start of the image sequence and their average speed from 84 to 96 hpi (Fig. 4). In this study, the location of the bacteria-containing macrophages in the first frame of the movie was set as the origin and we then tracked the migration of the macrophages during the following twelve hours. Our results show that both the maximum distance of migration and the average speed of macrophage migration in *tlr2*$^{-/-}$ was significantly higher than that in *tlr2*$^{+/+}$ (Fig. 4C and D). These results show that *tlr2* mutation affects macrophage migration after infection.

4 Tlr2-specific gene expression profiles after Mycobacterium marinum infection

To assess the general inflammation and specific immune responses in *tlr2* mutants, we studied mRNA expression levels of *il1b*, *tnfa*, *tnfb*, *irg1l* , and *tlr2* specific transcription factors *fosl1a* and *cebpb* in *tlr2*$^{-/-}$ and *tlr2*$^{+/-}$ larvae upon Mma20 infection by qPCR at 4dpi. Our results show that the expression levels of *il1b* and *tnf* in *tlr2*$^{-/-}$ larvae are significant reduced (Fig. 5A-C). The induction of *cebpb* and *fosl1a* was inhibited in *tlr2*$^{-/-}$ larvae (Fig. 5E, F). The expression of *irg1l* showed no significant difference between infection and non-infection in the *tlr2*$^{-/-}$ larvae (Fig. 5D). In previous work from our laboratory, Cxcl11-like chemokines were shown to play a crucial role in macrophage-mediated granuloma formation upon Mm infection (40). We therefore conducted qPCR to characterize the expression level of *cxcl11-like* genes, previously shown to be infection inducible (40), including *cxcl11aa* and *cxcl11ac* (Fig.5G and H). The expression level of gene *cxcl11aa* and *cxcl11ac* was significantly higher in *tlr2*$^{+/-}$ larvae than in *tlr2*$^{-/-}$ upon Mm infection (Fig. 5G, H). These results indicate that *tlr2* mutation results in a defective immune or inflammatory response to Mm infection.

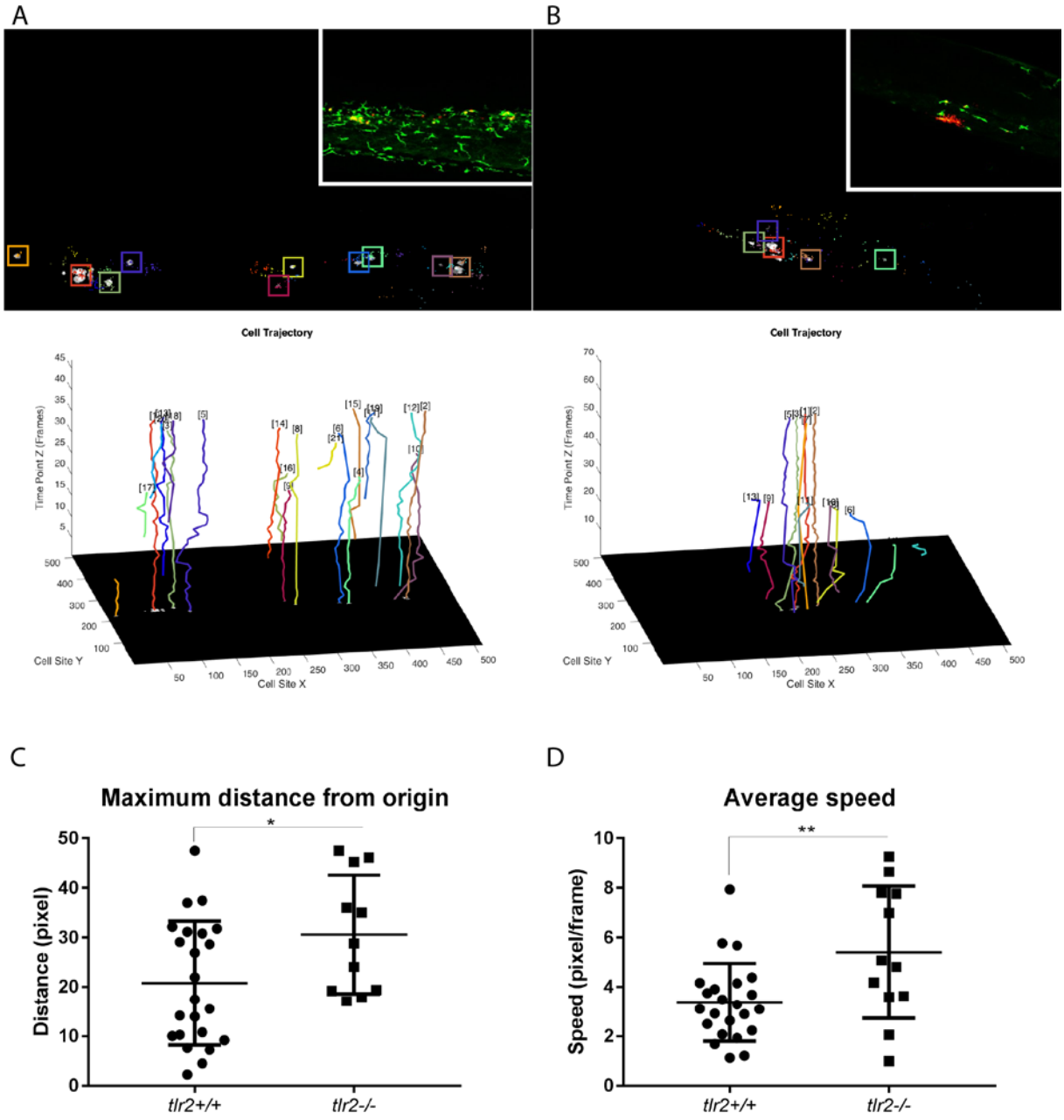

FIGURE 4 Cell tracking of macrophages in (A) *tlr2*$^{+/+}$/Tg(mpeg1:EGFP) and *tlr2*$^{-/-}$/Tg(mpeg1: EGFP) (B) larvae with Mm infection. Embryos were infected with ~150 CFU Mma20 strain at 28hpf, imaging was performed using a Nikon Eclipse Ti-E inverted microscope (40×) from 84hpi to 96hpi. There are 46 frames from *tlr2*$^{+/+}$ (A, bottom panel) and 70 from *tlr2*$^{-/-}$ (B, bottom-panel) that were analyzed, resulting in the reconstruction of 23 macrophage tracks in the wildtype and 12 in the mutant. The upper-panel in A and B shows the track of macrophages in 2D view, the insets are the original representative images and the bottom-panel shows that in 3D view. C and D, the maximum migration distance from the original site of infection in the first frame of the movie and the average speed of macrophages after phagocytosis of bacteria. The statistical significance of differences was determined by T-test. **p<0.01.

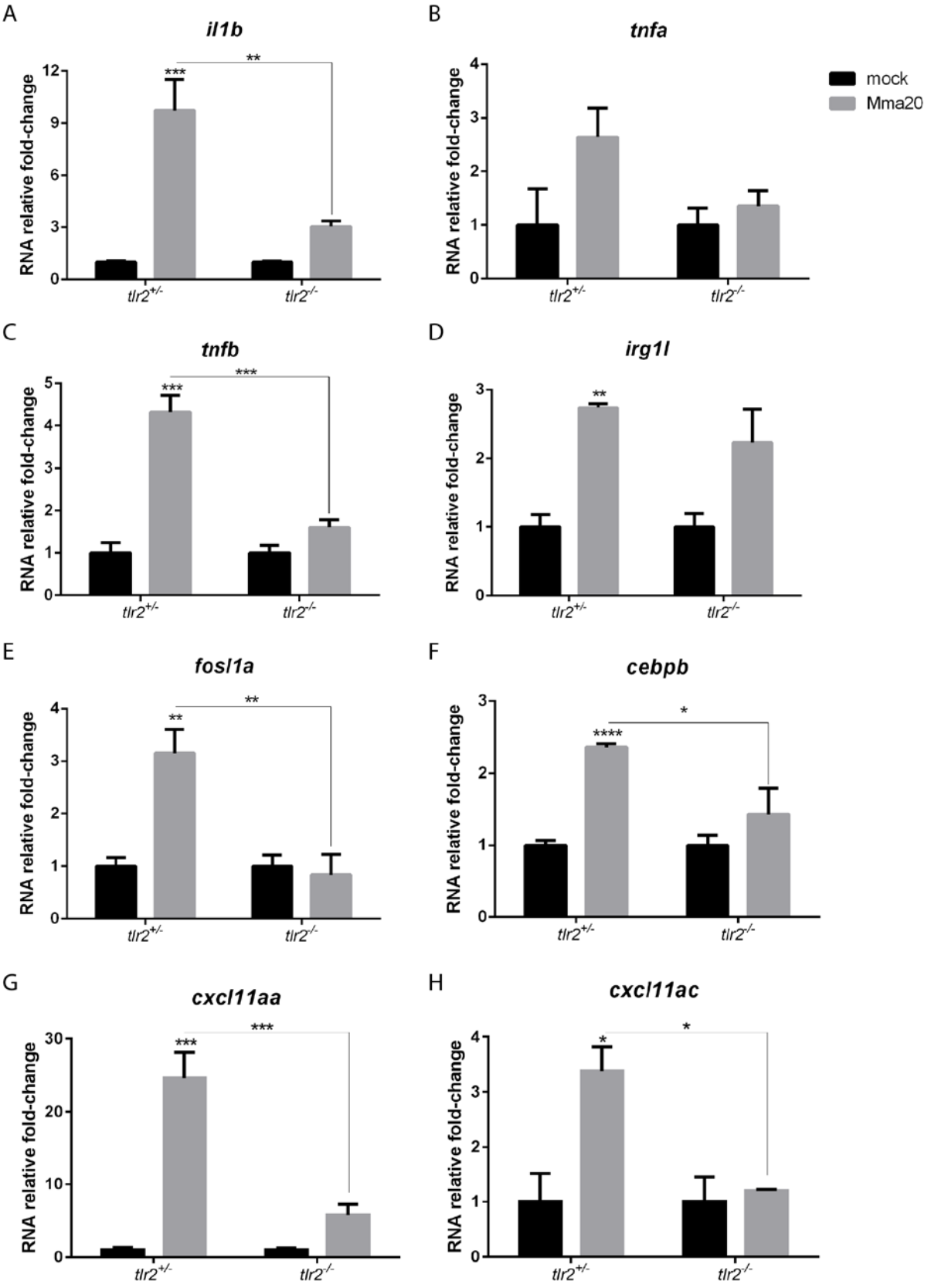

FIGURE 5 Immune genes expression in *tlr2*$^{+/-}$ and *tlr2*$^{-/-}$ fish lines infected with Mm. The expression levels of *il1b* (A), *tnfa* (B), *tnfb* (C), *irg1l* (D), *fosl1a* (E), *cebpb* (F), *cxcl11aa* (G) and *cxcl11ac* (H) were determined at 4dpi by qPCR. Data (mean ± SEM) are derived from at least three biological replicates

(n = 10 embryos per group) and expressed relative to their corresponding mock injection (PBS) control, which is set at 1. Statistical significance of differences was determined by two-way ANOVA with Sidak Multiple Comparison test as a post-hoc test. *p<0.05, **p<0.01, ****p<0.0001

To further study the function of *tlr2* in defense against mycobacterial infection, we performed RNAseq of *tlr2*$^{+/-}$ and *tlr2*$^{-/-}$ larvae infected with 150 CFU Mma20 at 4dpi and PBS-injected controls. We summarized the number of differentially expressed genes (DEGs) according to *p*-value (Fig. 6A, B). *tlr2*$^{+/-}$ induced an increased number of DEGs than *tlr2*$^{-/-}$ with Mma20 infection at any given *p*-value or false discovery rate less than 0.05, or any given fold-change with a *p*-value less than 0.05. The data also shows that most of the downregulation of genes caused by Mm infection in the control was abrogated by *tlr2* mutation (Fig. 6A, B). To further analyze these RNAseq data, we chose the genes with a threshold of a *p*-value less than 0.05 in *tlr2*$^{+/-}$ with Mma20 infection (1102 up- and 827 down-regulated genes, Fig. 6A). Then, for these 1102 and 827 genes, we calculated the ratio of the fold-change of *tlr2*$^{+/-}$ versus *tlr2*$^{-/-}$, after that, the genes with ratio greater than 2 or less than 0.5 were screened (Fig. 6C). As a result, 97 and 92 genes were selected as *tlr2* specific up- and down-regulated genes, respectively. We conducted gene ontology (GO) analysis (Fig. 6D, E) showing that genes that grouped into the immune system category are the most prominently deregulated (36%) in the whole *tlr2* up-regulated 97-gene set (Fig. 6D). Within this category we found genes involved in lysosome, chemotaxis, transcription regulation, diverse immunoglobulin domain-containing proteins (*dicps*) and other immune processes (Fig. 6D and 7A-E). For other process related categories, many up-regulated genes fell in the categories oxidation-reduction process, DNA repair, transcription regulation and apoptotic process regulation (Fig. S2). In the *tlr2* down-regulated 92-gene set, the immune related genes also were the largest portion (15%; Fig. 6E, 7F). The categories of non-immune related genes are listed in Fig. S3. Our results show that *tlr2* mutation leads to far less immune response genes that are upregulated upon Mm infection in zebrafish larvae.

To show the relationship between the genes that are differentially expressed, we constructed networks based on common expression targets in the 97 up- and 92 down-regulated genes (41). The involved networks with the up-regulated genes contain LIPC, NR0B2, HMGCR, ITLN1, FGF23, VDR, IRAK3 and TLR8 (Fig. S5). In the *tlr2*$^{+/-}$ control we observed positively regulated HMGCR, FGF23, VDR and TLR8, and negatively regulated NR0B2, whereas *tlr2*$^{-/-}$ shows an opposite regulation for most of them. However, LIPC and IRAK3, were positively regulated in both *tlr2*$^{+/-}$ and *tlr2*$^{-/-}$. For the 92 downregulated genes, a network containing NROB2 and MAFB was constructed (Fig. S6). NR0B2 and MAFB were positively regulated in *tlr2*$^{-/-}$ zebrafish and down regulated in *tlr2*$^{+/-}$.

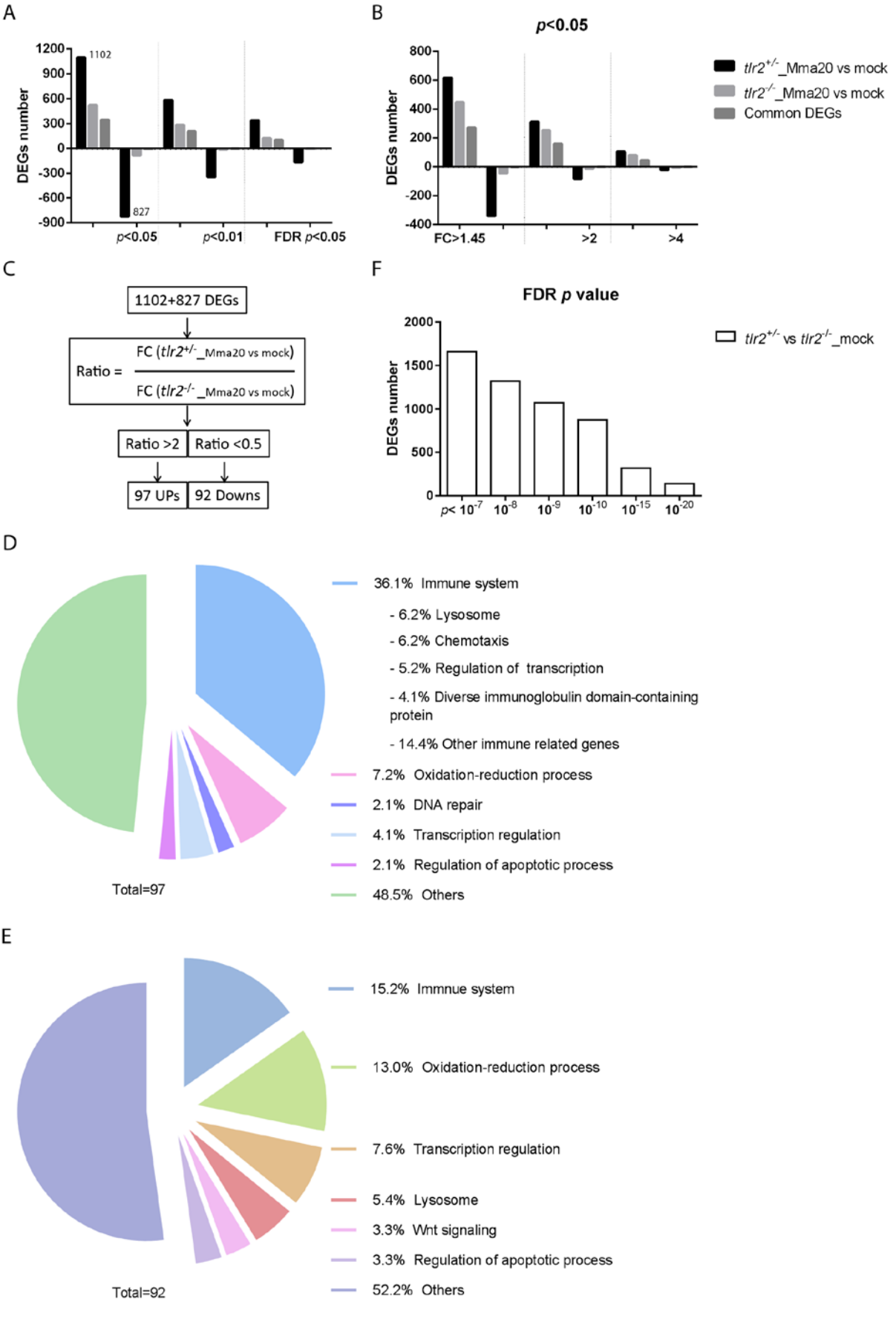
A
1200
900
600
300
0
-300
-600
-900
DEGs number
1102
827
p<0.05
p<0.01
FDR p<0.05
B
p<0.05
800
600
400
200
0
-200
-400
DEGs number
FC>1.45
>2
>4
tlr2+/-_Mma20 vs mock
tlr2-/-_Mma20 vs mock
Common DEGs
C
1102+827 DEGs
Ratio = FC (tlr2+/-_Mma20 vs mock) / FC (tlr2-/-_Mma20 vs mock)
Ratio >2
Ratio <0.5
97 UPs
92 Downs
F
FDR p value
2000
1500
1000
500
0
DEGs number
p< 10-7
10-8
10-9
10-10
10-15
10-20
tlr2+/- vs tlr2-/-_mock
D
36.1% Immune system
- 6.2% Lysosome
- 6.2% Chemotaxis
- 5.2% Regulation of transcription
- 4.1% Diverse immunoglobulin domain-containing protein
- 14.4% Other immune related genes
7.2% Oxidation-reduction process
2.1% DNA repair
4.1% Transcription regulation
2.1% Regulation of apoptotic process
48.5% Others
Total=97
E
15.2% Immnue system
13.0% Oxidation-reduction process
7.6% Transcription regulation
5.4% Lysosome
3.3% Wnt signaling
3.3% Regulation of apoptotic process
52.2% Others
Total=92

FIGURE 6 Overview of RNAseq results. A, B the number of DEGs of *tlr2*$^{+/-}$ and *tlr2*$^{-/-}$ strains infected with Mm compared to the control at different p-value and fold change. C, the work flow of screening genes of which the regulation by infection is dependent on *tlr2*. D, GO analysis of the 97 upregulated genes. E, GO analysis of the 92 down-regulated genes. FC, fold-change. F, the DEGs number of *tlr2*$^{-/-}$ versus *tlr2*$^{+/-}$ at different FDR p-value in the uninfected mock-injected condition.

