The unexplored functions of Toll-like receptor signaling: immunometabolism, development and microbiome interactions

Liu, L.

Chapter 6

Summarizing discussion

1. The different roles of TLR2, TIRAP and MyD88 in basal metabolism

In **Chapter 2**, it is shown that TLR2 is a regulator of vertebrate energy metabolism under unchallenged developmental conditions. Using transcriptomic and metabolomic profiling in zebrafish larvae, we demonstrated that deficiency of TLR2 leads to elevated glucose, lactate, succinate and malate levels, accompanied by transcriptional downregulation of key glycolytic and gluconeogenic enzymes, including *gpib*, *pfkma*, *pgk1*, *pgam2* and *pck2*. These alterations indicate that TLR2 maintains glucose homeostasis through transcriptional control of metabolic pathways. Beyond glucose metabolism, changes in amino acid abundance suggest broader impacts on metabolic regulation. Importantly, this study provides the first evidence that TLR2 shapes metabolism in the absence of infection, extending prior work that primarily focused on immunometabolic interactions during disease or pathogen challenge [1, 2]. The mechanistic basis for TLR2 activation under basal conditions remains unresolved but may involve signals derived from the microbiome [3]. This possibility is supported by accumulating evidence that microbial metabolites influence host immunometabolism and by previous findings that TLR2 regulates microbiota-suppressed transcription of *myd88* [4].

In **Chapter 3**, the role of TLR signaling in metabolism is further examined by comparing the distinct contributions of the adaptor proteins TIRAP and MyD88. This comparison shows that TLR2, TIRAP and MyD88 have specialized functions in host metabolism under unchallenged conditions. Metabolomic profiling shows that the *tirap* mutation results in reduced glucose levels, in contrast to the *tlr2* mutant larvae, which display elevated glucose levels. By comparison, the *myd88* mutant larvae exhibited a narrower metabolic signature, with only modest transcriptional changes in metabolism-related pathways and no detectable differences in glucose abundance.

Together, these results reveal the complex roles for TLR signaling in controlling basal metabolic homeostasis and suggest that the absence of TLR2, TIRAP and MyD88 disrupt both host transcriptional programs and metabolite profiles through distinct mechanisms. These insights provide a foundation for subsequent chapters, which explore how TLR signaling coordinates processes during tissue wounding, mycobacterial infection, and microbiome interactions.

2. The different roles of TLR2, TIRAP and MyD88 in inflammation

An important emerging theme from this thesis is that metabolic regulation by TLR signaling might be affecting immune cell behavior. In **Chapters 2** and **3**, we showed that TLR2 and its adaptor TIRAP distinctly affect glucose metabolism. These metabolic alterations may have direct or indirect consequences for immune cell function in inflammatory response during tissue injury. Immune cells such as neutrophils and macrophages rely on rapid metabolic rewiring, for instance glycolysis, to fuel migration, phagocytosis and effector functions [5-7]. In zebrafish tail wounding assays, TLR2 and MyD88 deficiency impaired leukocyte recruitment to the wounds [8]. TLR2 deficiency also resulted in increased free glucose levels, which might lead to reduced metabolic support for cell motility (**Chapter 2**). In contrast, TIRAP deficiency led to accelerated neutrophil migration upon tail wounding. This correlates with a decreased free glucose level and changes in transcriptional regulation of glycolysis that are distinct from the changes in a *tlr2* mutant (**Chapter 3**). Therefore, an opposite effect in migratory ability of neutrophils in the *tirap* and *tlr2* mutant larvae could be explained by differences in their functions in metabolic regulation. The difference of the effect of mutation of *tlr2* and *tirap* could be explained in several ways: (i) in the absence of TLR2, other TLR receptor might compensate for its loss of function (ii) although TIRAP is the only adaptor known to couple to TLR2, it is not impossible that other adaptors such as TRAM could also compensate for loss of function of TIRAP, thereby leading to very different downstream signaling effects. Our findings suggest that the adaptor-specific control of metabolism by TLR signaling is coupled to leukocyte migration dynamics, providing a mechanistic bridge between immunometabolism and inflammation. This integrative view positions the TLR2 signaling axis as a key regulator of both cellular energy states and immune cell motility.

