The Function of Toll-like receptor 2 in Infection and Inflammation
Hu, W.

Citation

Version: Publisher's Version
License: Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from: https://hdl.handle.net/1887/3247321

Note: To cite this publication please use the final published version (if applicable).
Chapter 1

General introduction:

The role of TLR2: from cell biology to therapeutic target
Chapter 1

1. Innate immunity and Toll-like receptors

1.1 Innate immunity

The host cells, rely on membrane-localized and germline-encoded pattern recognition receptors (PRRs) to initiate protective innate immune responses [2, 3]. PRRs recognize invading microbial pathogens through pathogen-associated molecular patterns (PAMPs) of the pathogens in combination with recognition of danger-associated molecular patterns (DAMPs) produced by infected or damaged tissues [2, 3, 15]. PRRs are comprised of four well-characterized groups, including Toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG)-I-like receptors (RLRs), the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and C-type lectin receptors (CLRs) [16]. TLRs and CLRs belong to transmembrane proteins, while RLRs and NLRs are cytoplasmic proteins [16]. TLRs are the most important and widely studied PRRs (See Fig 1).

![Figure 1 Publication counts for four PRRs in the NCBI database.](image)

**Key words:**
- Toll-like receptors
- C-type lectin receptors
- Nucleotide-binding oligomerization domain containing (NOD)-like receptors
- Retinoic acid-inducible gene-I (RIG)-I-like receptors

1.2 The structure of TLRs

The function of TLRs has been studied extensively in the last decades (Fig 1). Their capacity as a key control factor of innate immune responses makes them attractive therapeutic targets. TLRs are homologs of the *toll* gene that was first discovered to be involved in embryonic development in *Drosophila* [5, 6]. The investigation of TLRs became very intense after their function in defense against microbial infection in *Drosophila* and vertebrates was demonstrated [17]. TLRs are made up of an ectodomain, also known as the periplasmic extracellular domain,
Chapter 1

a cytoplasmic signaling domain, and a single transmembrane domain [3, 18]. The ectodomain of TLRs contains leucine-rich repeats (LRRs) and selectively recognizes PAMPs and DAMPs [3]. We summary the different PAMPs and DAMPs are recognized by the specific TLRs in Fig 2. The cytoplasmic signaling domain of TLRs comprises an evolutionary conserved Toll/IL-1 receptor (TIR) homology domain that is responsible for signal transduction [3]. Different species have different numbers of genes that encode TLRs. In the human genome, 10 TLRs are encoded, whereas the mouse and zebrafish genomes encode at least 12 and 17 TLRs, respectively [19-21]. TLRs can be divided into two subgroups based on their cellular location. TLRs are expressed either on the cell surface or in intracellular compartments. In humans, TLR1, 2, 4, 5, 6 and 10 are expressed on the cell surface, while TLR3, 7, 8 and 9 are localized in intracellular membranes [3, 22]. In mice, the cellular distribution of the conserved TLRs is assumed to be the same as the distribution in humans, while TLR12 is expressed on the cell surface and TLR13 is probably expressed within endosomes [22]. Interestingly, TLR11 can be expressed on both cell surface and intracellular compartments [23].

1.3 TLR2, an important member of the TLR family

After the identification of TLR2 in 1998, much progress has been made in our understanding of its function [9, 24]. Its functions, in the recognition of a large number of ligands, including PAMPs and DAMPs, are complicating the studies of the underlying recognition mechanisms (Fig 2). In addition, the ubiquitous distribution of TLR2 on various types of cells, e.g., immune, endothelial, and epithelial cells, also determines its wide range of functions [9]. Considering the broad functions of TLR2, the drive for the development of TLR2 related therapeutic targeted vaccine or treatment has accelerated in the last decades [7, 25, 26]. However, some studies on TLR2 are still controversial [7]. It is because of the complex functions of TLR2 that its role in immune regulation is not black or white [8]. For example, TLR2 plays a dual role in infection processes. TLR2 has been shown to play a protective role during infection by triggering a strong pro-inflammatory response which is considered beneficial for bacterial clearance [27, 28]. However, excessive inflammation caused by TLR2 can lead to tissue damage and even affect healing of damaged tissues [11]. The mechanisms of TLR2 signaling and its regulation are discussed in detail below.
2. Regulation of TLR2 signaling

2.1 TLR2 signaling pathway

The binding of the LRR domain in TLRs and its ligands stimulates the recruitment of adaptor proteins to interact with the intracellular TIR domain in TLRs to trigger the downstream cascades. Myeloid differentiation factor (Myd88) is a well-known adaptor protein that interacts with almost all TLRs except TLR3 [3]. TIR domain-containing adaptor protein (Tirap), which is also called Myd88 adaptor-like (Mal), is required in the TLR2/6 signaling via Myd88 while it is not necessary in the TLR2/1 signaling [29]. In addition to Myd88 and Tirap, other adaptor proteins in mammalian cells include TIR domain-containing adaptor protein inducing interferon-β (TRIF), TIR-containing adaptor molecule (TICAM), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo motif-containing protein (SARM) [30]. The recruitment of distinct adaptor proteins can trigger different downstream signaling pathways. Recent reviews have discussed in detail the known differences between downstream signaling pathways of the mammalian TLR receptors [3, 31, 32] and therefore we only briefly describe TLR2 signaling here and summarize it in Fig 3.