5 Enrichment analysis of Tlr2 specific genes after Mycobacterium marinum infection

To link our data to gene sets defined based on prior biological knowledge (including GO), we conducted Gene-Set Enrichment Analysis (GSEA) of the genes of which the ratios of Mm infection and control in *tlr2*$^{+/-}$ or *tlr2*$^{-/-}$ zebrafish are significantly different without considering fold-changes. This method derives its power by focusing on gene sets, that is, groups of genes that share common biological function, chromosomal location, or regulation (42). Because the numbers of predicted gene sets were too large for our analysis method (more than 1,000 with $p < 0.05$), we focused on the gene sets related to metabolic, immunological and inflammation pathways. The GSEA predicted 61 pathways in *tlr2*$^{+/-}$ and 67 pathways in *tlr2*$^{-/-}$ zebrafish responsive to Mma20 infection (Supplementary Table 2A and 2B). Interestingly, most of these pathways are common in *tlr2*$^{+/-}$ and *tlr2*$^{-/-}$, however some of them are detected as being anti-correlated in regulation, including some pathways underlying natural killer cell functions and omega-6-fatty acid metabolism (Supplementary Table 2C). We also performed Sub-Network Enrichment Analysis (SNEA) (43) to identify possible key genes that are responsible for the difference in response of the *tlr2*+/- and *tlr2*-/- group to Mm infection ($p < 0.05$). SNEA predicted 565 and 503 pathways for *tlr2*$^{+/-}$ and *tlr2*$^{-/-}$ zebrafish that are linked to the response to infection, respectively (Supplementary Table 2D and 2E), and 264 and 202 of them are specific for the response in *tlr2*$^{+/-}$ and *tlr2*$^{-/-}$ fish, respectively (Supplementary Table 2F). Since the RNAseq was conducted with the total RNA from whole body of zebrafish, it is not possible to define which pathways are involved in macrophage functions related to *tlr2* expression. Therefore, we downloaded DNA microarray data (GDS4781) of human macrophages transfected with *M.tuberculosis* from Gene Expression Omnibus (44). SNEA analysis of human macrophages shows that 659 pathways are linked to Mtb infection (Supplementary Table 2G). By comparing human SNEA result to *tlr2*$^{+/-}$ specific pathways in zebrafish, 56 pathways were defined as *tlr2*$^{+/-}$-specific (Supplementary Table 2H). Of these, the pathway of TLR8, which has the lowest *p*-value in both zebrafish and human enrichment, and its network is depicted as example in Figure S7. In general these analysis show that the *tlr2*-/- mutant has a very different immune response of which many pathways that are linked to human tuberculosis are differently responding.

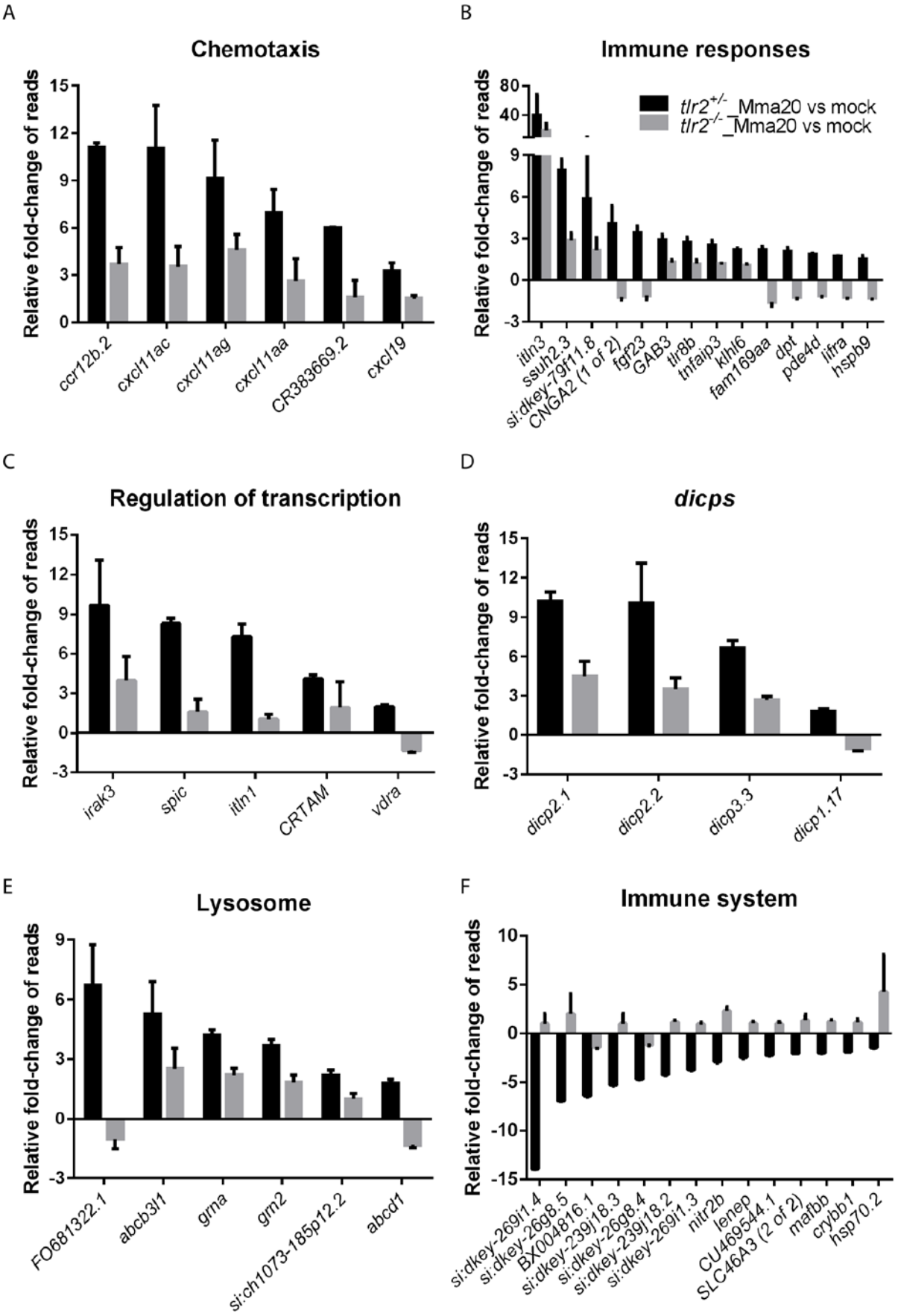
A
Chemotaxis
Relative fold-change of reads
15
12
9
6
3
0
ccr12b.2
cxcl11ac
cxcl11ag
cxcl11aa
CR383669.2
cxcl19
B
Immune responses
Relative fold-change of reads
80
40
9
6
3
0
-3
tlr2+/-_Mma20 vs mock
tlr2-/-_Mma20 vs mock
itln3
ssuh2.3
si:dkey-79f11.8
CNGA2 (1 of 2)
fgf23
GAB3
tlr8b
tnfaip3
klhl6
fam169aa
dpt
pde4d
lifra
hspb9
C
Regulation of transcription
Relative fold-change of reads
15
12
9
6
3
0
-3
irak3
spic
itln1
CRTAM
vdra
D
dicps
Relative fold-change of reads
15
12
9
6
3
0
-3
dicp2.1
dicp2.2
dicp3.3
dicp1.17
E
Lysosome
Relative fold-change of reads
9
6
3
0
-3
FO681322.1
abcb3l1
grna
gm2
si:ch1073-185p12.2
abcd1
F
Immune system
Relative fold-change of reads
10
5
0
-5
-10
-15
si:dkey-269i1.4
si:dkey-26g8.5
BX004816.1
si:dkey-239j18.3
si:dkey-26g8.4
si:dkey-239j18.2
si:dkey-269i1.3
nitr2b
lenep
CU469544.1
SLC46A3 (2 of 2)
mafbb
crybb1
hsp70.2

FIGURE 7 Overview of fold changes of representative genes selected from the gene categories resulting from GO-term analysis. A-E: *tlr2*-dependent genes with upregulation corresponding to Fig. 6D. F: *tlr2* specific genes with downregulation corresponding to Fig. 6E.

6 Comparison of gene expression profiles of tlr2 homozygote mutant with heterozygote

We compared basal levels of gene expression in the absence of infection between *tlr2* homozygote and heterozygote mutants. These results show that there is a large group of genes that are expressed differently even at extremely stringent *p*-values (Fig. 6F). To get possible indications whether the basal expression level differences of genes might be relevant to the Tlr2 pathway we performed GO analysis of a set of genes that were expressed differently with the very low FDR *p*-value of 10^{-10} (Supplementary Table 3). Our results show that there is a large enrichment of genes belonging to the GO terms related to neural development. In addition, we have also analyzed differences with a fold change criterion of two and FDR *p*-value of 0.05 (Supplementary Table 4). These analyses indicated that under the GO category transcription factor genes only two categories of genes including the c-Maf transcription factor (Supplementary Table 4) were present. Finally, the RNAseq analysis showed that the *mpeg1.1* gene is expressed approximately 2 fold higher in the $tlr2^{+/-}$ control than in the $tlr2^{-/-}$ mutant (Supplementary Table 5), consistent with the observation of reduced macrophage numbers in the *tlr2* mutant (Fig. 1D, Fig. S4).

Discussion

Our study created a systemic *tlr2* deficiency-Mm infection model in zebrafish larvae to study Tlr2 function in the innate immune system during mycobacterial infection. In alternative model systems for human tuberculosis that use mice, it is difficult to perform systemic studies at the genomic and microscopic level. As a result all the studies performed in mice have been carried out with isolated tissues. Furthermore, there are only a few RNA sequencing results of TB studies in rodents (45, 46), human serum (47), human pulmonary epithelial cells (48), and bovine systems (49-52). In zebrafish larvae it is possible to combine the advantage of studying the entire system using transcriptomics and zooming in on mechanisms of the infection process using fluorescence microscopy. Furthermore, as in rodents, the zebrafish offers excellent genetics tools for obtaining mutants such as the *tlr2* mutant described in this study. The deep sequencing data provided the chance to study the whole transcriptome profile in our mycobacterial infection model and revealed the panorama of *tlr2* signaling-related gene expression. Importantly, taking advantage of this model we

were able to document in vivo the impact that lack of Tlr2 has in the interaction between infecting bacteria and macrophages.

We have shown in this study that *tlr2* mutant leads to increased mycobacterial infection in zebrafish larvae. This result is similar to results from studies in Tlr2 knockout mice, where infection with Mtb resulted in increased lung infection and highly increased mortality (19, 20). However, in these works a role of Tlr2 could only be shown at high doses of infection, whereas at a low dose no effect was observed of the mutation (19,20). In the study of Drennan et al (20) it was shown that infection of the Tlr2 mutant leads to a decrease of the number of granulomas formed in the lungs. This is in line with our results where we found that the clustering of macrophages in pre-granuloma structures was strongly decreased. In addition, the study in mice showed that in the Tlr2 mutant there was an increased T cell response that was ineffective in combating progression of infection. Since in our larval system there is no adaptive response present, we can therefore not address this point and need to perform studies in adults in future studies. However, it is important that we can conclude that the role of Tlr2 in granuloma formation is not dependent on T cell activity. Furthermore, we can now also conclude that the function of Tlr2 in granuloma formation is at the very early steps of the infection process. We also observed that Tlr2 mutants in zebrafish larvae result in a decreased number of macrophages even in the absence of infection and this might attributed to the emergence and differentiation of hematopoietic stem and progenitor cells. He et al. (53) demonstrated that Tlr signaling-dependent inflammatory signaling is necessary and sufficient for hematopoietic stem and progenitor cells emergence in zebrafish, in the absence of infection or pathological inflammation. TLR2 agonist Pam3CSK4 was also shown to induce a large proportion of hematopoietic stem cells to express markers of the myelomonocytic lineage (54). On the other hand, Herman et al. (55) 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, but a loss of hematopoietic stem cell self-renewal capacity. We also observed that the Tlr2 mutation in zebrafish larvae induces an increased speed and the maximal distance of macrophage migration in the presence of infection (Fig. 4). These results might indicate that macrophages in the mutant might be less differentiated than those in the wildtype. These factors could contribute to the proliferation of infection.

Since the molecular mechanisms that underlie the role of TLR2 in the control of infection are still unknown we have performed RNAseq of entire larvae with our infection system. We also analyzed the basic expression levels of the mutant versus the control and noted a strong difference of the expression of a large group of genes in the absence of infection. Since little is known about the variations in basal gene expression levels in larvae from different crosses it is hard to speculate whether these differences are related to the Tlr2 mutation. Although we analyzed pools of 10 larvae there are

undoubtedly differences in development of individual larvae that are not filtered away by pooling and that will provide differences in organ development and metabolism that will lead to large variations in gene expression levels. In addition, also differences in the genetic makeup of the parents used for obtaining the batches of larvae are unknown and therefore it will not be possible to obtain solid conclusions. However, the largest category of genes that was significantly affected, namely neurological system process, might still be linked with a function of Tlr2. Many recent studies show that a mutation in Tlr2 in mice resulted in effects of neuronal development and responses to injury (56, 57). Some of these studies show a connection of Tlr2 in neuronal defects to be related to IL10 and possibly autophagy (58, 59). When focusing on the possible signaling pathways that could be involved we observed that there was a significant effects in the GO category of transcriptions factor, namely the c-Maf factors that totals up to 546 representatives that were affected (Supplementary Table 4). As members of Maf family transcription factors, c-Maf and Mafb are specifically expressed in monocyte and macrophage lineages (60, 61), in addition, c-Maf is also expressed in T helper cells (61). c-Maf was also reported to directly regulate IL-10 expression induced by LPS in macrophages (62). Double deficiency of Mafb/c-Maf promotes self-renewal of differentiated macrophages (63), which might indicate a link to the effect of the Tlr2 mutation on the number of macrophages in the absence of infection.

An analysis of the difference of induction and repression of genes during infection showed that that is a very pronounced effect of mutation of the Tlr2 gene. With respect to the number of genes affected, The strongest effect was observed on the genes that are down regulated during infection, since this category was strongly affected in the Tlr2 mutant (Fig. 6A, B). This category of genes includes several genes that are related to innate immune response and also many unknown genes. This indicates that Tlr2 has an important function in anti-inflammatory responses. This is in line with previous reports of studies in mice that showed a strongly decreased anti-inflammatory response in Tlr2 knockouts (64, 65). In a further analysis on the quantitative effects on the differences in expression levels of the genes that are affected in both the up regulated as down regulated groups we selected a group of genes that were most significantly affected for Gene Ontology analysis (Fig. 6D, E). This GO analysis showed many groups to be affected with the immune response as the biggest group. We also performed GSEA and SNEA analyses showing that the *tlr2*-/- mutant has a very different immune response than the heterozygote control line and that many signaling pathways that are linked to responses to human tuberculosis infection are differently responding. Most significantly, activation of the Tlr8 pathway was strongly affected (Fig. S7). This suggests that the Tlr2 signaling is strongly connected with Tlr8 function. TLR8 mutations (polymorphisms) increase susceptibility to mycobacteria (66, 67). These predictions from integrated transcriptome analysis are very valuable, and we would like to study them in our future work. In addition to

known immune genes we want to discuss three other very interesting categories of genes.

The vitamin D receptor pathway genes that are normally up-regulated during infection in zebrafish larvae were down regulated in the Tlr2 mutant. Furthermore, pathway analysis (Fig. S5 and S7) also implicated the expression of the Tlr8 pathway connected to vitamin D signaling as being strongly affected in the Tlr2 mutant. Vitamin D has been shown to be an important regulatory factor during tuberculosis (68) and has been linked previously to TLR2 function in studies in cell cultures (69).

Tlr2 showed to be essential for the up-regulation of a group of genes that encode the Diverse Immunoglobulin Domain-Containing Proteins (DICPS). This group is as novel multigene family encoding diversified immune receptors. Haire et al (70) reported that recombinant DICP Ig domains bind lipids and lipid extracts of different bacteria, including Mtb and Mm, a property shared by mammalian CD300 and TREM family members. In the down-regulated set also several DICP proteins appear to be dependent on Tlr2, such as *dicp1.17*, *dicp3.3*, that are linked to the GO term insulin-like growth factor binding. These correlations might relate to functions of TLR2 in other processes such as the control of diabetes type II by gut microbiota. However, the DICP gene family lacks easily recognizable genetic orthologues in mammals, making a translation to a function in mammalian tuberculosis and other diseases currently not yet possible (71).

Another highly relevant large category of genes of which the induction or repression during infection is dependent on Tlr2 includes the chemokines. In a previous study of our laboratory, Torraca et al. (40) demonstrated the function of the CXCR3-CXCL11 axis in macrophage recruitment and showed that disruption of this axis by *cxcr3.2*-mutation increases the resistance to mycobacterial infection. They also showed that Cxcr3.2 deficiency limited the macrophage-mediated dissemination of mycobacteria and attenuated the formation of granulomatous lesions and led to a reduction in the total bacterial burden (40). In our study, the *tlr2* mutant shows a significantly lower expression of *cxcl11aa* and *cxcl11ac* during Mm infection (Fig. 5). Considering the large number of other chemokines that are controlled by Tlr2 during infection, it is clear that the integrative network of connections cannot yet be understood from these expression studies and need more detailed functional analyses, e.g. by combinations of different mutations or directed studies on responses to chemokines as shown by Torraca et al. However, we can state that the phenotypes of the *tlr2* mutant, at the microscopic level, and the level of transcriptional control such as the mentioned effects on regulation of MafB/c-Maf and chemokines shows a clear connection with macrophage migration. This does not exclude that Tlr2 has many other functions in macrophage behavior during infection such as phagocytosis. For instance, Blander et al. (72) and Rahman et al. (73) showed that phagocytosis of bacteria and phagosome

maturation are impaired in the absence of TLR signaling. Therefore, the large number of unannotated genes of which the expression during infection is dependent on Tlr2 is also worth studying in more detail in future studies.

In conclusion, our study shows that TLR2, as a part of innate immunity, plays an important role in controlling mycobacterial infection as observed on the transcriptome and infection level. This may be mediated by several strategies, including macrophage migration and clustering, and other anti-mycobacterial effect like vitamin D signaling. The anti-inflammatory function of TLR2 as indicated by our study could be mediated by IL10. The gene *il10* is also controlled by other bacteria, yeasts or viruses to mediate immune evasion through TLR2 and therefore seems to have an opposing function as to what we report for mycobacterial infection (25, 26, 74). The TLR2 mutant is therefore highly suitable for further studies using the published infection models for other microbes and viruses in zebrafish larvae. This will make it possible to compare the possibly different roles of TLR2 in interactions with various microbes in one infection model.

Materials and methods

Zebrafish husbandry

The *tlr2*^{sa19423} mutant line (ENU-mutagenized) was obtained from the Sanger Institute Zebrafish Mutation Resource (Hinxton, Cambridge, UK) and shipped by the zebrafish resource Center of the Karlsruhe Institute of Technology. The mutant allele was identified by sequencing. Heterozygous carriers of the mutation were outcrossed twice against wildtype (*AB* strain), and were subsequently incrossed twice. Heterozygous fish of the resulting family were used to produce embryos. Homozygous mutants were outcrossed to the *Tg*(*mpeg1:EGFP*)^{gl22} transgenic line, and the offspring with GFP florescence were subsequently incrossed to produce *tlr2*$^{-/-}$ / *Tg*(*mpeg1:EGFP*) line.

All zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). Larvae were raised in egg water (60 g/ml Instant Ocean sea salts) at 28.5 °C. For the duration of bacterial injections, larvae were kept under anesthesia in egg water containing 0.02% buffered 3-aminobenzoic acid ethyl ester (Tricaine, Sigma-Aldrich, the Netherlands). The culture of zebrafish with mutations in immune genes was approved by the local animal welfare committee (DEC) of the University of Leiden (protocol 14198). All protocols adhered to the international guidelines specified by the EU Animal Protection Directive 2010/63/EU.

Bacterial strain preparation

The bacterial strain, *Mycobacterium marinum* m20 (Mma20) expressing mCherry fluorescent protein (75), was used in this study. For the infection to zebrafish larvae, the bacteria were prepared as previously described (76). The infection inoculum was prepared in 2% polyvinylpyrrolidone40 solution (CalBiochem, the Netherlands), and 150 colony-forming units (CFU) of bacteria were injected into the blood stream at 28 hours post fertilization (hpf) as previously described (77).

Ligands injection

Purified Pam3CSK4 (InvivoGen, France) and flagellin from *S. typhimurium* (Flagellin FliC VacciGrade™, Invitrogen, France) were diluted in 1 mg/ml and 100 µg/ml in sterile water, respectively. For injection, 1 nl of the ligand solutions were injected into the blood stream at 28hpf. Sterile water was injected as a control experiment. Injections were performed using a FemtoJet microinjector (Eppendorf, the Netherlands) equipped with a capillary glass needle.

Morpholino injection

Morpholino oligonucleotides (Gene Tools, Philomath, US) were diluted to desired concentrations in 1× Danieau's buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES[pH 7.6]) containing 1× phenol red (Sigma-Aldrich, the Netherlands). For knockdown experiments, *tlr2* ATG-morpholino (*tlr2* mo, 5'-AGTCATTG TTCCTACGAGTCTCATC-3') was injected with the optimal concentration at 0.5mM and 1nl volume per embryo at 1-2 cell stages. Control larvae were injected with the standard control morpholino (Ctrl mo, 5'-CCTCTTACCTCAGTTACAATTTA TA-3').

Immunohistochemistry

Larvae were fixed in 4% paraformaldehyde in PBS overnight at 4°C. Myeloperoxidase (Mpx) activity was labeled using TSA-Plus Systems (Perkin Elmer, Waltham, MA, US) as previously described (78).

Imaging and quantification of bacterial loads

Pools of 20 larvae were collected at 3- and 4-day post infection (dpi) and imaged by using the Leica MZ16FA Fluorescence Stereo Microscope (Leica Microsystems, info) equipped with the Leica DC500 camera (Leica Microsystems). Bacterial loads were analyzed using dedicated pixel counting software as previously described (79). Experiments were performed in triplicate.

Confocal microscopy imaging

Larvae (4 or 5 dpf) were embedded in 1% low melting point agarose (Sigma Aldrich), and image acquisition was performed by using a Leica TCS SPE confocal microscope

(Leica Microsystems) or a Zeiss LSM exciter on an Axio Observer confocal microscope (info) with 10 or 63 times objectives. Acquisition settings and area of imaging (in the caudal vein region) were kept the same across the groups for quantification of the bacterial burden.

Macrophage tracking

To visualize the granuloma formation and the leukocyte phenotype, *tlr2*$^{-/-}$ zebrafish were crossed with macrophage-specific reporter line *Tg*(*mpeg1:EGFP*), leading to *tlr2*$^{+/+}$/*Tg*(*mpeg1:EGFP*) and *tlr2*$^{-/-}$*Tg*(*mpeg1:EGFP*) fish lines. *tlr2*$^{+/+}$ and *tlr2*$^{-/-}$ - *Tg(mpeg1:EGFP)* embryos were injected with 150 CFU Mma20 at 1dpf. They were anesthetized and mounted in 1% low-melting agarose and subsequently imaged using a Nikon Eclipse Ti-E inverted microscope (40×) as described previously (80) from 84 hpi to 96 hpi. Images were taken every 6.5 min, extracted, and converted to movies using NIS-Elements AR software. ImageJ (NIH, Bethesda, ML, USA) was used to align all the frames. CellTracker (ver. 1.1, Copyright (©) 2015 Peter Horvath and Filippo Piccinini) was employed to analyze the moving tracks of infected macrophages.

RNA isolation, cDNA synthesis and qPCR

Total RNAs were extracted using TRIzol Reagent (Life Technologies) and purified using RNeasy MinElute Cleanup Kit (Qiagen, the Netherlands). The concentration and quality of RNAs were evaluated by NanoDrop 2000 (Thermo Scientific, the Netherlands). cDNAs were synthesized from 1 µg total RNAs and qPCR were performed by using the iScript™ cDNA Synthesis Kit (BioRad, the Netherlands) and iQ™ SYBR Green Supermix (BioRad) and normalized against the expression of *ppial* as a housekeeping gene (81). Results were analyzed using the ΔΔCt method (82). Primer sequences are described in Supplementary Table 1.

Deep sequencing and data analysis

Triplicates of 10 larvae of *tlr2*$^{+/-}$ and *tlr2*$^{-/-}$ with PBS (as control) or Mma20 injection, were homogenized in 300ul of TRIzol reagent, and total RNAs were purified as described above. RNAseq was performed using Illumina Hi-Seq 2500 as previously described (83). The RNAseq data were mapped on zebrafish genome (GRCz10 version) and tag counts were performed by Bowtie 2 using GeneTiles software (http://www.genetiles. com) (84, 85). Then, we performed normalization and gene expression analysis using the R package and DESeq2 (86). After statistical tests, we performed further bioinformatics analyses Gene-Set Enrichment Analysis (42), Sub-Network Enrichment Analysis (43) and Pathway Enrichment Analysis (87). For creating gene networks based on common regulatory targets, we used Pathway Studio 9.0 (Elsevier, Amsterdam, the Netherlands) as previously described (41).

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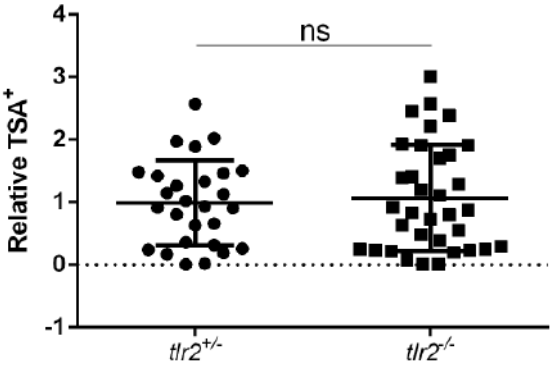
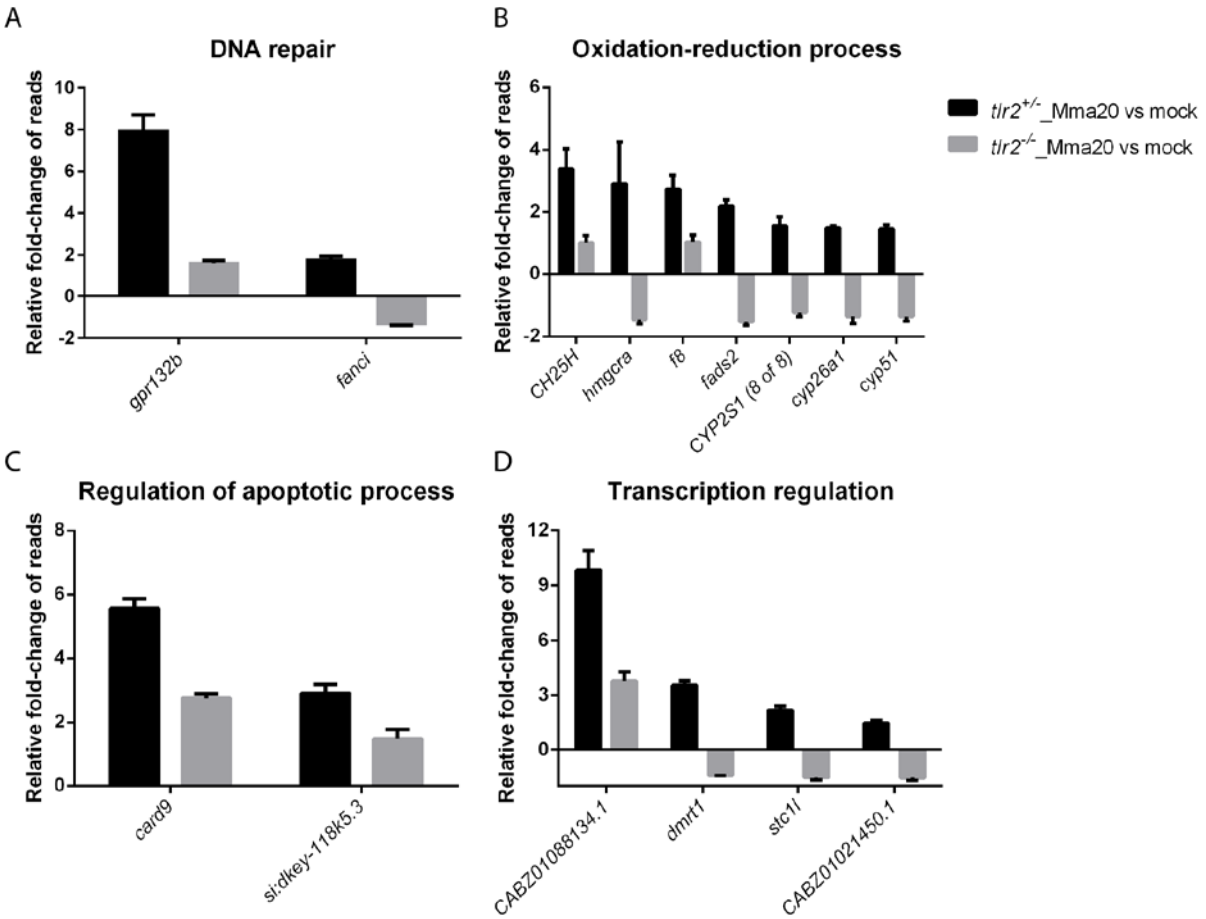
Supplementary data

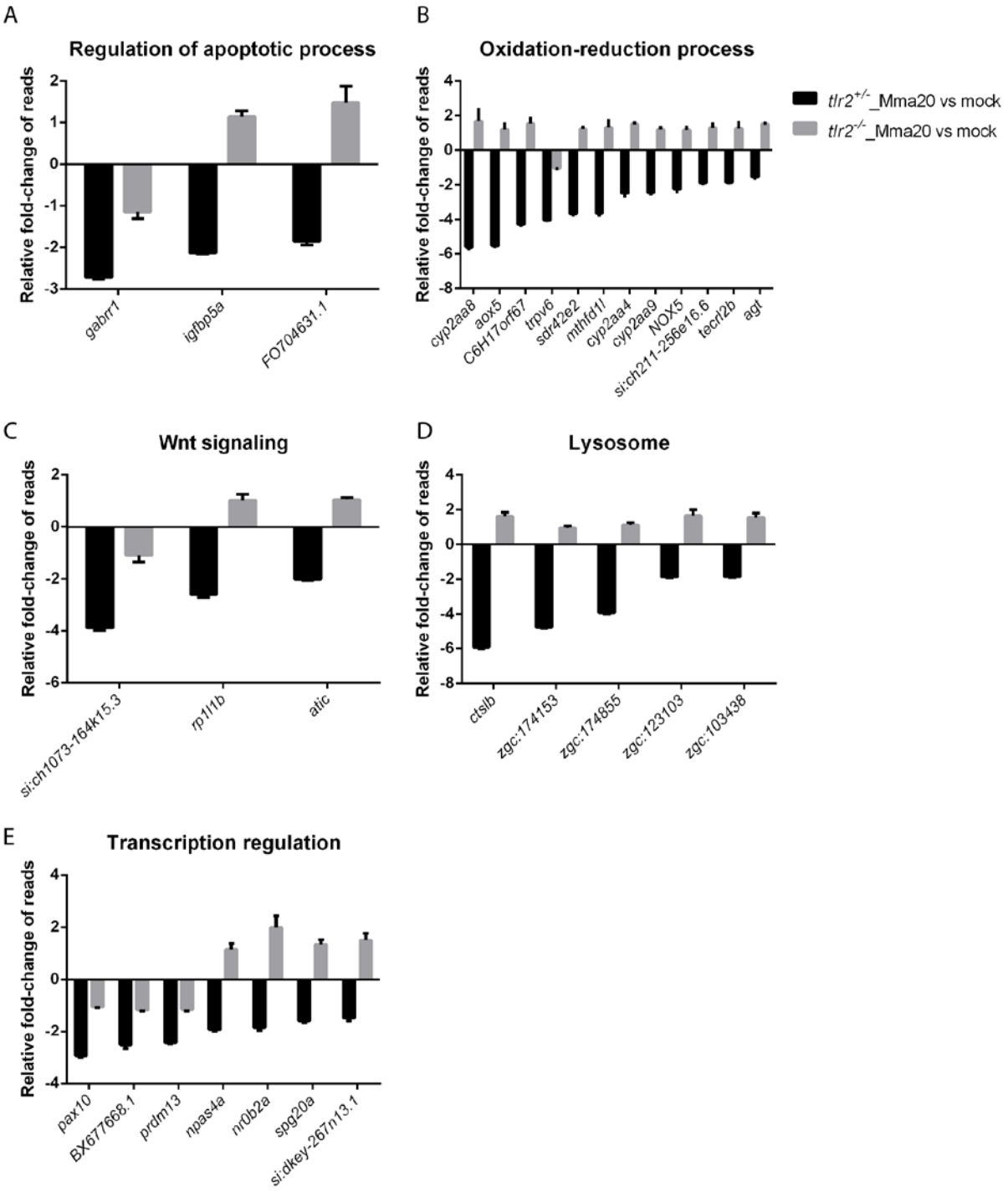
Supplementary figure 1 Relative pixel count analysis for TSA staining of neutrophils in 2dpf *tlr2*$^{+/-}$ and *tlr2*$^{-/-}$ embryos.



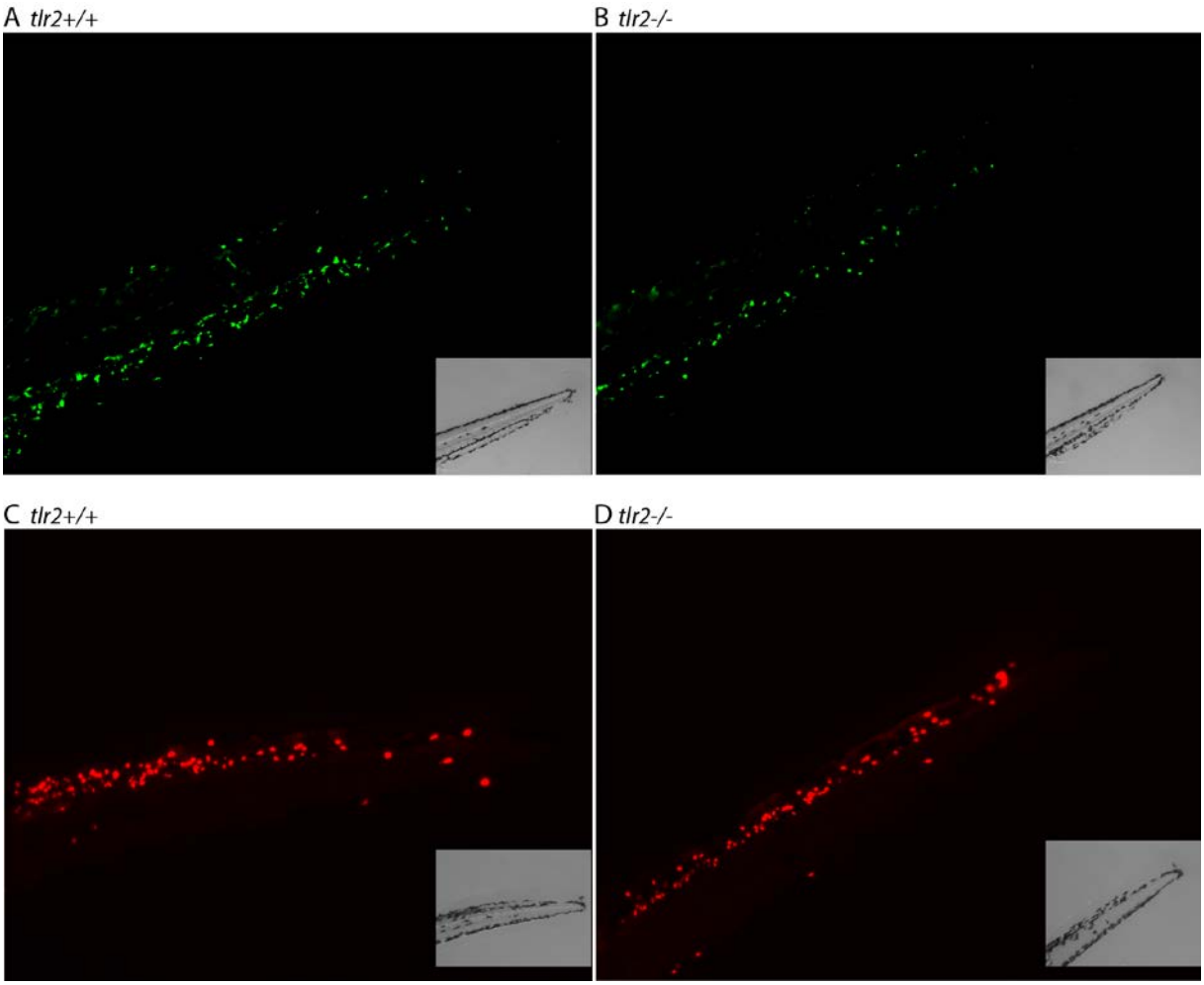
Supplementary figure 2 *tlr2*-dependent up regulation of other process related specific genes.



Supplementary figure 3 *tlr2*-dependent down regulation of other process related specific genes.