Beyond the perspective of metabolic regulation, our transcriptomic and metabolomic study in **Chapter 3** suggest several possible explanations for why the *tirap* mutant exhibits leukocyte behavior markedly distinct from the *myd88* and *tlr2* mutants in response to tissue wounding. First, among the three mutants examined, only *tirap* deficiency was associated with broad downregulation of ribosomal protein transcripts, suggesting a ribosomal stress response. Given the established roles of ribosomal proteins such as L13a in translational control of inflammatory mediators [9-12], these findings point to a link between Tirap signaling, ribosomal protein expression, and the regulation of inflammatory responses.

In addition, specifical alterations in calcium channel and myosin-related gene expression suggest a role for Tirap in calcium-dependent cytoskeletal regulation. Our transcriptomic analysis showed up-regulation of calcium channels and myosin-related gene expression specifically in the *tirap* mutant. Calcium ions act as pivotal second messengers that trigger actin-myosin interactions, not only in muscle cells but also across diverse cell types [13-15]. Myosin activity is a critical component of defense responses in myeloid cells [16]. Notably, reactive oxygen species (ROS) have been shown to negatively regulate *CACNA1S* expression in the context of *Mycobacterium tuberculosis* infection [17], and ROS are also key modulators of cell migration during tissue injury [18]. Therefore, the altered expression of *cacna1a* in the *tirap* mutant may be mechanistically relevant for its effects on neutrophil migration. Taken together, these findings suggest that Tirap contributes to the regulation of calcium-dependent myosin function, which could underlie the increased velocity of neutrophil migration observed in the *tirap* mutant following wounding.

Yet another explanation for the distinct effects of *tirap* deficiency on cell migration, compared with *myd88* and *tlr2* mutants, is its differential impact on cytokine and chemokine regulation. The *tirap* mutant uniquely displayed downregulation of *cxcl12a*, a chemokine with well-established roles in neutrophil responses to inflammation. Consistent with this, previous work has demonstrated that *cxcl12a* mutants exhibit increased neutrophil recruitment to wounds in zebrafish larvae [19]. Therefore, the enhanced neutrophil accumulation observed in *tirap* mutants may, at least in part, be attributable to reduced *cxcl12a* expression. However, the absence of an effect on macrophage migration in *tirap* mutants, unlike in *tlr2* and *myd88* mutants, remains unexplained. Addressing this gap will require higher-resolution approaches such as single-cell transcriptomics and lineage-specific functional studies to dissect the cell type-specific roles of Tirap in neutrophils, macrophages, and potentially T cells.

3. TLR2 regulates gut microbial diversity in larval and adult zebrafish

To further explore the role of TLR2 beyond immunity and understand the interaction between TLR2 and the microbiome, in **Chapter 4** we examined the impact of TLR2 on gut microbial composition across zebrafish developmental stages. Our data show that for the larvae at 5 days post fertilization, *tlr2* mutants exhibited increased microbial diversity and enrichment of genera such as *Chryseobacterium* and *Flectobacillus*. Functional predictions revealed

suppression of glycolysis pathways in the microbiota of *tlr2* mutants, paralleling reduced host glycolytic gene expression (**Chapter 2**) and highlighting bidirectional metabolic crosstalk between host and microbiome.

In adults, the trend was reversed. Wild-type zebrafish maintained higher microbial diversity, with enrichment of genera such as *Plesiomonas* and *Romboutsia*, whereas *Cetobacterium* dominated in *tlr2* mutants. Functional pathways also differed, with glycolysis and TCA cycle enriched in wild types, while glycan biosynthesis and pentose phosphate pathways were elevated in mutants. These results reveal the dynamic and stage-dependent influence of TLR2 on microbial diversity and metabolic potential.