After the interaction of TLR2 and its associated adaptor proteins, the IRAK complex is activated to recruit TRAF6. Activated TRAF6 triggers the activity of a complex of TAK1/TABs to stimulate both the activation of the MAPKs and the IKK complex (IKK1, 2, and IKK-γ, also named NEMO). The MAPKs family includes, but is not limited to JNKs and p38. The IKK complex promotes the nuclear translocation of NF-κB. In the end, the production of pro-inflammatory cytokines is induced by the AP-1 and NF-κB, which in turn controls inflammation and modulates cell survival and proliferation [9, 33].
TLRs and its ligands. TLRs can recognize PAMPs from invading microbial pathogens and DAMPs from infected or damaged tissue. TLR2, its heterodimers and TLR4 recognize pathogens through their cell wall surface components. TLR2 conjugates with TLR1 or with TLR6 to sense triacyl or diacyl lipopeptides, and lipoteichoic acid (LTA) on the cell wall of gram-positive bacteria, and mycobacteria [9, 34-36]. The process of the recognition of triacyl or diacyl lipopeptides by heterodimers needs the participation of accessory molecules. For example, CD14 and CD36 are well characterized as ligand delivery molecules to enhance TLR2 responses to ligands especially with a lower concentration of ligands, although the participation of these molecules is not essential [9, 36]. TLR4 can sense Gram-negative bacteria through the lipopolysaccharide (LPS) located on their outer membrane [35]. During this process, the formation of a complex of TLR4 with MD2 and CD14 is essential for recognizing LPS [35, 37]. TLR5 functions in the recognition of flagellin from bacterial surfaces. There is still relatively little knowledge about the function of TLRs in the recognition of DAMPs compared with its function in there cognition of PAMPs. TLR3, 7, and 9 have been reported to play a role to sense these nucleic acids released from damaged cells [38, 39]. It has been demonstrated that TLR2 and TLR4 can be activated by the intracellular proteins or extracellular matrix components released from damaged cells [38, 39]. It is controversial that DAMPs directly interact with extracellular TLRs, during this DAMPs recognition process [38]. It has been shown that recognition can be indirect for instance by the involvement of high-mobility group box 1 protein (HMGB1), which is a widely studied endogenous danger signal that induces inflammatory response through its interaction with DAMPs recognized by TLR2, TLR4 and TLR9 [40, 41].
2.2 Negative regulation in TLR2 signaling

Accumulating evidence has been reported that the activation of TLR2 signaling is a benefit for the host defense against invading pathogens [42-44]. Excessive activation of TLR signaling, can lead to over-expression of pro-inflammatory cytokines, which have been implicated in chronic inflammatory diseases, autoimmune diseases, and even aggravation of infection diseases [45-47]. For example, a deficiency of TLR2 in diabetic mice can accelerate wound healing, which indicates that excessive activation of TLR2 signaling might be harmful for wound healing [48]. Thus, it appears that TLR2 signaling needs to be tightly regulated by negative regulation mechanisms that are still poorly understood. Recent reviews have summarized many different mechanisms of negative regulation and their molecular components [49-52]. Negative regulators are including the following categories of molecules: ubiquitin ligases, deubiquitinases, transcriptional regulators, and microRNA, which induce different negative regulations [52]. The mechanisms inhibiting TLR2 signaling are based on (1) prevention of receptor-ligand binding; (2) dissociation of adaptor complexes; (3) inhibition of TLR2 downstream kinase signaling; (4) negative transcriptional regulation by TLR2 and other factors as described below [50, 51].