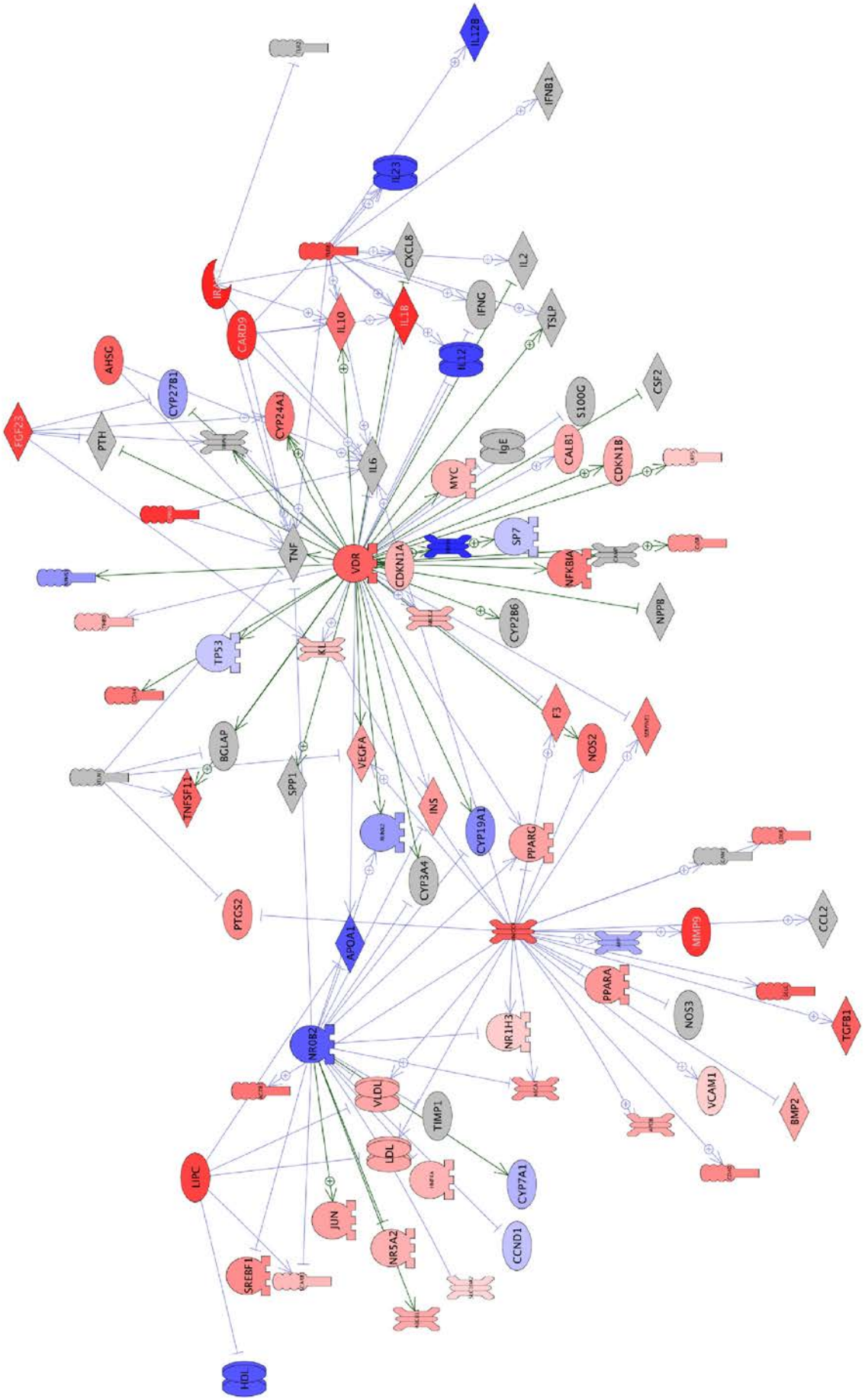


Supplementary figure 4 Representative images of Fig. 1D and 1E. Green signal represents macrophages, red signal represents neutrophils.



Supplementary figure 5 Sub-network enrichment analysis. The networks of common targets of the 97 up regulated genes (Fig.6D) in *tlr2*$^{+/-}$ (A) and *tlr2*$^{-/-}$ (B) with Mma20 infection. Red represents up regulation, blue represents down regulation and grey represents genes for which no expression was detected.

A


IL12B
IFNB1
IL23
CXCL8
IL2
IRF
IL10
IL18
IFNG
TSLP
CARD9
IL12
AHSG
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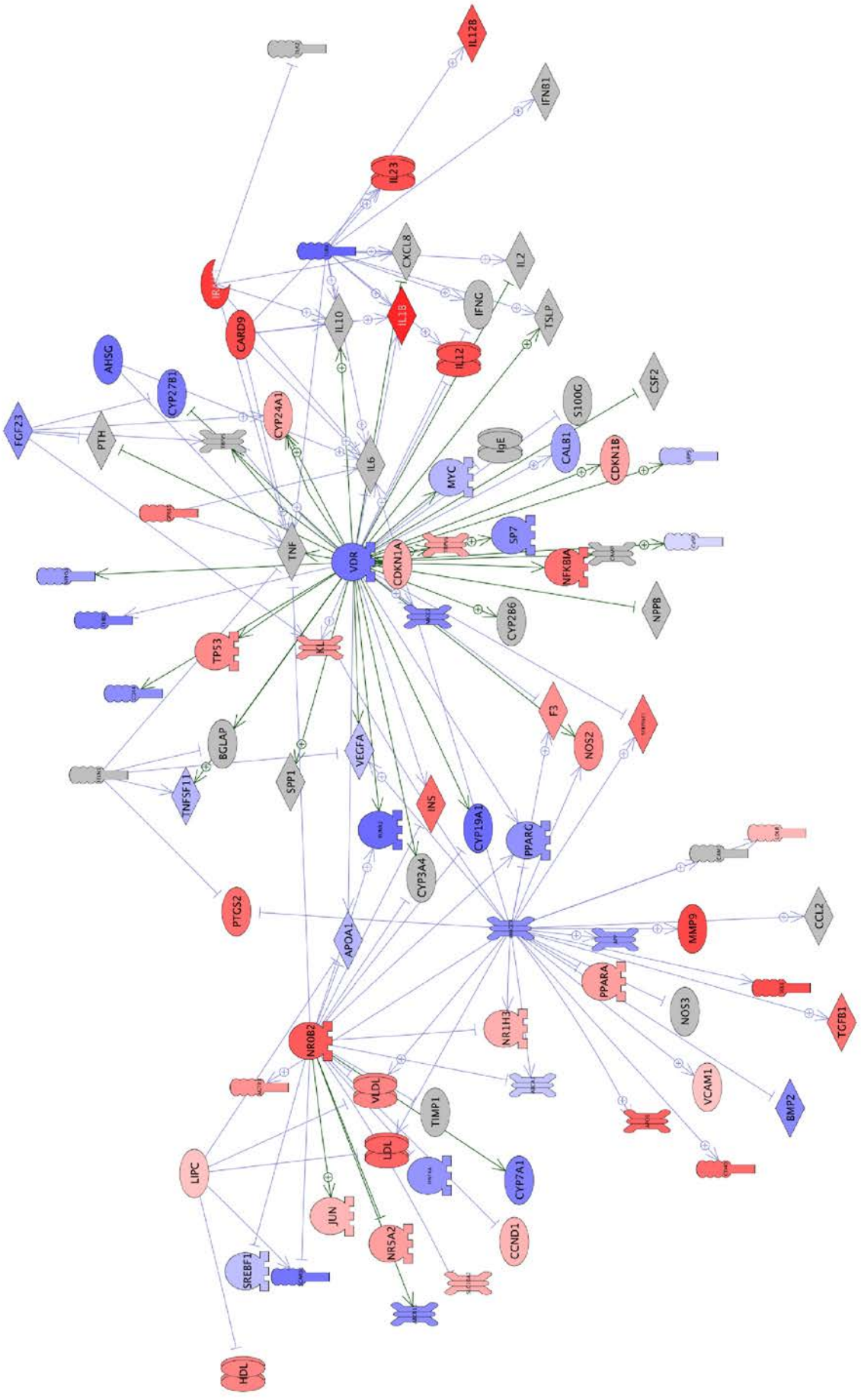
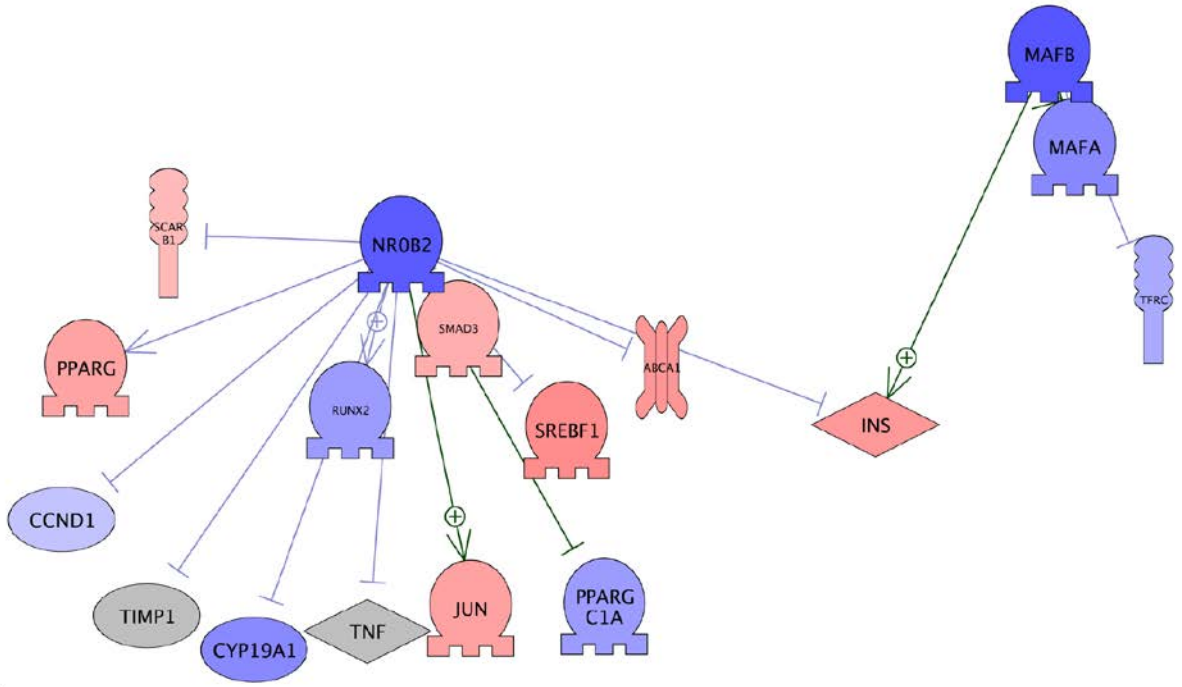
CSF2
S100G
FGF23
PTH
CYP24A1
IgE
CALB1
CDKN1B
IL6
MYC
TNF
VDR
CDKN1A
SP7
NFKBIA
NPPB
CYP2B6
TP53
KL
F3
NOS2
BGLAP
VEGFA
SPP1
TNFSF11
INS
CYP19A1
PPARG
CYP3A4
PTGS2
APOA1
MMP9
CCL2
PPARA
NOS3
TGFB1
NR1H3
NR0B2
VLDL
VCAM1
BMP2
TIMP1
LDL
LIPC
CYP7A1
JUN
NR5A2
CCND1
SREBF1
HDL

B

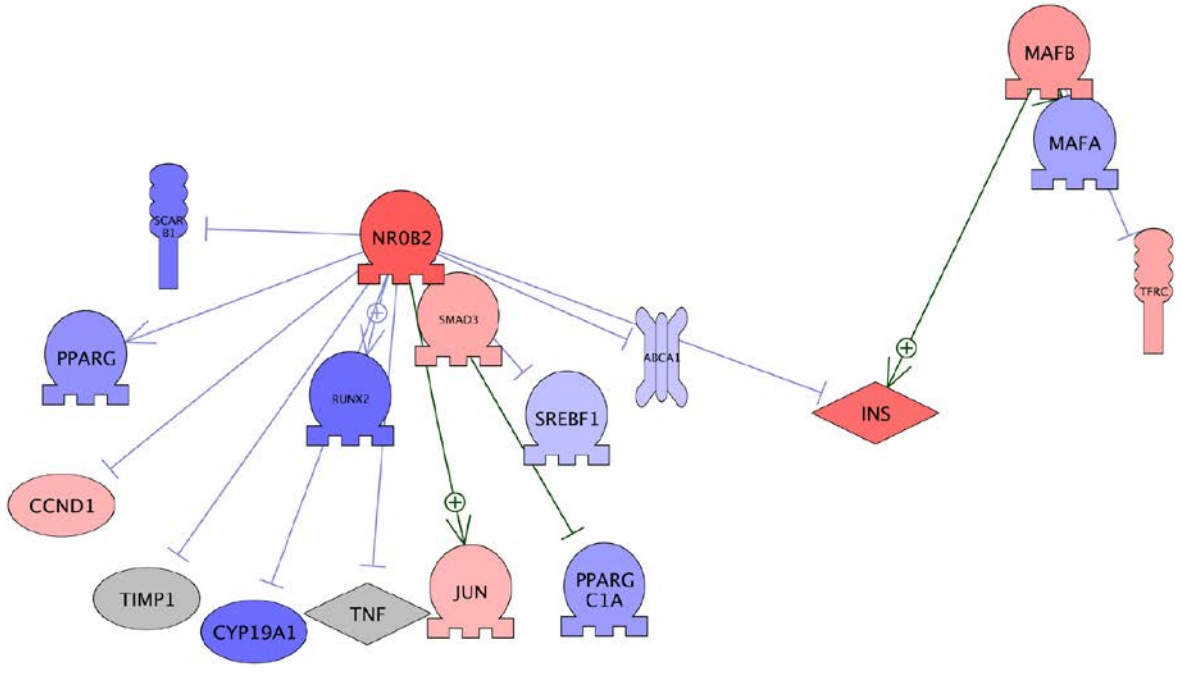

IL12B
IFNB1
IL23
CXCL8
IL2
IFNG
TSLP
IL1B
IL12
IL10
CARD9
AHSG
CYP27B1
FGF23
PTH
CYP24A1
IL6
IgE
MYC
S100G
CAL81
CSF2
CDKN1B
TNF
VDR
CDKN1A
SP7
NFKBIA
NPPB
CYP2B6
TP53
KL
BGLAP
TNFSF11
SPP1
VEGFA
F3
NOS2
INS
CYP19A1
PPARG
CYP3A4
PTGS2
APOA1
MMP9
CCL2
PPARA
NOS3
TGFB1
NR0B2
NR1H3
VLDL
TIMP1
VCAM1
BMP2
LDL
LIPC
CYP7A1
JUN
NR5A2
CCND1
SREBF1
HDL

Supplementary figure 6 Sub-network enrichment analysis. The networks of the common targets of the 92 down regulated genes (Fig. 6E) in *tlr2*$^{+/-}$ (A) and *tlr2*$^{-/-}$ (B) with Mma20 infection. Red represents up-regulation, blue represents down-regulation and grey represents genes for which no expression was detected.

A

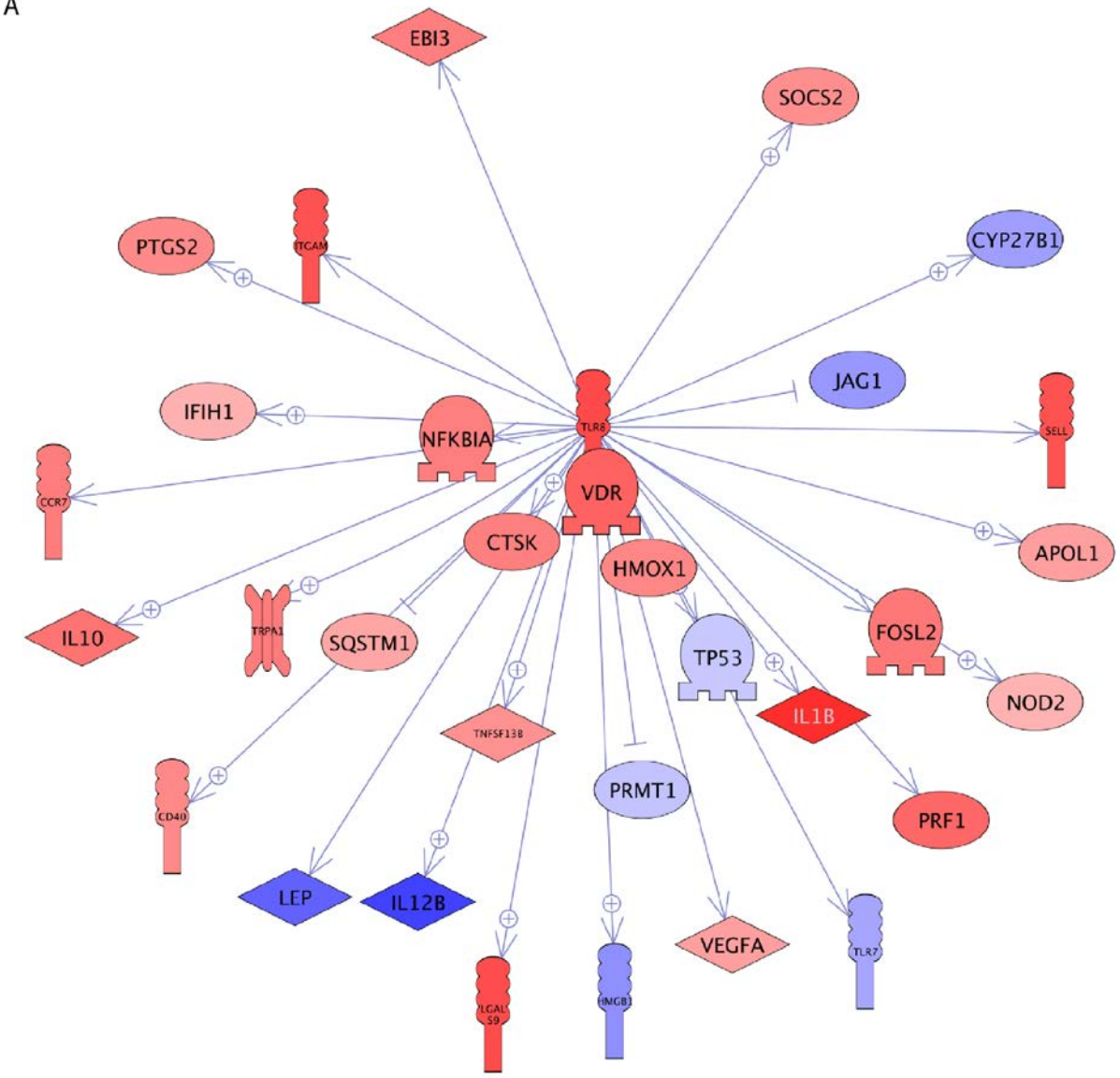


B

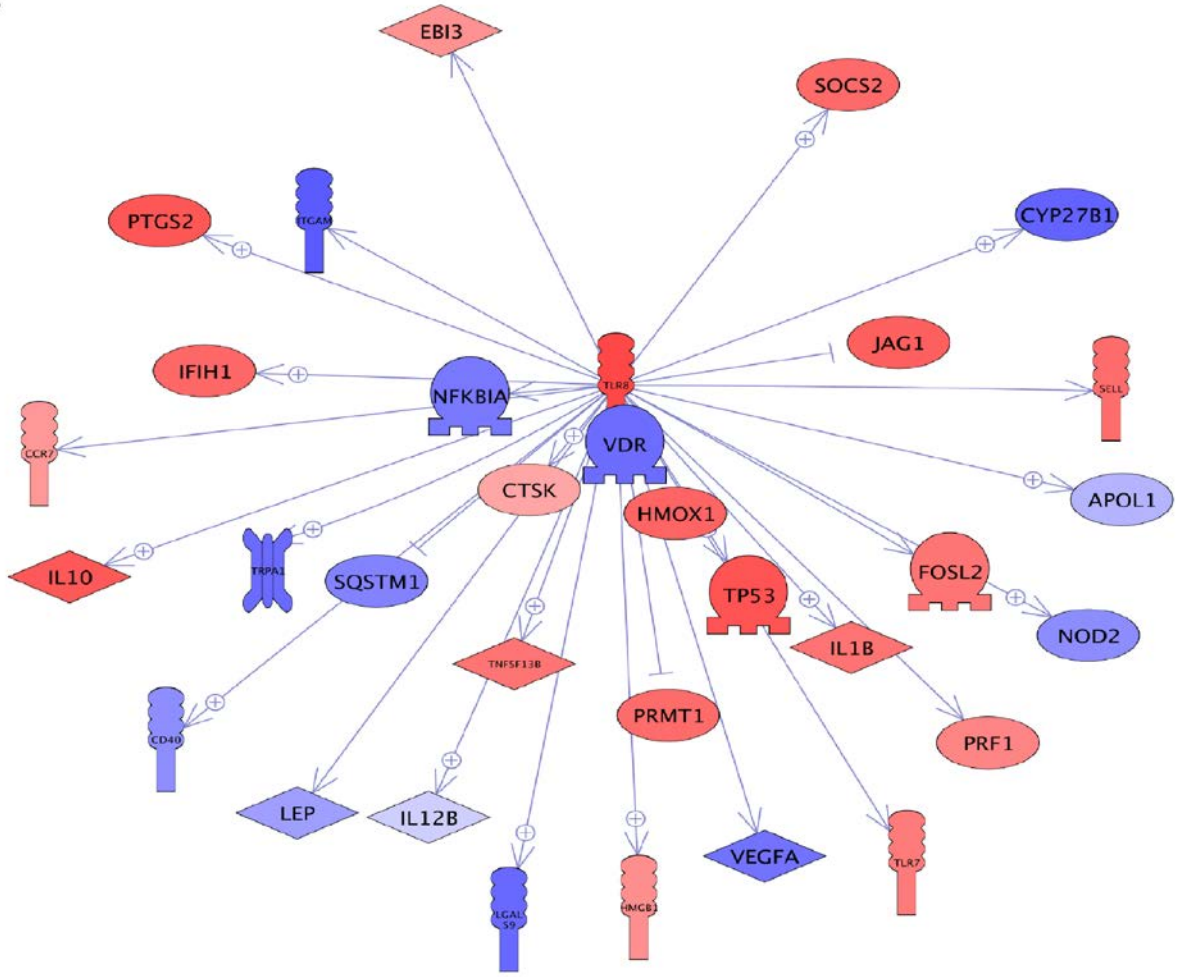


Supplementary figure 7 Sub-network enrichment analysis between zebrafish and human. The Tlr8 pathway in zebrafish (A) with Mm infection and human macrophages (B) with Mtb infection. Red represents up-regulation, blue represents down-regulation

A



B



Supplementary Table 1 List of primers

Supplementary Table 2 A-H Gene lists of GSEA and SNEA analysis

Supplementary Table 3 GO analysis of genes that have a different basal expression level in the absence of infection in the Tlr2 mutant versus the heterozygote control. Shown is the GO analysis of the group of 878 genes that were different with a FDR *p* value of 10^{-10} indicated in Fig. 6F.