Given the links between dysbiosis, barrier dysfunction, and inflammatory bowel disease, including Crohn's disease [20], our findings highlight TLR2 as a potential therapeutic target for many gut diseases. Modulating TLR2 signaling or leveraging TLR2-microbiome interactions may provide new opportunities to restore intestinal immune balance and improve disease outcomes.

4. The role of TLR2 and the microbiome in mycobacterial gut infection

4.1 TLR2 and the microbiome modulate mycobacterial localization and dissemination, and transcriptional responses during gut infection

In **Chapter 5**, we extend the role of TLR signaling beyond metabolism and inflammation to the context of host-pathogen and study the role of host-microbiome interactions in nontuberculous mycobacteria (NTM) gut infection and dissemination. Using zebrafish as an *in vivo* model of gastrointestinal infection with *Mycobacterium avium* and *M. marinum*, we demonstrated that TLR2 plays a central role in regulating mycobacterial localization and dissemination. Immersion infection assays showed that *M. avium* and *M. marinum* exhibit strikingly different colonization patterns in the zebrafish gut, with *M. avium* preferentially localizing to the posterior intestine and *M. marinum* to the anterior region in wild-type, microbiome-colonized larvae. These species-specific patterns were abolished in *tlr2* mutants or under germ free conditions, highlighting the critical role of both TLR2 signaling and microbiome-derived cues in determining spatial distribution of NTM. Given the clinical relevance of *M. avium* as an enteric pathogen, these observations underscore the need to elucidate the molecular determinants of localization, which are likely to involve complex

host-pathogen interactions mediated through the TLR2 pathway [4]. At the whole organism gene expression level, qRT-PCR analyses demonstrated distinct host responses to *M. avium* and *M. marinum*, with differences dependent on microbiome status. Interestingly, markers such as *fosl1a* and *cebpb*, which are downstream of TLR2, exhibited divergent expression profiles, suggesting that additional pathways and mediators contribute to strain-specific host responses [4]. Possible candidates for this include other TLR family members and host-derived factors from the intestinal mucosal environment, such as uromodulin, glycoprotein 2 (GP2), and omcins, which have been implicated in host-microbiome interactions in zebrafish and are conserved in mammalian systems [4]. These results point to a multilayered regulatory network in which TLR2 integrates microbial signals with mucosal factors and microbiome-derived cues to orchestrate the outcome of gut mycobacterial infections.

4.2. Macrophages contribute to the phagocytosis and dissemination of mycobacteria to distal tissues during gut infection

Our study in **Chapter 5** demonstrate that following immersion infection with *M. avium* and *M. marinum*, bacteria are not confined to the gut but also appear in peripheral tissues, including the tail. Blocking oral entry by embedding the head in agarose confirmed that bacteria cannot invade through intact skin, and gill colonization was rare, confirming the importance of the oral-gut route of bacterial entry. This finding aligns with previous reports that cutaneous diseases caused by mycobacterial skin infections usually result from hematogenous dissemination or spread from underlying wounding foci [21]. Besides, gut microinjection experiments clearly established the intestine as an efficient primary site of entry, from which mycobacteria subsequently disseminate systemically shortly after infection. How mycobacteria disseminate from the gut, particularly to distal tissues, remains an important question in understanding their pathogenesis. Live imaging revealed that bacteria detected in distal tissues were almost exclusively located within macrophages, supporting the view that these cells serve as principal vehicles for pathogen dissemination. This is consistent with earlier studies in mice and zebrafish model demonstrating that macrophages can phagocytose mycobacteria at mucosal surfaces and transport them to distal tissues via the circulation [22]. Our macrophage ablation experiments further substantiated this model, as dissemination was nearly abolished in the absence of macrophages.