Soluble TLR2, which is a smaller isoform of the TLR2 protein that has been reported to be secreted by human monocytes, has been characterized to compete with TLR2 located on cell membranes to bind with the ligands to inhibit TLR2 signaling [53, 54]. As a negative regulator that leads to dissociation of adaptor complexes it has been shown that a short form of Myd88 (sMyd88) is unable to bind with IRAK4 and thereby its expression can inhibit NF-κB activation [55]. Another described mechanism for inhibition adaptor signaling is the induction of TIRAP degradation by the suppressor of cytokine signaling 1 (SOCS1) [56]. Moreover, TRIF can be degraded by a disintegrin and metalloprotease 15 (ADAM15) in a TRIF-dependent pathway [57]. In terms of TLR2 downstream kinase signaling inhibition, Toll-interacting protein (TOLLIP) inhibits the TLR2 signaling by targeting with IRAK1 to suppress its phosphorylation or directly interacting with TLR2 [58, 59]. Thus, TOLLIP is widely utilized as an inhibitor to inhibit TLR2 signaling [59]. IRAK-M is another IRAK inhibitor, which belongs to the IRAK kinase family but cannot induce NF-κB activation [60]. In addition, tyrosine phosphatase SHP-1, CD300a, and CD300f are also reported as IRAKs inhibitors. SHP-1 inhibits IRAK1 activation by interaction with a kinase tyrosine-based inhibitory motif (KTIM) [61], and CD300a/f can associate with SHP-1 to inhibit signaling [62]. In addition to the inhibitors
targeting IRAKs, proteins bind with TRAF6, namely A20 (also called TNF-α induced protein 3, TNFAIP3), a non-receptor tyrosine kinase Sky, NLR family member X1 (NLRX1), and the cylindromatosis protein (CYLD) have also been shown to be negative regulators of TLR2 signaling [63]. Furthermore, A20 and NLRX1 can also block the activation of the IKK complex [64]. The last category is the negative regulatory of transcription. The transcription of some pro-inflammatory genes, like IL-6, are negatively regulated by transcription factor 3 (ATF3) [65], A TLR-inducible IκB protein (IκBNS) [66], and B-cell CLL/lymphoma 3 (Bcl-3) [67]. It is not yet known how these negative regulators are controlled. It has been hypothesized that Myd88 might be involved in a feedback loop that could be under control of TLR2 or other Toll like receptors [68]. Therefore, it is very likely that TLR2 is an important control factor of negative regulation of transcription of genes involved in inflammation.
Figure 3 A brief overview of the TLR2 signaling pathway in mammals [9, 33]. To be noted, the shown TLR2 signaling components are not exclusive for this TLR receptor, and the phosphorylation and ubiquitination processes are not mentioned in this figure. TLR2 or its heterodimers are ubiquitously located on the cell membranes. The TLR2 signaling activation through TLR2/1 requires the participant of accessory molecule CD14, while TLR2/6 requires CD36. The TLR2 signaling pathway is activated after TLR2 ligand-recognition (PAMPs or DAMPs). Subsequently, the adaptor proteins, Myd88 and Tirap/Mal, are recruited. After a series of cascades involving NF-κB and MAPKs, various transcription factors are activated to induce pro-inflammatory cytokines.
Chapter 1

3. TLR2 function in mycobacterial infection studies

3.1 Tuberculosis and non-tuberculosis disease

Tuberculosis (TB) is a communicable disease, which is caused by infection with *Mycobacterium tuberculosis* (Mtb) [69]. At present, TB remains one of the top 10 diseases which give rise to death and currently its death toll is higher than that caused by other major infectious disease such as malaria, AIDS or COVID-19 [69]. It has been reported that in recent years, incidence and death of TB are falling, but it is not reaching any of the global TB eradication goals set by the WHO [70]. Currently, in the COVID-19 pandemic situation the numbers of deaths due to tuberculosis are rapidly rising due to a lack of diagnostic and treatment capacity [69]. Nontuberculous mycobacteria (NTM) diseases are defined as caused by mycobacterial pathogens other than Mtb and *Mycobacterium leprae* [71]. Besides TB, NTM infection diseases have recently attracted wide attention because the disease prevalence of NTM infection diseases is increasing sharply since 2000 [72]. It is hard to combat TB or NTM infection incidence due to the rapid increase in multi-drug resistant mycobacterial strains [69, 73]. Therefore, there is an urgent need to discover novel preventive or therapeutic strategies for TB and NTM infection diseases. Currently, host-directed therapies (HDT) are one of the most promising strategies to combat NTM infectious diseases by making the NTM antibiotic treatment regimens more effective [73, 74]. In mycobacterial infection, TLR2 is a key receptor to recognize mycobacteria, modulate immune cells recruitment, modulate phagocytosis, and trigger pro-inflammatory responses to eliminate the invading mycobacteria [9]. Thus, it is helpful to discover new host directed therapeutic targets by better understanding the mechanism of TLR2 modulating the host-mycobacterial interactions.

3.2 TLR2 recognizes mycobacterial components

As we described in this introduction, TLR2 plays a crucial role in recognizing bacteria such as Mtb through their cell wall components [75]. TLR2 lipoprotein ligands of the cell surface of Mtb include 19-kDa lipoprotein (Rv3763, LpqH), 24-kDa lipoproteins (Rv1270c, LprA and Rv1411c, LprG), and 38-kDa glycolipoprotein (PhoS1). Other categories of TLR2 ligands include lipoarabinomannan (LAM), lipomannans (LM), phosphatidylinositol dimannoside (PlMs) and trehalose dimycolate (TDM), and mycobacterial heat shock protein 70 (HSP70). The TLR2 ligands from Mtb are briefly summarized and described in Table 1 [76]. As we described in this introduction these ligands activate macrophages by activating NF-κB through TLR2 (Fig 3). However, prolonged TLR2 signaling triggered by these ligands might help Mtb
Chapter 1

to evade immune surveillance. For example, long-term exposure of macrophages to LpqH, LprG, LprA, PhoS1, LM, and PIM leads to IL-10, IL-4, and TGF-β expression, which in turn inhibits the activation of macrophages [76]. Furthermore, it has been demonstrated that prolonged TLR2 signaling activated by LpqH and LprG inhibits the expression of MHC class II molecules and exogenous antigen processing for presentation to CD4+ T cell, which can be a basis for Mtb immune evasion (Fig 3) [77-80].