Supplementary Table 4 List of transcription factors under GO category in absence of infection

Supplementary Table 5 List of basal expression levels of gene *mpx* and *mpeg1*

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3

CHAPTER 4

Characterization of zebrafish Traf6 and Ikk2 mutants

4

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Abstract

The Traf6 and Ikk2 proteins are partners in the signal transduction pathway underlying innate immunity and are involved in multiple pathological processes. Furthermore, they are also important for development, as shown by the fact that the total deficiency of Traf6 or Ikk2 in rodents is lethal. We analyzed zebrafish Traf6 and Ikk2 mutants with a stop codon in the reading frame of the protein coding sequences (*traf6*$^{sa244/sa244}$ and *ikk2*$^{m10/m10}$). We characterized the influences of these *traf6* and *ikk2* mutations on immunity and development. In the case of *ikk2* we also tested the response to hyperinsulinemia, since Ikk2 has previously been implicated in insulin signaling. Both mutants did not show any visible abnormal organ phenotype during embryonic or later developmental stages, and the adults were also capable of breeding. There were no consistent indications for effects on leukocyte phenotype 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. For analysis of immune responses we infected the mutants with *Mycobacterium marinum* and measured bacterial proliferation. In the case of the *traf6* mutant no significant difference with the wildtype in susceptibility to infection could be observed and the surprising lack of phenotypes of this mutant is discussed. The *ikk2*$^{m10/m10}$ larvae showed a higher bacterial burden than *ikk2*$^{+/+}$ larvae upon *M. marinum* infection. In addition, in the *ikk2* mutant the insulin resistance induced by hyperinsulinemia was modulated. We also studied the inflammatory response induced by flagellin in the *ikk2* mutant through activating Tlr5 signaling. Unexpectedly, we found that in the *ikk2* mutant a significantly higher expression of *il1b* was induced 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 induced by flagellin. 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.

Introduction

Toll like receptors (TLRs) are an important class of pattern recognition receptors (PRRs). TLRs recognize conserved pathogen-associated molecular patterns (PAMPs) from microbial components and danger-associated molecular patterns (DAMPs) from the host itself. Thereby they initiate signaling pathways by the activation of NF-κB or AP-1 transcription factors and finally activate inflammatory and immune responses. Tumor necrosis factor receptor associated factor 6 (TRAF6), plays a critical role in mediating these processes. After signal transduction is initiated from the Toll/IL-1R (TIR) domain of TLRs, TRAF6 induces recruitment of MYD88 and IRAK1/4 to form a

receptor complex. Subsequently, TRAF6 dissociates from this receptor complex, and associates with TAK1, TAB1 and TAB2 to form a new complex. This new complex then translocates from the plasma membrane to the cytosol and is ubiquitinated by ubiquitin ligases UBC13 and UEV1A. Finally, it actives the MAPKs and the IKK complex (IKK1, IKK2 and NEMO), resulting in AP-1 and NF-κB activation (1, 2). In addition to functioning in the TLR/IL-1R signal transduction pathway, TRAF6 also interacts directly with TGFβRI/ALK5 (3, 4) and proteins from the TNF-receptor family, such as CD40 and TRANCER, also known as RANK (1, 5), that in turn activate the PI3K and downstream Akt/PKB pathway.

In mammals TRAF6 is broadly expressed in various tissues and cells, but most immune cells restrict their activity through selective expression of receptors used to activate TRAF6 (6). Moreover, TRAF6 has been shown to be critical for developmental processes (7, 8), bone metabolism (9) homeostasis (10), apoptosis (11) and cancer (12, 13). For instance, Traf6 knockout mice showed a high mortality within three weeks after birth, accompanied by severe defects in the encephalon and neural tube and abnormal regulation of the central nervous system (9, 14, 15). It has also been demonstrated that TRAF6 is important for the immune system, since Traf6-deficient chimeras generated a progressive lethal inflammatory disease associated with massive organ infiltration and a dominant Th2-type polarized autoimmune response (16). TRAF6 regulates the critical processes required for maturation, activation, and development of dendritic cells (DCs) (14). Mice that are deficient for Traf6 specifically in their regulatory T cells, developed allergic skin diseases, arthritis, lymphadenopathy and hyper IgE phenotypes (17). Furthermore, TRAF6 is a key component in the signaling cascade downstream of C-type lectin receptors and is a critical mediator of the anti-fungal immune response (18). TRAF6 is also required for production of IL-12 in control of parasite infections (19).

In the canonical NF-κB signaling, the IKK complex is activated by the TRAF6/TAK1/TAB1/ TAB2 complex. As the key catalytic subunit in the IKK complex, IKK2, also named as IKKβ or IKBKB, is essential for NF-κB activation in response to pro-inflammatory stimuli. Knockout of Ikk2 is lethal in mice (20, 21), but conditional knockout of Ikk2 can even reverse some pathological diseases and lesions induced by the IKK2/NF-κB signaling pathway. For instance, Ikk2 conditional knockout or inhibition in mice attenuates laser-induced choroidal neovascularization, a leading cause of blindness in the elderly (22), and reduces liver necrosis and inflammation from ischemia/reperfusion injury (23). However, mice with epidermis-specific deletion of Ikk2 develop a severe TNF-mediated inflammatory skin disease (24). Furthermore, Ikk2 has also been shown to be required for a proper response to infection in mice (25-28). For instance, previous studies indicate that selective ablation of lung epithelial Ikk2 impairs pulmonary Th17 responses and delays the clearance of Pneumocystis, an atypical fungal pathogen that causes severe, often fatal pneumonia in

immunocompromised patients (25). The pharmacological inhibition of IKK2 prevents human cytomegalovirus replication and virus-induced inflammatory response in infected endothelial cells (26). Interestingly, Ikk2 has also been implicated in insulin sensing, where heterozygous deletion of Ikk2 protected against the development of insulin resistance during high-fat feeding in obese ob/ob mutant mice (29). Moreover, inactivation of Ikk2 prevents insulin resistance in skeletal muscle by blocking fat-induced defects in insulin signaling (30).

Zebrafish has become an important vertebrate model for studying human diseases, such as cancer (31, 32), tuberculosis (33, 34), microbial infections (35, 36), neurodegeneration (37, 38), and developmental and metabolic diseases (39, 40). Their small size, transparent larvae and short generation time of 3-4 months are beneficial for the screening and imaging of transgenic lines. The excellent imaging tools and applicability of microinjection of disease inducing agents allow the study of the function of Traf6 and Ikk2 in different disease models in zebrafish. TRAF6 shows a highly conserved structure from mammals to zebrafish (5, 41, 42). In a previous study from our laboratory it was shown that Traf6 has a dynamic role as a positive and negative regulator in a zebrafish-Salmonella infection model, based on transcriptome profiling by a combination of microarray analysis and whole transcriptome deep sequencing of Traf6 morphants (43).

In this study, to further analyze the role of Traf6 and Ikk2 upon *Mycobacterium marinum* (Mm) infection, we conducted infection experiments using *traf6* and *ikk2* mutant zebrafish. We found that the *traf6* mutant display an absence of phenotypes that was not expected from results of previous studies. Specifically, we could not demonstrate an effect of the mutation in the defense of larvae to mycobacterial infection. Since we are not able to show any specific phenotype resulting from the mutation in Traf6 we consider it likely that it does not represent a null mutant, perhaps because of residual activity of a truncated protein or of the result of suppression by other mutations. In contrast, the *ikk2* mutant shows higher bacterial burden than wildtype upon infection with Mm. Furthermore, the *ikk2* mutant shows a reduction in the number of macrophages and neutrophils in the absence of infection. In addition, our studies revealed that *ikk2* mutation led to blocking of the insulin resistance induced by hyperinsulinemia. This indicates that the *ikk2* mutation might represent a null allele of this gene.

Results and discussions

Studies of a Traf6 mutant

1 Description of the traf6 mutation

The *traf6*^{sa244} mutant carries a guanine to thymine (G to T) point mutation that generates a premature stop codon located at the nineteenth amino acid close to the N-terminus of the RING domain (Fig. 1A). Theoretically, this leads in the homozygous situation to a complete absence of the Traf6 protein and prevents Traf6-mediated downstream signaling. To get rid of many of the background ENU mutations in the *traf6* mutant zebrafish, we conducted outcrosses between the wildtype and this mutant line (Fig. S1). The offspring from the fifth generation (F5) were used to conduct experiments in our study.

2 traf6 mutation affects the survival rate of zebrafish larvae

We performed an incross between the F0 generation of Traf6 heterozygotes, and only one male Traf6 homozygote offspring survived. This homozygote male was outcrossed to wildtype (AB/TL) to obtain the F2 generation of Traf6 heterozygotes (Fig. S1). Two groups of *traf6*$^{sa244/sa244}$, and *traf6*$^{+/+}$ larvae from the third generation (F3) (Fig. S1) were compared under normal embryo raising conditions to test for differences in unchallenged survival during development. Up to 7 dpf there was no difference in survival between mutants and wildtypes (Fig. 1B). After this period, *traf6*$^{sa244/sa244}$ larvae showed a significantly decreased survival rate (75% survival) compared with *traf6*$^{+/+}$ larvae (95% survival) (Fig. 1B). The steady mortality rates of *traf6*$^{sa244/sa244}$ larvae ceased at around 20 dpf. Thus, under our raising conditions, 8-20 dpf was a crucial period for raising *traf6*$^{sa244/sa244}$ larvae in this generation. Subsequent development of *traf6*$^{sa244/sa244}$ was normal with the larvae reaching adulthood in a normal time span leading to adults with a normal fertility rate at the expected time period. Furthermore, *traf6*$^{sa244/sa244}$ adults did not display any increased mortality rate compared with wildtype individuals and heterozygotes, or showing pathological features linked with infection.

In mice, Traf6 deficiency is known to influence bone development, for example Traf6-/- mice exhibit severe osteopetrosis and are defective in osteoclast formation (44) and Traf6 is involved in TNF-alpha-induced osteoclastogenesis (45). We therefore measured the body size of *traf6*$^{sa244/sa244}$ and *traf6*$^{+/+}$ zebrafish larvae at 5dpf (Fig. 1C). We found that there is no significant difference of the body size between *traf6*$^{sa244/sa244}$ and *traf6*$^{+/+}$ larvae.

3 traf6 mutation does not prevent activation of TLR signaling in zebrafish larvae

The premature stop codon in the mutant allele is predicted to lead to the disruption of almost the complete reading frame encoding the Traf6 protein. The resulting deficiency in the capacity of cells to induce the MYD88/IRAKs receptor complex and

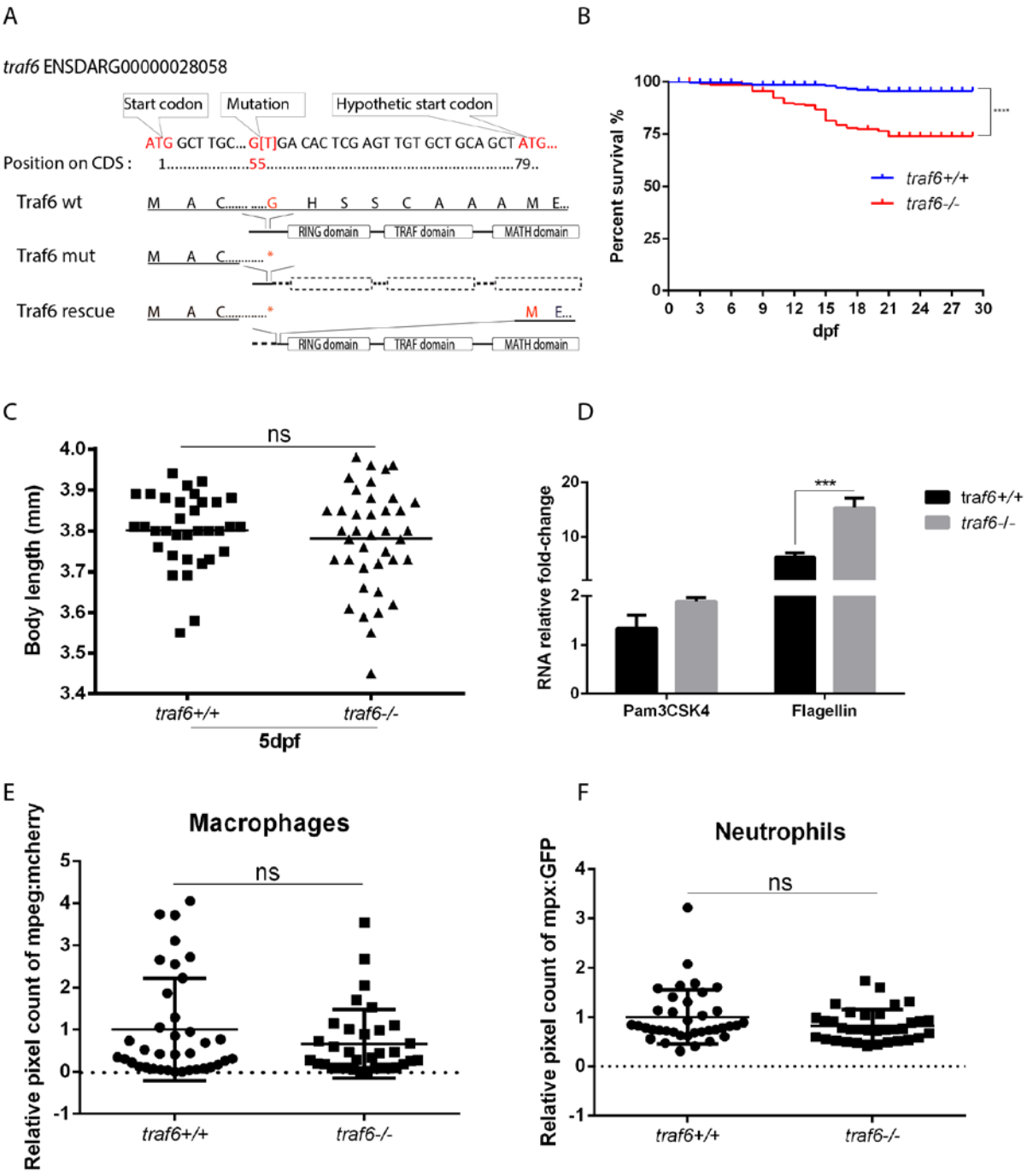

FIGURE 1 Description of the Traf6 mutant. A, mutant sequence and protein structure. A point mutation (G to T) in the codon for the nineteenth amino acid close to the N-terminus of the RING domain of zebrafish *traf6* introduces a premature stop codon. Nucleotide and amino acid positions are indicated with respect to the translation start codon. B, survival assays. Percentage survival during the first 20 days of development (under unchallenged conditions) is shown for two groups (n=70×3 per group) of *traf6*$^{sa244/sa244}$ mutants (red line) and *traf6*$^{+/+}$ wildtype (blue line), grown in three separate tanks. The groups are the F3 offspring of *traf6*$^{sa244/sa244}$ and *traf6*$^{+/+}$ siblings born from heterozygous parents. At 21 dpf, 95% of *traf6*$^{+/+}$ survived, 75% of *traf6*$^{sa244/sa244}$ mutants survived. The asterisks indicate the significant difference between wild-type and mutant survival (P<0.0001), tested

with a log rank test. C, body size measurement. Zebrafish larvae were imaged by stereo microscopy, and the body sizes were measured from *traf6*$^{+/+}$ and *traf6*$^{sa244/sa244}$ (n>30). D, *traf6*$^{sa244/sa244}$ and *traf6*$^{+/+}$ embryos were injected at 27 hpf with 1 ng Pam3CSK4 or 0.1 ng flagellin and expression levels of *il1b* were determined at 1hpi by qPCR. Data (mean ± SEM) are combined from at least three biological replicates (n=10 embryos per group) and expressed relative to their corresponding mock injection (water) control, which is set at 1. Statistical significance of differences was determined by two-way ANOVA with Sidak Multiple Comparison test as a post-hoc test, ***p<0,001. E-F, relative fluorescence pixel-count analysis of whole body number of mCherry-labeled macrophages and GFP-labeled neutrophils in 2dpf *traf6*$^{+/+}$*/Tg(mpeg1:mcherry/mpx:gfp)* and *traf6*$^{sa244/sa244}$*/Tg(mpeg1:mcherry/mpx:gfp)* embryos (n>30) were performed based on stereo fluorescence imaging. No significant differences were observed in both macrophages and neutrophils phenotype with a T-test.

activate TAK1/TABs, is expected to inhibit all TLR downstream signaling, including MAPK- and IKK complex-induced AP1 and NF-κB activation.

To confirm whether this *traf6* mutation blocks the downstream TLR signaling pathway, we analyzed the expression level of the inflammation marker gene *il1b* in zebrafish embryos upon injection with the TLR2 agonist Pam3CSK4 and the TLR5 agonist flagellin. Pam3CSK4 and flagellin were injected into the blood island of zebrafish embryos at 27 hpf. One hour post injection (hpi), we collected samples and conducted qPCR. We injected water (solvent for Pam3CSK4 and flagellin) as a control for which the gene expression level was set at 1 (Fig. 1D). In *traf6*$^{sa244/sa244}$ the expression level of *il1b* unexpectedly showed a significantly higher induction than that in *traf6*$^{+/+}$ upon flagellin injection, which remains unexplained (Fig. 1D). There was no significant difference of *il1b* expression between *traf6*$^{sa244/sa244}$ and *traf6*$^{+/+}$ upon Pam3CSK4 stimulation (Fig. 1D). These results show that the *traf6* mutation in our study does not block the TLR downstream signaling as we expected.