3.3 TLR2 is associated with susceptibility to various mycobacteria

The structural integrity of TLR2 is crucial for defense against invading pathogens. Single nucleotide polymorphisms (SNPs) in human TLR2 have been reported to associate with increased susceptibility to infectious diseases [81]. For example, one of the TLR2 polymorphisms, Arg753Gln has been demonstrated to lead to higher susceptibility to TB [82]. Moreover, the TLR2 polymorphism R753Q impairs the activation of TLR2 signaling upon M. smegmatis infection [83]. These studies indicate that TLR2 plays a protective role in mycobacterial infection diseases, although a small number of studies found no effect of TLR2 polymorphism [8, 84, 85]. Thus, an animal model for TLR2 polymorphisms is needed to investigate the functions of the polymorphisms in tuberculosis.

Mice is a widely used animal model to study the function of TLR2 in resistance to mycobacterial infection. The evidence for the role of TLR2 in defense against NTM infection is still limited, and no correlation has been found between TLR2 polymorphism and human susceptibility to NTM infection until now [86]. Interestingly, TLR2−/− mutant mice were more susceptible to M. avium infection [87]. It has been demonstrated that TLR2 deficient mice, but not TLR4 deficient mice, were more susceptible to a high dose Mtb infection than wild type mice [88-90]. However, the results of the studies of the role of TLR2 in low-dose Mtb infection are controversial. As a result, it is not clear at which infectious stages TLR2 functions in defense against Mtb infection: it is undecided whether it functions at an acute infectious stage or chronic infectious stage. Some researchers hypothesize that TLR2 plays a protective role during Mtb chronic infection while it does not affect Mtb acute infection [89, 91, 92]. In contrast, Tjärnlund et al. demonstrated that TLR2 has a function in Mtb acute infection (at 3 weeks post infection, wpi) but not in Mtb chronic infection (at 8 wpi) [27]. Interestingly, other studies found no significant differences between TLR2 defective mice or wild type mice upon low-dose Mtb infection, in both acute or chronic infection [88, 93, 94]. The reasons for these different results may be because (1) different Mtb strains were used. Most researchers used the Mtb H37Rv
Chapter 1

strain, while some studies utilized the Mtb Kurono strain or the Mtb Erdman strain. (2) They application of different infection methods. Aerosol challenging is the most extensively used method, but some studies also use intranasal (i.n.), intravenous (i.v.), or intratracheal infection methods (i.t.). (3) Differences in the definition of acute or chronic infection. For example, how long is an infection considered a chronic infection? In some studies, 8 weeks are considered as a chronic infection, while other studies 21 weeks is taken as a threshold. In summary the lack of standardization in mice studies has given rise to many uncertainties as to the function of TLR2 in defense against tuberculosis.

3.4 Macrophage-mycobacterium interactions mediated by TLR2

Macrophages are not only the primer cells to recognize the invasion of mycobacteria, but are also the main cellular components of granulomas [95]. TLR2 plays an essential role in mediating the interaction of macrophages and mycobacteria. At the early infection stage, TLR2 enhances the entrance of Mtb bacteria into macrophages by binding PE_PGR33, a mycobacterial protein from the Mtb [96]. The binding of TLR2 and PE_PGR33 can activate macrophages by inducing the expression of TNF-α and some other pro-inflammatory cytokines (Table 1), while it can also trigger the PI3K pathway that can impair the macrophage antimicrobial responses [96]. In addition to promoting inflammatory responses, TLR2 also plays a role in promoting apoptosis of macrophages [97], which is an important defense mechanism of the host against intracellular pathogens. For example, Sánchez et al. has reported that the apoptosis triggered by Mtb infection is depending on the TLR2 signaling pathway [98]. In addition, it has been demonstrated that the apoptosis induced by ESAT-6 is a TLR2-dependent event [99]. ESAT-6, an abundantly secreted protein of Mtb is an important virulence factor. Furthermore, TLR2-dependent microRNA-155 (miR-155) expression is required to elicit macrophage apoptosis by Mycobacterium bovis BCG [100]. The antimicrobial activity of macrophages is an essential function of the host to combat invading mycobacteria and is mediated by TLR2 [101]. In human macrophages, the stimulation of TLR2 by mycobacteria results in the upregulation of Cyp27B1 and VDR, which have a function in the induction of transcription of antimicrobial factors, like the antimicrobial peptide cathelicidin [102, 103]. To be noted, mouse macrophages and human macrophages utilize different mechanisms to kill intracellular Mtb through TLR2 activation [28]. TLR2 activation leading to killing of intracellular bacilli is an iNOS-dependent process in mouse macrophages, whereas human macrophages do not depend on it [27]. Thus, other animal models are needed to confirm the
Chapter 1

TLR2-mediated mechanisms of triggering macrophage antimicrobial activity. There is only one *in vitro* study describing how mycobacteria can directly control macrophage migration by rearranging the cytoskeleton via activation of TLR2 [104]. Konowich et al. have demonstrated by using various chimeric mice that TLR2 signaling in hematopoietic cells plays a role in controlling bacterial burden and granuloma integrity, while TLR2 signaling in non-hematopoietic cells may play a role in promoting granulomatous inflammation and bacterial dissemination [92]. Furthermore, Carlos et al. found that TLR2<sup>−/−</sup> mice displayed increased bacterial burden, diminished myeloid cell recruitment, and defective granuloma formation [90]. Interestingly, the adoptive transfer of TLR2 positive mast cells into these TLR2<sup>−/−</sup> mice reversed the phenotype [90]. In conclusion, TLR2 participates in mediating macrophage-mycobacteria interactions in many ways during phagocytosis, apoptosis, antimicrobial activity, cell recruitment, and granuloma formation. But, the mechanisms underlying these function of TLR2 are still not clear and need to be further studied.

3.5 Neutrophil-mycobacterium interactions mediated by TLR2

In addition to macrophages, neutrophils are innate immune cells that have an important function in defense against mycobacterial infection. A large number of neutrophils can be detected in TB lesions and in the sputum of TB patients, which indicates that neutrophils play a crucial role during Mtb infection [105, 106]. There is consensus that neutrophils are activated upon mycobacterial infection via TLR2-mediated recognition of LAM on the surface of bacteria (Table 1) [107]. However, the reports on the function of TLR2 in regulation of the recruitment of neutrophils during mycobacterial infection are contradictory. In TLR2<sup>−/−</sup> mutant mice, the bacterial burden after Mtb infection was increased compared to the wild type, and this was accompanied by increased neutrophil influx in the lungs and tissue damage [108]. Conversely, after alveolar epithelial cells were infected by *Mycobacterium bovis BCG in vitro*, the recruitment of neutrophils was significantly reduced by blocking TLR2 [109]. Moreover, injection of non-mannose-capped lipoarabinomannnan (AraLAM), which is a TLR-ligand from *Mycobacterium smegmatis*, led to a stronger reduction of neutrophils influx in the pulmonary compartment in TLR2<sup>−/−</sup> mice than compared to WT mice [110]. These results demonstrate that it is important to study, the function of TLR2 in neutrophils migration in further detail.

3.6 Therapeutic targeting of TLR2 signaling in mycobacterial infection disease

TLR2, as one of the most important representatives of PRRs, can recognize many mycobacterial components which are known as PAMPs. Some of these TLR2 ligands constitute the main
protein component of TB vaccines or adjuvants [96]. For example, the ESAT-6 and PPE18 proteins (Rv1196) are important components of the M72/AS01 and H56/IC31 vaccine candidates [96]. In addition, the mycobacterial MPT38 and PE_PGRS33 proteins have been reported to be TLR2-targeted secreted proteins that are promising pulmonary TB vaccines [96, 111]. At present, *Mycobacterium bovis Bacille Calmette and Guérin* (BCG) remains the only available vaccine for TB, but it is only validated for prevention of TB in children [112, 113]. Furthermore, there is no effective vaccine for infection disease caused by NTM strains.

TLR2 plays a dual role to trigger both pro-inflammatory and anti-inflammatory responses after infection. Thus, modulation of TLR2 signaling has become a popular approach for the design of host-directed therapeutics against infectious diseases. A recent review has described in detail how TLR2 could be used as a therapeutic target to cure bacterial infections [114]. TLR2 ligands from mycobacteria constitute a large group of natural TLR2-agonists and TLR2-antagonists which we summarize in Table 1. These TLR2 agonists or antagonists not only can be used to study the function of TLR2 in infectious diseases, but also provide new possibilities as therapeutic that target TLR2 signaling to treat hyper-inflammation and auto-inflammatory diseases. For example, recombinant PPE18 protein (rPPE18), which is a TLR2 ligand derived from Mtb, has been demonstrated to be a promising novel therapeutic to control sepsis [115]. Because rPPE18 significantly decreases the secretion of serum pro-inflammatory cytokines and reduces organ damage in mice infected with high doses of *E. coli* bacteria [115].

4. **Zebrafish as a model to study the innate immune system**

4.1 **General advantages of the zebrafish larval model**

In the last decades, extensive disease models have been established for using zebrafish larvae to study hematology [116, 117], oncology [118] and other pathogenic processes [119]. Zebrafish models contributed to uncovering pathogenic mechanisms and to the discovery and efficacy screening of innovative drugs [120, 121]. As an animal model, zebrafish possesses various advantages. The zebrafish larvae already have a functional innate immune system within 5 days post-fertilization, when the adaptive immune system is still not functional, providing a great advantage for studying the mechanisms of acute inflammation[122]. Moreover, its optical transparency and small size are the most significant advantages of the zebrafish embryos and larvae, because it provides an ideal *in vivo* system to directly observe cell-cell or cell-microbe interactions [122]. This is very difficult to achieve in other vertebrate
models. In addition, the large number of zebrafish offspring makes it possible for omics studies of large groups of larvae. In this thesis, we make use of these advantages of zebrafish larvae to investigate the role of the innate immune system in inflammation and defense against mycobacteria.