4 Macrophage and neutrophil phenotype in traf6 mutant larvae

GM-CSF and M-CSF promote macrophage proliferation, survival and differentiation through TRAF6-dependent AKT activation (46-50). A recent publication reported that the inhibition of TRAF6-mediated AKT activation is involved in macrophage proliferation regulated by CKIP (51), so our prediction is that a *traf6* mutation may influence the number of leukocytes in zebrafish. To study this we crossed *traf6*$^{sa244/sa244}$ into double transgenic *Tg(mpeg1.1:mCherryF/mpx:GFP)* fish, allowing the visualization of macrophages and neutrophils respectively. First, we analyzed leukocyte number by fluorescence pixel-count analysis of macrophages and neutrophils in 2dpf *traf6*$^{+/+}$*/Tg(mpeg1.1:mCherryF/mpx:GFP)* and *traf6*$^{sa244/sa244}$*/Tg(mpeg1.1:mCherryF/mpx:*

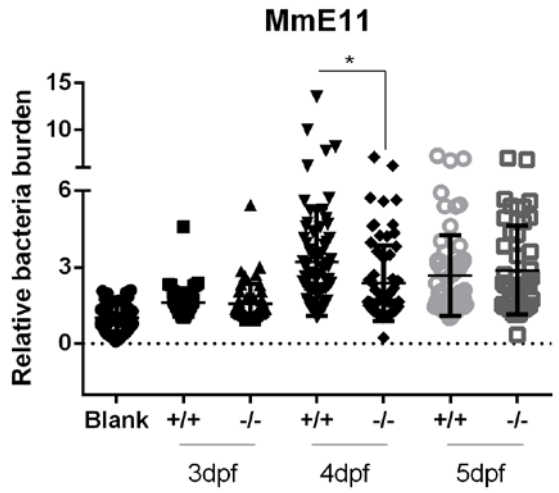

FIGURE 2 Quantification of bacterial burden in *traf6*$^{+/+}$ and *traf6*$^{sa244/sa244}$ larvae. Embryos were infected at 64-128 cell stage with ~40 CFU MmE11 strain. COPAS analysis were used for quantification of bacterial burden. Data (mean ± SD) are combined from two individual yolk infection assays . Statistical significance of differences was determined by one-way ANOVA with Tukey's Multiple Comparison method as a post-hoc test. *p<0,05, **p<0,01, ***p<0,001.

GFP) embryos. We observed no significant difference in the number of macrophages and neutrophils in the mutant (Fig. 1E-F).

5 Mycobacterium marinum infection in traf6 mutant larvae

We performed two individual yolk infection assays with *Mycobacterium marinum* E11 (MmE11) strain in the *traf6* mutant. We injected 64-128 cell stage embryos with ~40 cfu MmE11 by yolk injection, and conducted COPAS analysis at 3, 4 and 5 dpf (Fig. 2). The *traf6* mutant showed a decreased bacterial burden at 4dpf but not at 3 and 5 dpf (Fig. 2). Therefore we conclude that the *traf6*^{sa244}mutant allele does not have a major effect on host defense against Mm.

6 Western blot analysis of the Traf6 mutant

Considering the unexpected results of the phenotypic screening of the Traf6 mutant we tried to confirm that the mutation leads to a complete absence of Traf6 protein in zebrafish. Therefore we performed western blot analysis in 1dpf *traf6* morphants and 4dpf wt and mutant larvae (Fig. 3) using a commercially available monoclonal antibody against human TRAF6 protein. There was one band with a size close to the positive control TRAF6 protein from human Jurkat cells (58 kDa). The size of the predicted Traf6 protein in zebrafish is 62 kDa. The relative expression of this band that might represent zebrafish Traf6 was compared between different zebrafish samples using ImageJ analysis. The results showed that there was no significant difference of protein expression between wildtype and *traf6* morphants or *traf6* mutant larvae. One of the explanations for this result is that this protein band is not representing the Traf6

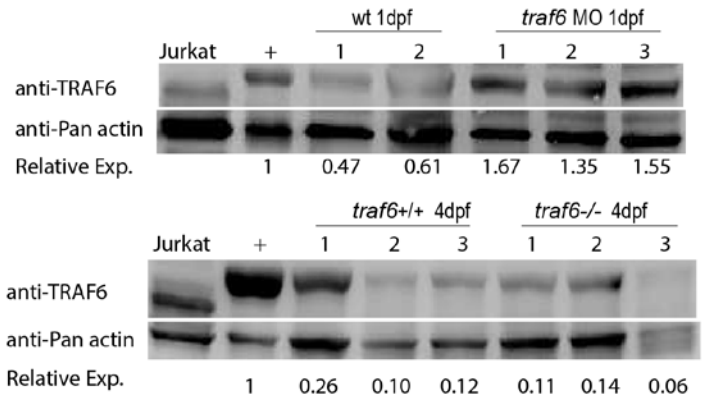

FIGURE 3 Western blot analysis of Traf6. Western blot results of whole 1 dpf embryos (*traf6* morphants and wildtype, upper two-panels) and 4 dpf larvae (*traf6* mutant and wildtype, lower two-panels) are shown. Relative expression data (mean ± S.E.M.) are determined from two (wt) or three (*traf6* MO, *traf6*+/+, *traf6*-/-) biological replicates (n=100 embryos (1dpf)/group (upper panel), n=15 larvae (4dpf)/group (lower panel)) and expressed relative to the positive control (+, whole protein from 4dpf AB/TL wildtype fish line), which is defined as 1. A protein sample of human Jurkat cells is included as a control for the antibody specificity. MO, morphants; wt, wildtype.

protein and that the antibody was not able to detect zebrafish Traf6 due to an aspecific background band. This is argued by the absence of an effect in the samples from treatment with a Traf6 translation blocking morpholino, which was previously shown to affect embryo development as well as the expression of immune effector genes downstream of Traf6 signaling (43). The other explanation is that the *traf6* mutation does not result in a null mutant at the protein level and that the *traf6* morpholino treatment isnot sufficiently penetrant to show an effect at the protein level.

7 Conclusion

In conclusion we can propose two possible explanations for the shown results.

Firstly, the point mutation from G to T at position 55 bp after the predicted start codon of Traf6 does not lead to a null phenotype *traf6* mutant. We hypothesize that 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 at the position 79 bp in the coding sequence (Fig. 1A) that if used as an alternative start codon would lead to a slightly shorter Traf6 protein. Although various of the amino acids in the resulting deletion are conserved in the mammalian counterparts, the entire RING domain would not be affected and therefore it is conceivable that the resulting protein 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 unclear.

Secondly, 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 homologs 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 signaling pathways. This would be similar to the published suppression of mutants of Ikk2 by mutations in Tnfr1 (52). Li et al (52) showed that mice double mutants of $Tnfr1^{-/-}/Ikk2^{-/-}$ can survive until one month rather than die around 12.5-13.5 days like the $Ikk2^{-/-}$ mutant. They demonstrated that the rescue of embryonic lethality of Ikk2 deficiency by inactivation of Tnfr1 is caused by the inhibition of Tnfa-induced hepatocyte apoptosis. If Traf6 protein indeed is essential for development in zebrafish (as suggested by our previous morpholino results (43)) it is very much possible that we have selected for suppressor mutations during our incrossing schemes. An indication for this is the higher levels of lethality of larvae resulting from incrosses at the third generation after 7 days of development post fertilization (Fig.1B). For instance, it is possible that the larvae that died represent a population in which the suppressor mutation was lost or that the surviving population have optimized the effect of the suppressor mutation via epigenetic regulatory mechanisms.

In order to resolve these questions and to proceed with further studies of Traf6 in zebrafish it is needed to construct new mutants for comparative studies. Fortunately, during the time that this study was performed the new CRISPR/CAS technology has been made suitable for the efficient generation of targeted mutations also in zebrafish (53, 54). However, this study also illustrates that a limiting factor in zebrafish mutant studies is the lack of reliable antibodies against the studied signaling proteins. The availability of good antibodies against zebrafish Traf6 protein will remain extremely important for future studies of the function of this protein using new mutant lines.

Studies of an Ikk2 mutant

1 General characterization of ikk2 expression and ikk2 mutant zebrafish embryos

To show the transcription expression pattern of *ikk2* in zebrafish embryos, we collected RNA from wildtype (AB/TL) embryos to perform a time course qPCR series at different stages, which included oblong (3.7 hpf), germ ring (5.7 hpf), bud (10 hpf), 20-somite (19 hpf), 24 hpf, 48 hpf, 60 hpf and 72 hpf (Fig.4A). We found that the expression level of *ikk2* starts to show a stabilized expression from the 20-somite stage (19 hpf) until the last time point (72 hpf) of the assay (Fig.4A).

An *ikk2*^{m10} mutant was obtained from Dr. Vladimir Korzh's lab. These researchers developed this mutant by zinc finger nuclease-mediated mutagenesis. The *ikk2*^{m10} mutant carries an insertion mutation from AC to ATGC that creates a premature stop codon (Fig. 4B). The position of this stop codon located in the N-terminus of the kinase domain of zebrafish Ikk2 leads to a gene reading frame shift at the codon of the tenth amino acid and introduces a premature stop codon at the predicted sixty first amino acid. Technically, this should lead to a complete absence of the Ikk2 protein and prevent downstream signaling. Korzh et al. found that homozygote mutants carrying this predicted null allele showed survival rates comparable to wildtype from the one-cell stage until three months post fertilization, but decreased body lengths at nine months of age (unpublished data).This is in agreement with a previous study that shows that endothelial-specific Ikk2 knockout mice were ~25% smaller than the control (55). We measured the body length of zebrafish *ikk2* mutants at 3dpf, and found that, compared to their siblings control *ikk2*$^{+/+}$, *ikk2*$^{m10/m10}$ showed a significant shorter body length (Fig. 4C). This result is therefore in good agreement with the earlier unpublished studies of Khorz et al. and shows that this phenotype is already detectable at a very early stage of development.

To determine the RNA expression pattern of *ikk2* in the *ikk2* mutant, total RNA was isolated from embryos and qPCR was performed at 20-, 33-, 48- and 72 hpf. We found that the *ikk2* mutant showed a significant upregulation of the *ikk2* mRNA level at 3dpf (Fig. 4D). This suggests that the *ikk2* mutant induces an overexpression of *ikk2* at the transcriptional level in zebrafish early stage development or that the *ikk2*^{m10} mRNA is more stable than the wild type *ikk2* mRNA. This may indicate a negative feedback regulation of Ikk2 on its own expression level.

To determine whether the *ikk2* mutation inhibits NF-κB activation, we injected TLR5 agonist, flagellin, into the blood island of zebrafish embryos at 28 hpf and checked *il1b* expression at 1hpi as previously described (56). We found that *il1b* shows a significant upregulation in the *ikk2* mutant, and that the induction of the expression level was even much higher than in *ikk2*$^{+/+}$ larvae (Fig. 4E). This is a surprising result since the *ikk2*^{m10} mutation is predicted to lead to the absence of the whole Ikk2 protein and

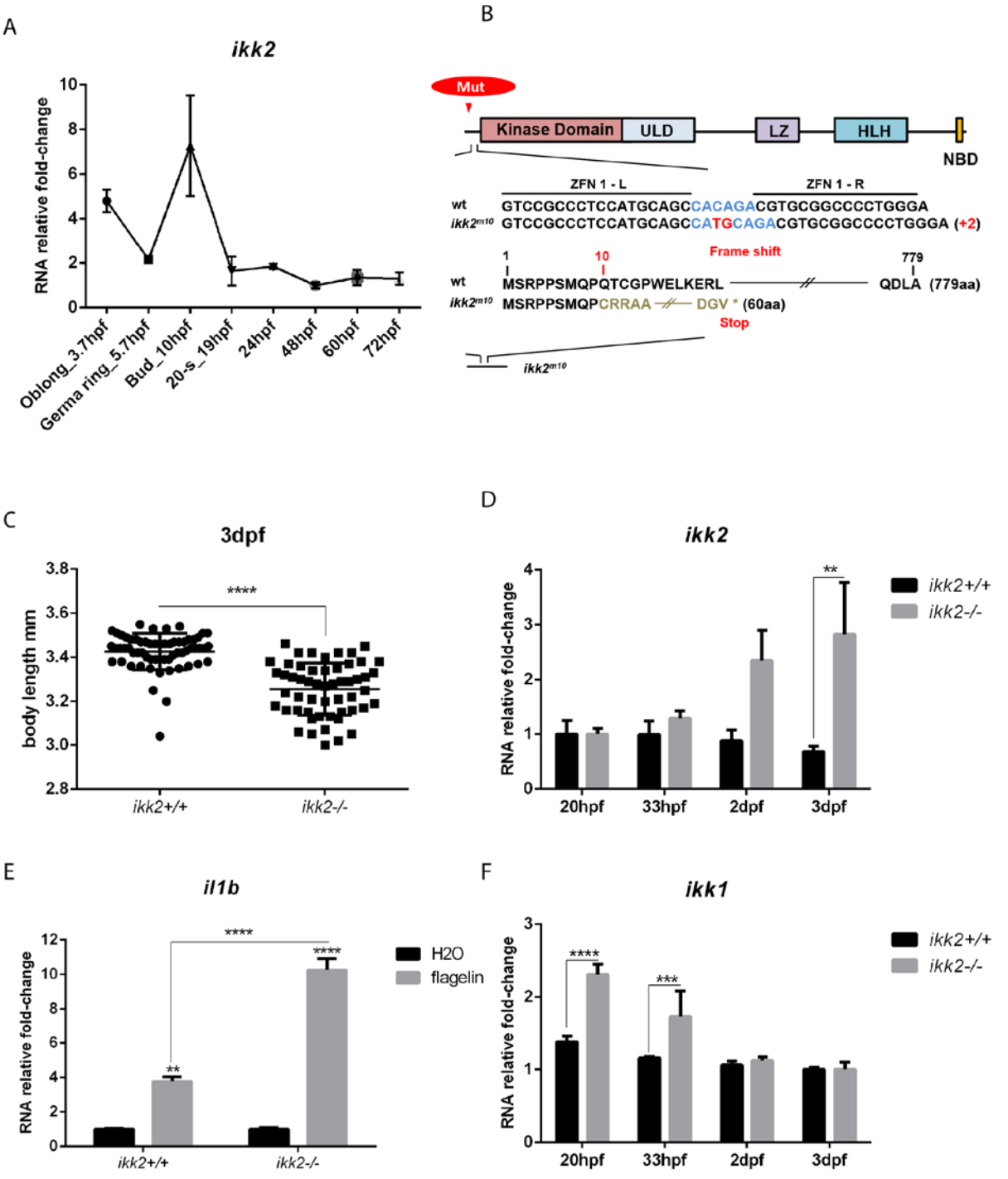

FIGURE 4 Characterization of *ikk2* and *ikk1* mRNA expression in the *ikk2*^{m10} mutant. A, mRNA expression analysis of *ikk2* from 3.7 to 72hpf in wildtype (AB/TL). B, *ikk2* mutant sequence and protein structure. An insertion mutation of two bases (AC to A**TG**C) in the N-terminal sequence of the kinase domain of zebrafish Ikk2 leads to a frame shift at the codon for the tenth amino acid and introduces a premature stop codon at the sixty first amino acid. The truncated protein lacks the whole Ikk2 protein. Nucleotide and amino acid positions are indicated with respect to the mutation position. C, larvae of *ikk2*$^{+/+}$ and *ikk2*$^{m10/m10}$ were imaged using stereo microscopy and their body length were measured at 3dpf. Statistical significance of differences was determined by *t*-test,

****p<0,0001. D, the mRNA expression of *ikk2* from 20hpf to 3dpf in *ikk2*$^{+/+}$ and *ikk2*$^{m10/m10}$ larvae. E, *il1b* mRNA expression in *ikk2*$^{+/+}$ and *ikk2*$^{m10/m10}$ embryos at 1hpi after flagellin blood island injection. F, the mRNA expression of *ikk1* from 20hpf to 3dpf in *ikk2*$^{+/+}$ and *ikk2*$^{m10/m10}$ larvae. Statistical significance of differences in D-F was determined by two-way ANOVA with Sidak Multiple Comparison test as a post-hoc test, ***p<0,001, ****p<0,0001.

should disrupt its function of phosphorylating IκB molecules, the inhibitors of NF-κB transcription factors. These disruptions will subsequently inhibit the release and nuclear translocation of NF-κB.

2 the Ikk2 mutation induces a temporary upregulation of Ikk1

Theoretically, phosphorylation of IKK2 is necessary for activation of the canonical NF-κB pathway (57) and IKK1, also known as IKKα or CHUK, is necessary for activation of the non-canonical or alternative pathway (58). Functioning as one of the catalytic subunits of the IKK complex, IKK1 shows a similar structure as IKK2 (59). A previous study in human lymphoma cells showed that IKKα may directly phosphorylate IκBα under conditions of IKKβ inhibition contributing to cell survival. Therefore, we studied the gene expression level of *ikk1* in *ikk2*$^{m10/m10}$ embryos and larvae. As the results show (Fig. 4F), the expression level of *ikk1* was significantly upregulated at 20 and 33hpf in *ikk2*$^{m10/m10}$ compared to *ikk2*$^{+/+}$ embryos, but after that it decreased to the normal level as shown in the *Ikk2*$^{+/+}$ larvae at 2 and 3dpf. These results suggest the hypothesis that *ikk2* deficiency may lead to *ikk1* overexpression in zebrafish embryos at an early stage, which induces a compensatory activation of the canonical NF-κB pathway.

3 The ikk2 mutant shows a deficient phenotype of leukocytes and is more susceptible to infection with Mycobacterium marinum than the wildtype

To characterize the phenotype of immune cells in *ikk2* mutant embryos, we performed a combination of TSA and L-plastin immunostaining in embryos at 2 and 3dpf (Fig. 5A, B). Cells labeled with TSA are neutrophils, and those labeled with L-plastin represent all leukocytes (i.e. neutrophils and macrophages). We found that there is a significant decrease in the number of neutrophils at 2dpf but not at 3 dpf (Fig. 5A, B). Furthermore, the total number of leukocytes seemed reduced at both 2 and 3 dpf, although not reaching the significance threshold (Fig. 5A, B). To confirm these result, we conducted qPCR to detect the transcript level of the genes *mpx* and *mpeg1* (Fig. 5C, D), which are marker genes of neutrophils and macrophages respectively. The expression level of these two genes showed a significant downregulation in the *ikk2*

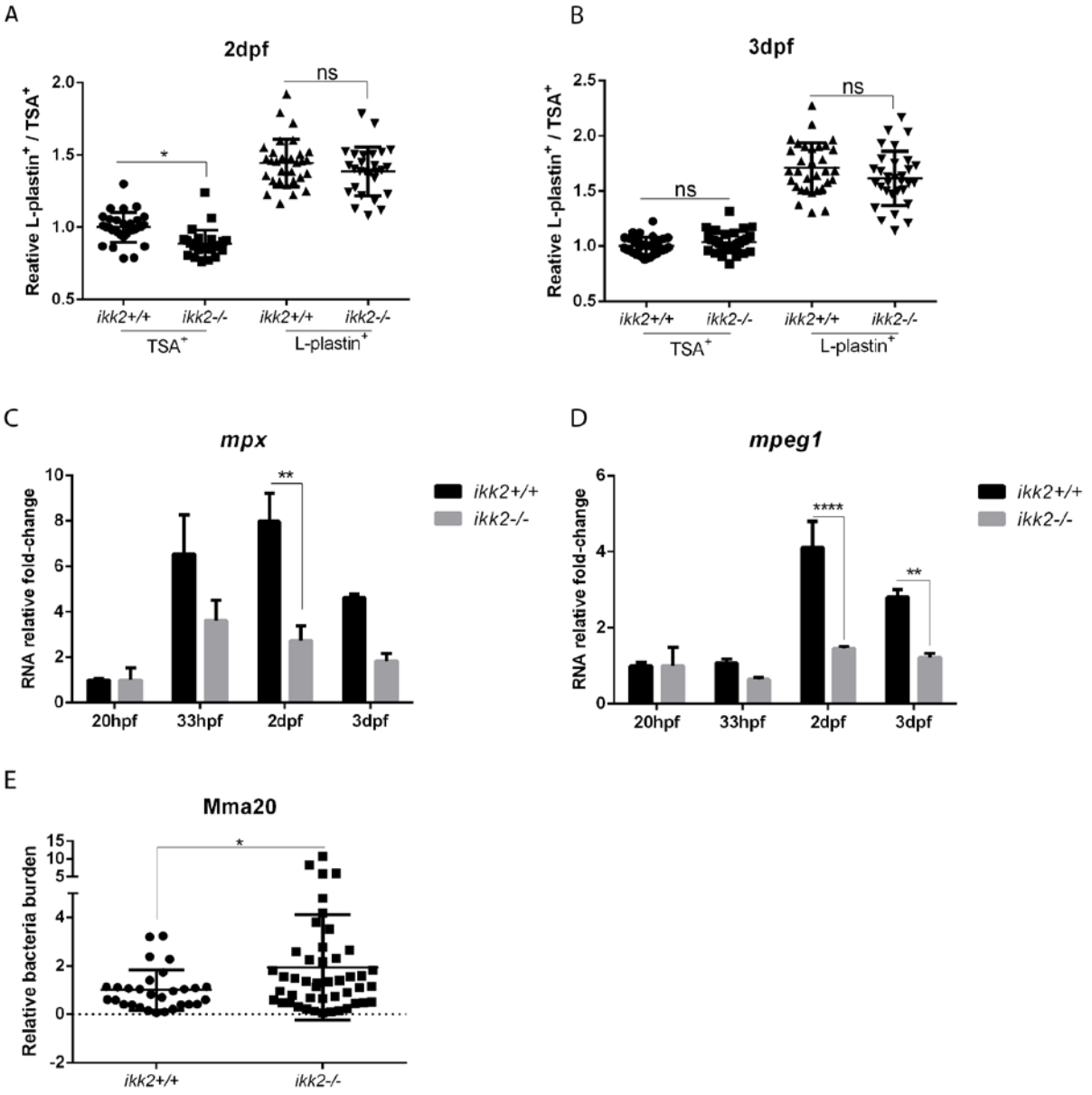

FIGURE 5 Study of leukocytes and mycobacterial infection in the Ikk2 mutant. A, B, quantification of leukocyte numbers. The number of neutrophils and macrophages was determined by performing whole mount L-plastin immuno-histochemistry (total leukocytes) combined with TSA staining (neutrophils) in 2 and 3dpf zebrafish larvae (n>=27). Statistical significance of differences was determined by *t*-test, *p < 0.05, **p<0,01. C, D, mRNA expression levels of *mpx* and *mpeg1* were determined by qPCR from 20hpf to 3dpf. E, quantification of bacterial burden. i*kk2*$^{+/+}$ and *ikk2*$^{-/-}$ embryos were infected at 28hpf with ~150CFU Mma20 strain. Stereo fluorescence images of Mm infected embryos at 4dpi were used for quantification of bacterial fluorescence pixels. Statistical significance of differences was determined by *t*-test, *p < 0.05.

mutant at 2 and 3dpf (Fig. 5C, D). Our results indicate therefore that in the *ikk2* mutant both neutrophil and macrophage development is affected.

Considering the deficiency of macrophages and neutrophils in the *ikk2* mutant at an early developmental stage, we performed an infection assay with *Mycobacterium marinum* m20 (Mma20) through blood island injection at 28hpf and detected the bacterial burden at 4dpi. The result shows that the *ikk2*$^{m10/m10}$ mutation promotes an increase of bacterial proliferation compared to the sibling control (Fig. 5E).

The effect of a mutation of Ikk2 on immune responses was also demonstrated in rodents. For instance, compared to the wildtype , mice with Ikk2 deficiency in lung epithelial cells exhibited a delayed onset of Th17 and B cell responses in the lung and delayed fungal clearance. Importantly, delayed Pneumocystis clearance in Ikk2-deficient mice was associated with an exacerbated immune response, impaired pulmonary function, and altered lung histology. These data demonstrate that Ikk2-dependent lung epithelial cell responses are important regulators of pulmonary adaptive immune responses and are required for optimal host defense against Pneumocystis infection (25).

4 The mutation of ikk2 modulates the insulin resistance induced by hyperinsulinemia

Mice with Ikk2 deficiency in myeloid cells retain global insulin sensitivity and are protected from insulin resistance (60). Zebrafish has been shown to be a good model to study acute insulin resistance induced by hyperinsulinemia in a previous study from our laboratory (61). To study how the *ikk2* zebrafish mutant responds to hyperinsulinemia, we injected insulin into the caudal aorta at 4dpf, and the second injection was conducted 240 minutes later following the protocol published previously. A glucose measurement was performed at 0, 30 and 240 minutes after the first injection and 30 minutes after the second injection. The results show a typical insulin resistance in wildtype larvae after the second injection, where they exhibited a significant higher glucose level (Fig. 6A). The result also show that there was no upregulation of the glucose level in *ikk2*$^{m10/m10}$ after the second insulin injection (Fig. 6C). We outcrossed the *ikk2*$^{m10/m10}$ mutant with the wildtype (AB/TL) leading to an offspring called *ikk2*$^{+/-}$. We found that in this heterozygote the glucose level increased after the second insulin injection as in the wildtype control (Fig. 6B).

To further study the role of IKK2 in insulin resistance, we measured the transcriptional level of the *phosphoenolpyruvate carboxykinase 1* gene (*pck1*), which is well known to be inhibited by insulin in mammals and zebrafish (62). As expected we found that the expression level of *pck1* in the wildtype was decreased after insulin injection (Fig. 6D). For the *ikk2*$^{+/-}$ with AB/TL background, analysis of *pck1* expression

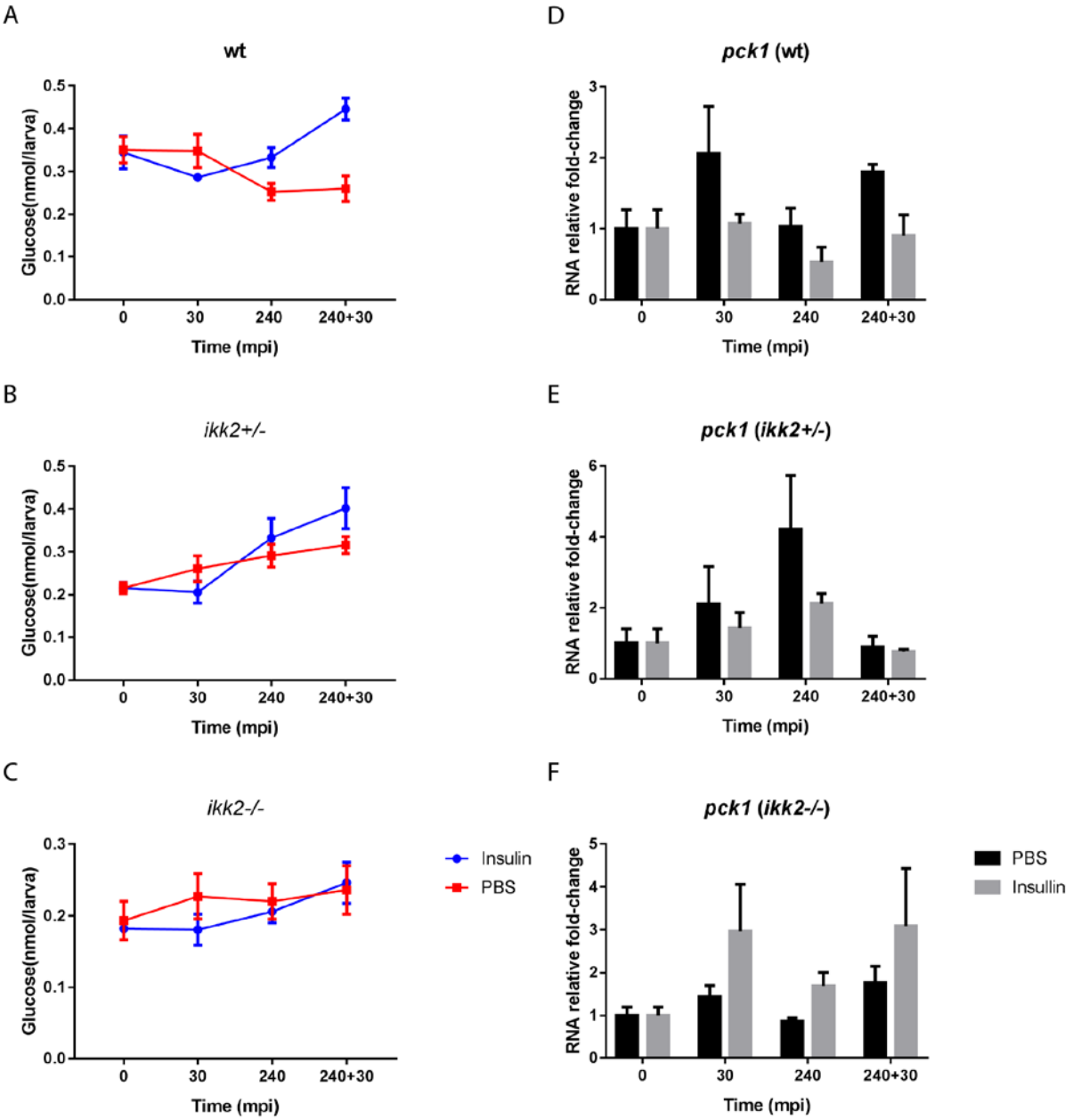

FIGURE 6 Studies of responses to hyperinsulinemia in the *ikk2* mutant. A-C, Zebrafish larvae from wildtype (A), *ikk2*$^{+/-}$ (B) and *ikk2*$^{-/-}$ (C) were injected with 1nl human recombinant insulin at 100 nM in the caudal aorta at 4 dpf and received a second injection at 4 hpi (240 min). Control larvae were injected with PBS. Samples for glucose measurements were taken at 0, 30, and 240 min after the first injection and 30 min after the second injection. Values in the scale are indicated in minutes post injection (mpi). Data (mean ± SE) are combined from 3 biological replicates (n= 4 or 5 larvae per group). D-F, mRNA expression level of *pck1* from wildtype (D), *ikk2*$^{+/-}$ (E) and *ikk2*$^{-/-}$ (F) were determined by qPCR at the same time points as the glucose measurements.

showed a similar result as in the wildtype (Fig. 6E). In contrast, for the *ikk2*$^{m10/m10}$, the expression level of *pck1* showed an increase after the insulin injection (Fig. 6F). These results indicate that the inhibition of *pck1* induced by insulin is reversed in the *ikk2* mutant.

Our zebrafish Ikk2 mutant has demonstrated its value for studying a role of IKK2 in responses to insulin. The mechanism underlying these results might be related to the degradation of S6K protein in the absence of IKK2 activation (63). This suggestion is based on a previous report of Um et al. (64) who reported that knockout of S6K protected mice from obesity-induced insulin resistance. However, the conclusion that Ikk2 deficiency reverses insulin resistance has also been challenged by the study of Rohl et al (65), who showed that Ikk2 expression in mice skeletal muscle is not essential for obesity-induced insulin resistance in mice (65). Considering these conflicting results, additional studies of the function of IKK2 in insulin resistance are necessary. We expect that our zebrafish mutant will be of great use for such studies.

General conclusions

TLR signaling is involved in a multitude of physiological and pathological processes. The derivative question is how TLR signaling can be involved in so many processes? Therefore the mediators in TLR signaling have become major research targets. In the last decade, construction of mutations in rodent models became a common strategy to study the function of important genes in TLR signaling. However, the global deficiency of most of them has been shown to lead to a lethal phenotype in mice, and therefore the zebrafish model becomes a prominent candidate for further studies because of its suitability for studies at the embryonic and larval stages. For example, in our study the *Ikk2* mutant was shown to be a potential model to study the mechanism of acute insulin resistance in the larval stage. This is based on our conclusion that in the *ikk2*$^{m10/m10}$ mutant the acute insulin resistance induced by hyperinsulinemia was modulated. Furthermore, the Ikk2 zebrafish mutant showed defects in development and immunity, such as decreased body length, lower leukocyte numbers, lower expression level of marker genes of immune cells and higher bacterial proliferation upon Mm infection. Unexpectedly, we found that the *ikk2* mutant induced an overexpression of *ikk1*, which may play a compensatory role in NF-κB activation and *il1b* expression. In future research, the Ikk2 deficiency model provides a chance to further study the interaction between the innate immune system and metabolic diseases.

In addition, we can also expect that an effective mutant of Traf6 can be generated in the near future, which not only can be applied in studies of immune-related diseases but also metabolic diseases, like diabetes, as well. This is expected because it has been

reported that blocking Cd40-Traf6 signaling is a therapeutic target in obesity-associated insulin resistance in mice (66).

Materials and methods

Zebrafish husbandry

The *traf6*^{sa244} mutant allele was identified by sequencing of an ENU-mutagenized zebrafish library. The mutant line was obtained from the Sanger Institute Zebrafish Mutation Resource. We performed an incross between the Fo generation of Traf6 heterozygotes, and only one male Traf6 homozygote offspring survived. Outcrosses of the heterozygote lines unfortunately did not survive and therefore we could only continue with the incrossed homozygote line. The *ikk2*^{m10} mutant allele was obtained from Dr. Vladimir Korzh from the Institute of Molecular and Cell Biology (Singapore). Heterozygous and homozygous carriers of the mutation were outcrossed twice with the wildtype (AB/TL strain), and were subsequently incrossed twice. Homozygous fish of the resulting family were used to produce embryos.

Wildtype zebrafish of the AB/TL strain and strains *traf6*$^{sa244/sa244}$, *traf6*$^{+/+}$, *traf6*$^{sa244/sa244}$/*Tg*(*mpeg1.1:mCherryF/mpx:GFP*), *traf6*$^{+/+}$/*Tg*(*mpeg1.1:mCherryF/mpx:GFP*), *ikk2*$^{m10/m10}$ and *ikk2*$^{+/+}$ strain were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin. org). Embryos and larvae were raised in egg water (60g/ml Instant Ocean sea salts) at 28.5 °C. For the duration of bacterial injections, larvae were kept under anesthesia in egg water containing 0.02% buffered 3-aminobenzoic acid ethyl ester (Tricaine, Sigma-Aldrich, the Netherlands). The culture of zebrafish carrying mutations that might cause immune deficiencies was approved by the local animal welfare committee (DEC) of the University of Leiden. All protocols adhered to the international guidelines specified by the EU Animal Protection Directive 2010/63/EU.

Bacterial strain preparation

The bacterial strains, *Mycobacterium marinum* (ATCC#BAA-535) m20 (Mma20) and E11 (MmE11), containing the plasmid pSMT3-mCherry (67), were used for the infection of zebrafish larvae. For the infection assay, bacteria were prepared as previously described (68), where the injection inoculum was prepared in 2% polyvinylpyrrolidone40 (PVP40) solution (CalBiochem), and 150 colony-forming units (CFU) of Mma20 were injected into the blood stream at 28 hours post fertilization (hpf) as previously described (68). For the yolk injection, 40 CFU of MmE11 were injected into the yolk around the 64 cell-stage as previously described (68).

Ligands injection

Purified Pam3CSK4 (InvivoGen, France) and flagellin from *S. typhimurium* (Flagellin FliC VacciGrade™, Invitrogen, France) were diluted in 1 mg/ml and 100 µg/ml in sterile water, respectively. For injection, 1 nl of the ligand solutions were injected into the blood stream at 28hpf. Sterile water was injected as a control experiment. Injections were performed using a FemtoJet microinjector (Eppendorf, the Netherlands) equipped with a capillary glass needle.

Insulin injection

To inject PBS and human recombinant insulin (Sigma–Aldrich, the Netherlands), 1nl was injected into the caudal aorta of 4 dpf zebrafish larvae using a glass capillary.

Glucose measurements

Glucose measurements were done using a fluorescence-based enzymatic detection kit (Biovision, Inc., Mountain View, CA, USA) as described previously (69).

Morpholino injections

Morpholino oligonucleotides (Gene Tools) were diluted to desired concentrations in 1× Danieu's buffer (58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6mM Ca $(NO_3)_2$, 5.0mM HEPES (pH 7.6)) containing 1× phenol red (Sigma-Aldrich). For knockdown experiments, *traf6* ATG-morpholino (*traf6* mo, 5'-GCCTATACTGCTGCTTCCTGTAA AG-3') was injected with the optimal concentration at 0.5 mM and 1nl volume per embryo at 0~2 cell stage. Control larvae were injected with the standard control morpholino (Sc mo, 5'- CCTCTTACCTCAGTTACAATTTATA-3').

Immunohistochemistry

Embryos or larvae were fixed in 4% paraformaldehyde in PBS overnight at 4°C. Mpx activity was detected with TSA (TSA plus kit, Fluorescence Systems, Perkin Elmer Inc., Waltham,MA) as previously described (70). Immunostaining with L-plastin antibody (71) and the secondary antibody was as described (72).

Infection assays and imaging

Zebrafish larvae were staged at 28 hours post fertilization (hpf) by morphological criteria (73), and ~150 CFU of Mma20 m-cherry strain were injected into the blood island under the dorsal aorta, sterile PBS injection using as negative control. Injections were controlled using a FemtoJet microinjector (Eppendorf) and a micromanipulator with pulled microcapillary pipettes. Pools of around 20 larvae were collected at 3- and 4-day post infection (dpi) and performed imaging on a Leica MZ16FA Fluorescence Stereo Microscope. Bright field and fluorescence images were generated with a Leica DC500 (DFC420C) camera. Bacterial loads were analyzed using dedicated pixel

counting software as previously described (74). Experiments were performed in triplicate.

COPAS analysis

Zebrafish larvae were injected with ~40 CFU of MmE11 m-cherry strain at 64 cell stage, and the bacteria burden was measured with COPAS at 3, 4 and 5dpf as previously described (75).

Western blot analysis

Western blot analysis were performed as previously described (61).Whole homogenized zebrafish 1dpf embryos (n=100/sample) or 4dpf larvae (n=15/sample) were prepared for protein isolation. The protein concentration was determined using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The extracted protein samples were subjected to SDS–PAGE and transferred onto nitrocellulose membranes (Bio-Rad). The primary antibody is anti-TRAF6 (Abcam, EP591Y), and Pan-actin (Cell Signaling, no. 4968) was used as a loading control. Finally, the bands were quantified by densitometry using ImageJ Software (National Institutes of Health, Bethesda, MD, USA). The bands were quantified by densitometry using ImageJ 64 Software (National Institutes of Health).