4.2 Confocal real-time imaging as a tool for investigating cell function in zebrafish larvae

Cell migration is an important physiological parameter for many pathological processes, including inflammatory responses, immune defense and metastasis of malignant tumor cells. Single-cell tracking using confocal real-time imaging is one of the most popular methods to analyze cell migration. Transgenic zebrafish larvae with fluorescently labeled cells are highly suited for non-invasive real-time imaging because of their transparency at early developmental stages. To visualize and quantify the trajectories of cell migration, a large number of algorithms and software programs have been developed in the last decades. In this thesis we also have developed new automated methods for cell tracking (chapter 3). Notwithstanding these efforts, for much biological research it is needed to track the cell movements manually. In the discussion chapter of this thesis, we will discuss future research directions that could make such time-consuming steps of cell migration research less of a bottleneck.

Overview of this thesis

TLR2 plays a pivotal role in triggering the innate immune responses in inflammation and infection. This makes TLR2 an attractive therapeutic target for developing cures to many diseases. However, its dual role in inflammation and infection makes it very difficult to use the available results from basic research for the development of clinical trials. In addition, it is still not clear and controversial what is the function of TLR2 in regulating phagocytic cell migration. In this thesis, we used the advantage of the transparent zebrafish larval model to observe the dynamics of cell migration \textit{in vivo} under various conditions. We not only aimed to acquire new insights into the function of Tlr2 in regulating cell migration in inflammation and infection, but also aimed to extend our understanding of the role it thereby plays in host defense against pathogens.

The role of TLR2 in host defense against Mtb is still controversial in reports based on rodent \textit{in vivo} studies. In addition, its function in host innate immunity during infection is still not clear.
Moreover, there is still a lack of research of the systemic transcriptome regulation of TLR downstream signaling in a whole animal model after infection. Therefore, in Chapter 2, we study the function of tlr2 in defense against *Mycobacterium marinum* (Mm) infection in zebrafish by measuring infection phenotypes and corresponding transcriptome responses. We show that infection of a tlr2 mutant in zebrafish larvae leads to a higher Mm bacterial burden, accompanied with a lower number of granulomas and increased extracellular bacterial growth, compared to wild type siblings. These results suggest that Tlr2 plays a protective role in defense against mycobacteria at early infection stages. To obtain explanations and genetic markers for further studies of the effect of the tlr2 mutation on infection we performed deep RNA sequencing to study the whole transcriptome profile in our mycobacterial infection model at the systems level. From this RNAseq analysis, we found that the role of Tlr2 in controlling mycobacterial infection can be explained by several mechanisms, like reduction of mycobacterial dissemination by dampening of CXCR3-CXCL11 signaling, and modulation of anti-mycobacterial activity by regulating vitamin D signaling.

In a previous study, Torraca et al. found the CXCR3-CXCL11 axis signaling executes an important function in regulating macrophage recruitment in zebrafish larvae [123]. In chapter 2, we found that the expression of *cxcl11aa* and *cxcl11ac* is significantly decreased in the tlr2 mutant infected with *M. marinum*. Considering that the expression of many chemokines is controlled by Tlr2, we hypothesized that Tlr2 is a key factor in the control of chemokine expression in order to regulate cell recruitment in innate immunity. To test this hypothesis, in Chapter 3, we first investigated the function of Tlr2 and Myd88 in leukocytes migration behavior upon tissue damage by using a zebrafish tail wounding model. In this chapter, live fluorescent imaging was performed to study the effect of the tlr2 mutation and myd88 mutation on leukocyte migration upon tail wounding. We observed reduced numbers of recruited neutrophils and macrophages at the wounding area in both tlr2 mutants and myd88 mutants, compared to their wild type sibling controls. Extensive mathematical analyses have been performed of the cell migration trajectories in the zebrafish larvae upon wounding. Through these analyses, we demonstrated that both tlr2 and myd88 control the migration direction of neutrophils upon wounding. Furthermore, in both the tlr2 and the myd88 mutants, macrophages migrated more slowly toward the wound edge.

The migration of leukocytes is important during the infection process for bacterial clearance, containment, dissemination, and granuloma formation at the early mycobacterial infectious
stage [124-127]. However, the role of Tlr2 in modulating leukocyte migration in infection is still not clear. In chapter 3, we show that tlr2 is involved in regulating leukocyte migration in response to inflammatory signaling. Thus, we hypothesize that tlr2 could also be involved in the regulation of migratory behavior of macrophages and neutrophils to the sites of mycobacterial infection. To test this hypothesis, in Chapter 4, we studied the function of tlr2 during the infection with two different NTM mycobacterial species with special attention to the responsive cell migration behavior. In this chapter, *M. marinum* Mma20 strain and *M. avium* MAC 101 strain were used to study the function of tlr2 in infection. *M. marinum* infected zebrafish larvae is a recognized model for tuberculosis infection, whereas *M. avium* was never studied in zebrafish before. Thus, we first developed a zebrafish larval infection model for studying *M. avium* infection. The results show that *M. avium* bacteria can infect zebrafish larvae effectively leading to the formation of granulomas. Moreover, we compared the innate immune response of zebrafish larvae to infection with these two different species of NTM, specifically with regard to the bacterial burden, granuloma-like cluster formation, and transcriptomic gene expression profiles. Subsequently, we utilized this model to study the function of tlr2 in regulating leukocyte migration using a tail fin infection method.

In the last Chapter 5, we summarize the findings from the thesis and discuss the challenges and perspective for further research of TLR signaling by using the zebrafish larval model. In addition, we briefly discuss some unpublished results from ongoing studies into the function of TLR2 in system metabolism.
<table>
<thead>
<tr>
<th>Species</th>
<th>Ligand(s)</th>
<th>Ligand(s) abbreviation</th>
<th>PRRs</th>
<th>Accessory molecules</th>
<th>Observations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lipoproteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>19-kDa lipoprotein (Rv3763)</td>
<td>LpqH</td>
<td>TLR2/1; CD14</td>
<td>CD14</td>
<td>Inhibits MHC- expression and antigen processing; IFN-γ-induced genes is inhibited by prolonged LpqH stimulation</td>
<td>[77-79]</td>
</tr>
<tr>
<td></td>
<td>24-kDa lipoprotein (Rv1270c)</td>
<td>LprA</td>
<td>TLR2/1; CD14/CD36</td>
<td>CD14/CD36</td>
<td>Induces cytokine response and regulates APC function</td>
<td>[79, 128]</td>
</tr>
<tr>
<td></td>
<td>24-kDa lipoprotein (Rv1411c)</td>
<td>LprG</td>
<td>TLR2/1; TLR2</td>
<td>CD14</td>
<td>Long-term exposure of LprG inhibits the processing of MHC-II antigen; Short-term exposure of LprG induces the production of TNF-α</td>
<td>[79, 80]</td>
</tr>
<tr>
<td></td>
<td>24-kDa lipoprotein (Rv1016c)</td>
<td>LpqT</td>
<td>TLR2</td>
<td>Unknown</td>
<td>Induces TLR2-dependent apoptosis in macrophages and inhibits MHC- expression and antigen processing</td>
<td>[129]</td>
</tr>
<tr>
<td></td>
<td>38-kDa glycolipoprotein</td>
<td>PhoS1</td>
<td>TLR2/1, TLR4</td>
<td>Unknown</td>
<td>Activates the ERK1/2 and p38 MAPK signaling, which in turn induces TNF-α and IL-6 expression</td>
<td>[79, 130]</td>
</tr>
<tr>
<td><strong>Lipoglycans/Glycolipids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>Lipoylated and glycosylated Mtb lipoprotein (Rv2873)</td>
<td>MPT83</td>
<td>TLR2</td>
<td>unknown</td>
<td>Mpt83 induced cytokine production was decreased in the TLR2 deficient mice</td>
<td>[131]</td>
</tr>
<tr>
<td>Lipoarabinomannan</td>
<td>LAM</td>
<td>TLR2/1; TLR2</td>
<td>CD14</td>
<td>Mtb LAM induces the production of pro- and anti-inflammatory cytokine to activate neutrophils</td>
<td>[75, 107]</td>
<td></td>
</tr>
<tr>
<td>Arabinosylated lipoarabinomannan</td>
<td>AraLAM</td>
<td>TLR2</td>
<td>Unknown</td>
<td>Induces the pro-inflammatory responses</td>
<td>[132]</td>
<td></td>
</tr>
<tr>
<td>Lipomannans</td>
<td>LM</td>
<td>TLR2/1; TLR2</td>
<td>CD40/CD86</td>
<td>Induces TNF-α and NO secretion to activate macrophages</td>
<td>[133, 134]</td>
<td></td>
</tr>
<tr>
<td>phosphatidylinositol dimannoside</td>
<td>PIM2/6</td>
<td>TLR2</td>
<td>Unknown</td>
<td>Induces the expression of TNF-α to activate macrophages</td>
<td>[75, 135]</td>
<td></td>
</tr>
<tr>
<td>Trehalose dimycolate</td>
<td>TDM</td>
<td>TLR2</td>
<td>CD14/MARCO</td>
<td>Induces NF-kB signaling</td>
<td>[136]</td>
<td></td>
</tr>
</tbody>
</table>
Table 1 Continued. Mycobacterial ligands of TLR2