Acknowledgments

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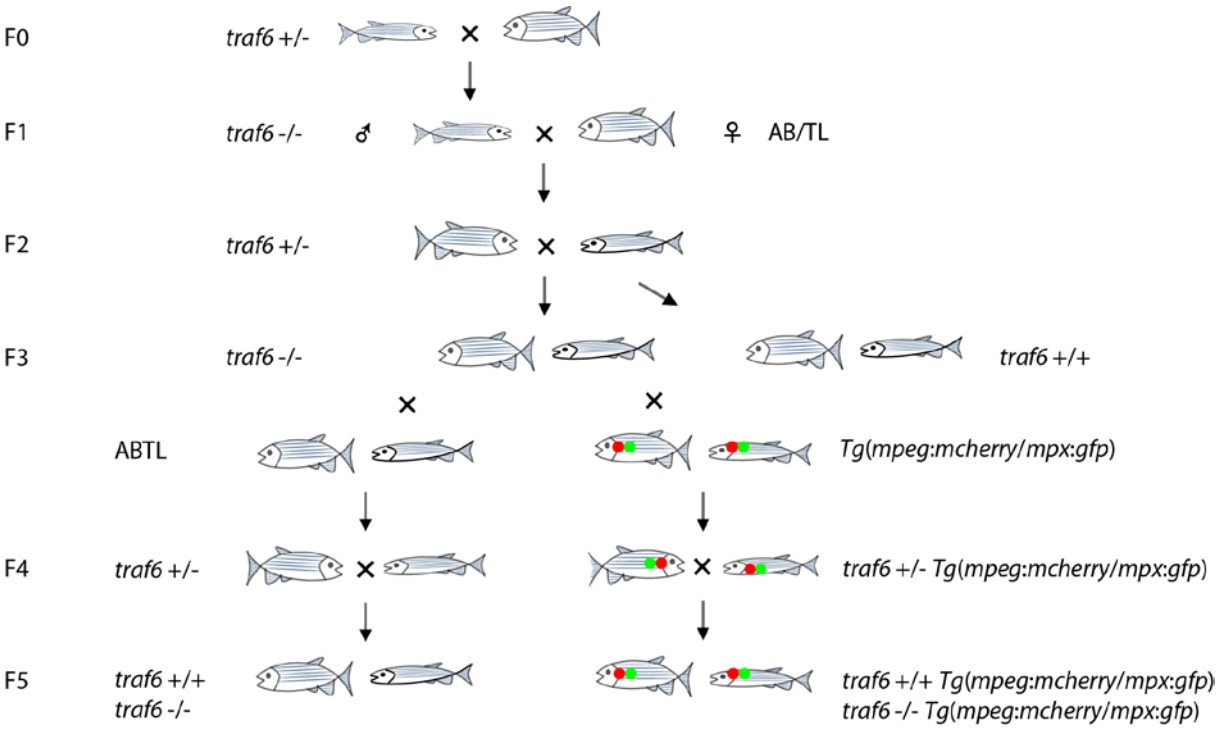
Supplementary data

Supplementary figure 1 Overview of the process of outcrosses and incrosses of the Traf6 mutant



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CHAPTER 5

Summary and general discussion

5

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).

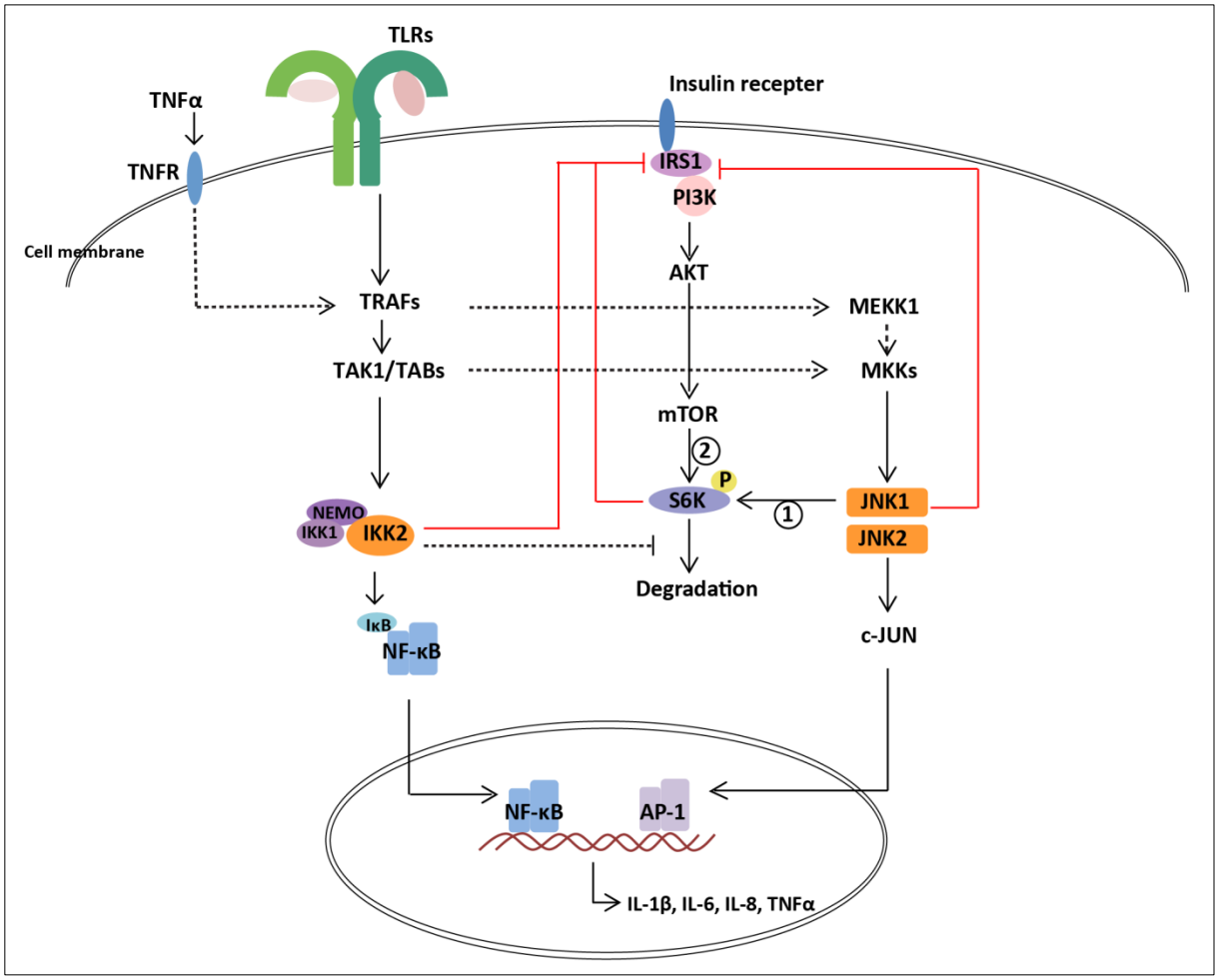

FIGURE 1: The interaction between TLR and insulin signaling in mammals. IKK2 and JNK1 modulate insulin signaling through targeting IRS1 and S6K. S6K negatively regulates IRS1 through phosphorylating Serine 307 and Serine 636/ Serine 639 sites on IRS1, which are involved in obesity- and/or diet-induced 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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Nederlandstalige samenvatting

In dit proefschrift beschrijf ik mijn studies naar de zebravisorthologen van de menselijke TLR5- en TLR2-receptorgenen. Dit zijn genen van het aangeboren immuunsysteem die verantwoordelijk zijn voor, respectievelijk, de herkenning van bacterieel flagelline en een breed spectrum aan bacteriële celwandcomponenten. Een belangrijk aandachtspunt van dit proefschrift is het verschil in het netwerk van genen dat door TLR5- en TLR2-signaaltransductie op transcriptioneel niveau wordt aangestuurd. Bovendien heb ik onderzoek gedaan naar andere genen die een centrale rol spelen in de TLR-signaaltransductie, waaronder TRAF6 en IKK2. Om de biologische functies van deze immuungenen te bestuderen heb ik onderzocht hoe zebravislarven reageren op infectie met de vispathogeen *Mycobacterium marinum*, een naaste verwant van *Mycobacterium tuberculosis,* de bacterie die bij de mens tuberculose veroorzaakt. Om inzicht te verkrijgen in de systeembiologie van genregulatie door TLR-receptoren heb ik gebruik gemaakt van nieuwe mogelijkheden voor genoombrede analyse van genexpressie, genaamd transcriptoomanalyse, waarbij alle geproduceerde RNA-moleculen worden gekwantificeerd met behulp van sequentiebepaling. Ook heb ik verschillende genetische methoden gebruikt om de activiteiten van genen te verminderen (knockdown) of uit te schakelen (mutatie) om daarmee de functies van genen in de TLR-signaaltransductie verder te onderzoeken.

In **hoofdstuk 2**, wordt aangetoond dat signaaltransductie door Tlr2 in zebravislarven op dezelfde manier functioneert als in zoogdiercellen. Dit is getest door analyse van de regulatie van genexpressie van bekende immuungenen na stimulatie van zebravisembryo's met lipopeptiden. Voor deze studie hebben we gebruik gemaakt van een stof genaamd Pam3CSK4. Dit is een synthetisch lipopeptide dat het geacyleerde aminozuuruiteinde van bacteriële lipoproteïnen nabootst en dat in experimenten met zoogdiercellen vaak gebruikt wordt om TLR2 te activeren. De transcriptoomprofielen die het gevolg zijn van de signaaltransductie door TLR2 en TLR5 na stimulatie door Pam3CSK4 en flagelline zijn bepaald met behulp van diepgaande sequentieanalyse van RNA-moleculen. De resultaten laten zien dat er een relatief kleine overlap is tussen de reacties op transcriptoomniveau na stimulatie door Pam3CSK4 en flagelline. De overlap omvat een groep van bekende immuunsysteemfactoren waarvan voorheen al aangetoond was dat ze geïnduceerd worden door flagelline, zoals de genen *il1b*, *tnfa*, *irak3*, *mmp9*, *cxcl-c1c* en *il8*. Opvallend is dat de genen *il6* en *il10*, die geassocieerd zijn met een anti-inflammatoire reactie, veel sterker geïnduceerd worden door Pam3CSK4 dan door flagelline. Een relatief veel grotere groep genen vertoonde een volledig verschillende reactie op Pam3CSK4 of flagelline, waaronder een groep genen waarvan de transcriptie alleen reageert op één van de twee behandelingen. De groep genen die specifiek alleen reageert op de stof Pam3CSK4 omvat een aantal transcriptiefactoren die meer dan twee keer omhoog gaan in expressie zoals *cebpb*, *fosb*, *nr4a1* en *egr3*. Er is vervolgens aangetoond dat de activering van de groepen van genen die specifiek reageren op behandeling met Pam3CSK4 of flagelline geblokkeerd wordt door het

uitschakelen van, respectievelijk, Tlr2 of Tlr5. Deze studie toont daarmee aan dat toediening van Pam3CSK4 of flagelline aan zebravislarven specifiek de signaaltransductie door Tlr2 of Tlr5 kan activeren met als gevolg een aantal reacties waarvan sommige algemeen zijn voor beide behandelingen en andere uitsluitend met één van de behandelingen optreden.

Om de functie van Tlr2 in de afweer van de gastheer tegen infectie door *M. marinum* verder te bestuderen hebben we, zoals beschreven in **hoofdstuk 3**, een infectiestudie uitgevoerd met zebravislarven die gemuteerd zijn in dit gen. De Tlr2-specifieke genen die in hoofdstuk 2 waren geïdentificeerd vertonen een significante verhoging van de transcriptie na infectie met *M. marinum* in het wildtype maar echter niet, of minder sterk, in de Tlr2-mutant. Bovendien bevatten ongeïnfecteerde Tlr2-mutanten een verminderd aantal macrofagen, wat mogelijk veroorzaakt wordt door een afwijking in de productie en differentiatie van hematopoietische stamcellen. Na infectie met *M. marinum* vertoont de mutant een verhoogde proliferatie van bacteriën en een gereduceerd aantal granulomen. De resultaten wijzen er ook op dat macrofagen in de Tlr2-mutant een hogere migratiesnelheid hebben, aangezien de maximale afstand van migratie van geïnfecteerde macrofagen vanaf een infectiebron groter was dan in de heterozygote controle. Dit zou mogelijk verklaard kunnen worden doordat Tlr2-deficiënte macrofagen minder actief zijn in fagocytose. Vervolgens zijn de transcriptoomreacties op infectie bepaald in de Tlr2-mutant en de heterozygote controle met behulp van RNA-sequentieanalyse. De resultaten tonen aan dat de mutatie van Tlr2 als gevolg heeft dat er veel minder verandering in de transcriptieniveaus van immuungenen plaatsvindt na infectie met *M. marinum*. Daarnaast zijn andere genen betrokken bij diverse processen, zoals oxidatie-reductie, DNA-herstel, transcriptionele regulatie en apoptose, afhankelijk van Tlr2 voor hun transcriptionele reactie op infectie. De interacties tussen de genen waarvan de regulatie afhankelijk is van Tlr2 zijn onderzocht met een methode voor de analyse van genetische netwerken. Een voorbeeld van een resultaat dat met deze netwerkanalyse is gevonden is dat de signaaltransductiemechanismen die door vitamine D worden gereguleerd normaalgesproken worden geactiveerd als gevolg van infectie, maar in de Tlr2-mutant juist worden uitgezet. Dit is interessant omdat al eerder was aangetoond dat vitamine D een belangrijke regulerende factor is tijdens de ontwikkeling van tuberculose en ook in studies met celculturen in verband is gebracht met de functie van Tlr2. Samenvattend kan uit deze resultaten geconcludeerd worden dat Tlr2 een rol speelt als beschermende factor tegen infectie met mycobacteriën.

Om de functies te bestuderen van factoren die een rol spelen in de signaaltransductie door de Tlr-receptoren is er in **hoofdstuk 4** een analyse uitgevoerd van zebravisstammen met mutaties in de genen *traf6* en *ikk2*. Deze mutaties betreffen puntmutaties die leiden tot een stopcodon in het begin van de eiwitleesramen van deze genen. Beide mutanten vertoonden geen zichtbare orgaanafwijkingen tijdens de

embryonale en latere ontwikkelingsstadia en de volwassen vissen waren ook in staat zich normaal voort te planten. Deze resultaten waren onverwacht omdat knaagdiermutanten waarin dezelfde genen zijn uitgeschakeld niet levensvatbaar zijn.

In het geval van de Traf6-mutant bleek er geen significant verschil met het wildtype wat betreft ontwikkeling van leukocyten en het verloop van het proces van infectie met mycobacteriën. Dit leidde tot de hypothese dat de bestudeerde mutatie in het *traf6*-gen niet resulteert in een uitschakeling van de functie op eiwitniveau. Dit is mogelijk het geval doordat er een alternatief startcodon dichtbij de mutatiepositie aanwezig is dat gebruikt kan worden om de eiwittranslatie opnieuw op te starten. Hoewel het ontbrekende gedeelte van dit hypothetische resulterende eiwit wel aanwezig is in de homologen van Traf6 in zoogdieren, is het overblijvende gedeelte mogelijk nog functioneel omdat dit het zogenoemde RING-domein bevat, dat bekend staat als een essentieel domein in het N-terminale gedeelte van het Traf6-eiwit. Het is echter niet bekend of de translatiemechanismen in de zebravis in staat zijn opnieuw op te starten na een stopcodon. Een andere mogelijke verklaring voor het gebrek aan fenotype van de Traf6-mutant is dat de mutatie gecompenseerd wordt door een andere mutatie in het genoom die het resultaat is van de gebruikte mutagenesemethode. Een aanwijzing voor deze verklaring is dat we slechts één homozygote mutant verkregen na het inkruisen van de eerste generatie heterozygote stamouders. Deze andere mutatie kan geleid hebben tot een verkeerde regulatie van naaste verwanten van het Traf6-eiwit die mogelijk de functie van Traf6 kunnen overnemen. Het is daarom noodzakelijk om nieuwe mutanten in het *traf6*-gen te construeren om deze mogelijkheden verder te onderzoeken.

Larven van de Ikk2-mutant vertoonden een significante afname in lichaamsgrootte, aantallen leukocyten en transcriptionele expressie van merkergenen van macrofagen en neutrofielen. Verder vertoonde deze mutant een toegenomen bacteriële proliferatie na infectie met *M. marinum* vergeleken met het wildtype. Een onverwachte waarneming was dat het bekende immuungen *il1b* een hogere expressie had na injectie van flagelline dan in de controle. Dit resultaat kan mogelijk verklaard worden doordat de expressie van het gen dat codeert voor Ikk1 verhoogd is in de Ikk2-mutant en dat dit gen een compenserende rol speelt om het *il1b*-gen aan te zetten. Deze hypothese kan verder getest worden door de constructie van dubbelmutanten van Ikk1 en Ikk2. Er is in voorgaand onderzoek aangetoond dat Ikk2 een rol speelt in insuline-signaaltransductie in zoogdieren. In overeenstemming hiermee hebben wij gevonden dat de resistentie tegen insuline die wordt geïnduceerd door een overmaat aan insuline gemoduleerd is in de Ikk2-mutant van de zebravis.

De hoofdconclusie die uit deze studies kan worden getrokken is dat TLR-signaaltransductie een essentiële rol speelt in de immuniteit en het bewaren van een natuurlijk metabool evenwicht. De transcriptoomprofielen die het resultaat zijn van

activering van verschillende TLR-receptoren zijn in dit proefschrift op systeemniveau beschreven. Hiermee is aangetoond dat er naast algemene effecten ook specifieke effecten zijn die door de TLR2- en Tlr5-receptoren worden geactiveerd en die nog niet eerder bekend waren (**Hoofdstuk 2**). Door gebruik te maken van een Tlr2-mutant is inzicht op het niveau van transcriptie en cellulaire reacties vekregen in de rol van Tlr2 bij de afweer van zebravislarven tegen infectie met *M. marinum* (**Hoofdstuk 3**). Deze Tlr2-mutant is erg geschikt voor toekomstige studies naar andere microbiële infecties, naar de reactie van de gastheer op de microbiële darmflora en voor onderzoek naar metabole afwijkingen zoals diabetes type II waarbij het immuunsysteem een belangrijk rol speelt. In de studie beschreven in **hoofdstuk 4** is gebleken dat de Ikk2-mutant naast een functie in de aangeboren afweer tegen mycobacteriële infectie ook een rol speelt in de regulatie van het metabolisme, zoals aangetoond door het effect van de mutatie op insulineresistentie die wordt geïnduceerd door een overmaat aan insuline. Zoals bediscussieerd in **hoofdstuk 5** biedt het werk in dit proefschrift daarom nieuwe mogelijkheden om de koppeling tussen het aangeboren immuunsysteem en de controle van metabole afwijkingen verder te bestuderen.

Curriculum Vitae

Shuxin Yang was born on November 18th, 1985 in Chifeng, China. She grew up there and graduated from Hongqi high school of Chifeng in 2004. Afterwards, she started her undergraduate study in Jilin University and majored in Veterinary Medicine. She obtained her bachelor degree in 2009 and started her postgraduate study in Microbiology and Immunology under the supervision of Prof. Dr. Liancheng Lei in the same university with a full scholarship. In 2012, she got her MSc degree and obtained a CSC scholarship for a PhD study at Leiden University. There she worked in the Institute of Biology under the supervision of Prof. Dr. Herman Spaink, Prof. Dr. Annemarie Meijer and Dr. Rubén Marín-Juez. During her PhD program, she focused on the study of Toll-like receptor (TLR) signaling function in the innate immune system in zebrafish larvae, as described in this thesis. In 2017, she will start her postdoctoral research in University College London under supervision of Dr. Gillian Tomlinson.

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