<table>
<thead>
<tr>
<th>Species</th>
<th>Ligand(s)</th>
<th>Ligand(s) abbreviation</th>
<th>PRRs</th>
<th>Accessory molecules</th>
<th>Observations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Others</strong></td>
<td>Heat shock protein 70</td>
<td><strong>HSP70</strong></td>
<td>TLR2</td>
<td>Unknown</td>
<td>Inhibits the secretion of IL-6 in TLR2-deficient macrophages</td>
<td>[137]</td>
</tr>
<tr>
<td>55-kDa flavin containing monoxygenase (Rv3083)</td>
<td><strong>MymA</strong></td>
<td>TLR2</td>
<td>CD40/CD80/CD86/HLA-DR</td>
<td>Upregulates the expression of TLR2 and its co-simulatory molecules. Activates macrophage by inducing TNF-α and IL-12.</td>
<td>[138]</td>
<td></td>
</tr>
<tr>
<td>PE_PGRS proteins (Rv1818c) Secreted antigenic targets of 6-kDa (ESAT-6) family proteins (Rv1198)</td>
<td><strong>PE_PGRS33</strong></td>
<td>TLR2</td>
<td>CD14</td>
<td>Contributes to Mtb enter macrophage by interacting with TLR2</td>
<td>[139, 140]</td>
<td></td>
</tr>
<tr>
<td><strong>M. tuberculosis</strong></td>
<td>PE/PPE protein (Rv1196)</td>
<td><strong>PPE18</strong></td>
<td>TLR2</td>
<td>Unknown</td>
<td>Interacts with TLR2 to produce IL-10 and SOCS3 to in turn inhibit TLR2 signaling</td>
<td>[142, 143]</td>
</tr>
<tr>
<td>PE/PPE protein (Rv1789)</td>
<td><strong>PPE26</strong></td>
<td>TLR2</td>
<td>CD80/CD86</td>
<td>Activates macrophage by inducing pro-inflammatory cytokine TNF-α, IL-6 and IL-12</td>
<td>[144]</td>
<td></td>
</tr>
<tr>
<td>PE/PPE protein (Rv1808)</td>
<td><strong>PPE32</strong></td>
<td>TLR2</td>
<td>Unknown</td>
<td>Induces both anti-inflammatory cytokine IL-10 and pro-inflammatory cytokines TNF-α and IL-6</td>
<td>[145]</td>
<td></td>
</tr>
<tr>
<td>PE/PPE protein (Rv3425) Leucine-responsive regulatory protein</td>
<td><strong>PPE57</strong></td>
<td>TLR2</td>
<td>CD80/CD86</td>
<td>Activates macrophage by inducing pro-inflammatory cytokine TNF-α, IL-6 and IL-12</td>
<td>[146]</td>
<td></td>
</tr>
<tr>
<td><strong>M. avium</strong></td>
<td>Glycopeptidolipids</td>
<td><strong>GPLs</strong></td>
<td>TLR2, TLR4</td>
<td>Unknown</td>
<td>Promotes the activation of macrophages depended on a TLR2 and MyD88 manner. TLR2 senses GPLs needs its specific acetylation and methylation;</td>
<td>[148-150]</td>
</tr>
<tr>
<td><strong>M. abscessus</strong></td>
<td>TLR2-enriched fraction</td>
<td><strong>TLR2eF</strong></td>
<td>TLR2</td>
<td>Unknown</td>
<td>TLR2eF mildly protects Mab infected ΔF508 mice and its littermates</td>
<td>[151]</td>
</tr>
<tr>
<td>Glycopeptidolipids</td>
<td><strong>GPLs</strong></td>
<td>TLR2</td>
<td>Unknown</td>
<td>The switch of Mab from the smooth to the rough morphotype depends on the present of bacterial surface GLPs.</td>
<td>[152]</td>
<td></td>
</tr>
<tr>
<td><strong>M. smegmatis</strong></td>
<td>Phosphoinositol-capped LAM</td>
<td><strong>PILAM</strong></td>
<td>TLR2/TLR1</td>
<td>Unknown</td>
<td>High affinity binding to TLR2 and strong pro-inflammatory response</td>
<td>[133, 135]</td>
</tr>
<tr>
<td>Arabinosylated lipoarabinomannan</td>
<td><strong>AraLAM</strong></td>
<td>TLR2</td>
<td>CD14?</td>
<td>The lung inflammation induced by AraLAM is diminished in TLR2 deficiency mice</td>
<td>[110]</td>
<td></td>
</tr>
<tr>
<td>Dimannoside hosphatidyl-myo-inositol mannosides</td>
<td><strong>PIM2/6</strong></td>
<td>TLR2</td>
<td>Unknown</td>
<td>Induces the expression of TNF to activate primary macrophages</td>
<td>[135]</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 1

Reference


Chapter 1


Chapter 1


Chapter 1


78. Pai RK, Convery M, Hamilton TA, Boom WH, Harding CV. Inhibition of IFN-gamma-induced class II transactivator expression by a 19-kDa lipoprotein from Mycobacterium tuberculosis: a potential mechanism for...


Chapter 1


Chapter 1


Chapter 1