Previous studies have shown that *M. tuberculosis* and *M. marinum* preferentially recruit and infect permissive macrophages while evading microbicidal subsets. One host strategy to counteract this immune evasion is the TLR2-dependent recruitment of microbicidal macrophages [22]. Consistent with this, our results demonstrate that TLR2 deficiency significantly reduces the number of macrophages harboring *M. avium* and *M. marinum*, underscoring the importance of TLR2 in regulating macrophage function during mycobacterial infection. To further explore this mechanism, future studies should focus on distinguishing permissive from microbicidal macrophages *in vivo*. Advanced approaches such as single-cell transcriptomics and ultrastructural electron microscopy will be particularly valuable for resolving macrophage heterogeneity and for uncovering how TLR2 shapes host-pathogen interactions within the gut microenvironment.

4.3. Macrophage behavior and TLR2-microbiome interactions during mycobacterial gut infection

In **Chapter 5**, we demonstrate that TLR2 plays a pivotal role in regulating macrophage recruitment and motility during intestinal mycobacterial infection. Using gut microinjection of *M. avium* and *M. marinum*, we observed that *tlr2* mutants displayed reduced macrophage accumulation at infection sites and diminished migratory speed. These findings are consistent with earlier zebrafish tail fin infection models and extend the role of TLR2 to natural gut-associated infection [23]. Furthermore, macrophage motility defects were also evident after PBS injection in *tlr2* mutants, indicating a broader role for TLR2 in wounding responses and aligning with findings from previous zebrafish tail fin wounding models [8]. Notably, this effect was dependent on microbiome colonization. Wild-type larvae under conventionalized conditions showed enhanced macrophage motility compared with germ free larvae, whereas this microbiome-dependent modulation was absent in the *tlr2* mutants. These results suggest that TLR2 signaling is required for microbiome-mediated modulation of innate immune cell dynamics at the systems level. Analogous studies have shown that *M. tuberculosis* initiates infection in the relatively sterile lower airways, rather than in the microbe-rich upper tract, where microbiota-derived signals promote recruitment of microbicidal macrophages through TLR-dependent pathways [24]. Our findings support this perspective and extend it to the intestinal context, highlighting a potential mechanism by which the microbiome, through TLR2 signaling, modulates macrophage behavior and influences host responses to

mycobacterial infection. To further examine transcriptional responses, we analyzed *mmp9* gene, a marker linked to granuloma formation during *M. marinum* infection [25]. Induction of *mmp9* was partly dependent on TLR2, suggesting that macrophages may be recruited to gut epithelial sites through TLR2-dependent MMP9 secretion, possibly in coordination with other TLRs such as TLR8 [3, 25]. Future studies using single-cell transcriptomics and emerging in situ bacterial transcriptome approaches will be critical to dissect the complex interplay between host macrophages, invading mycobacteria, and the TLR2-microbiome axis.

5. Additional data on the function of TLR2: Absence of TLR2 and the microbiome facilitate the proliferation of mycobacteria after systemic infection

In addition to oral-gut infection, we examined the contribution of TLR2 and the microbiome to systemic host defense using blood injection of nontuberculous mycobacteria through the Duct of Cuvier which is not included in this thesis but is relevant for this discussion section. This approach bypasses the intestinal barrier and directly exposes circulating immune cells to infection. Using microinjection of *M. avium* and *M. marinum* into Duct of Cuvier, we observed that *tlr2* mutants displayed higher Mma20 bacterial burden compared with the wild type, aligning with findings from previous zebrafish caudal vein infection through blood island injection [26]. Moreover, consistent with our gut infection data, both *tlr2* mutant and germ free larvae exhibited enhanced bacterial proliferation and burden following systemic injection with MAC 101 and Mma20, underscoring the protective role of TLR2 and the microbiome in restricting mycobacterial growth (Figure 1 and 2). These findings indicate that TLR2-dependent signaling provides a conserved defense mechanism overarching distinct routes of infection, while the microbiome enhances systemic resistance by modulating innate immune response. Together, these results complement our gut infection model and highlight the broad protective functions of the TLR2-microbiome axis in controlling mycobacterial pathogenesis.

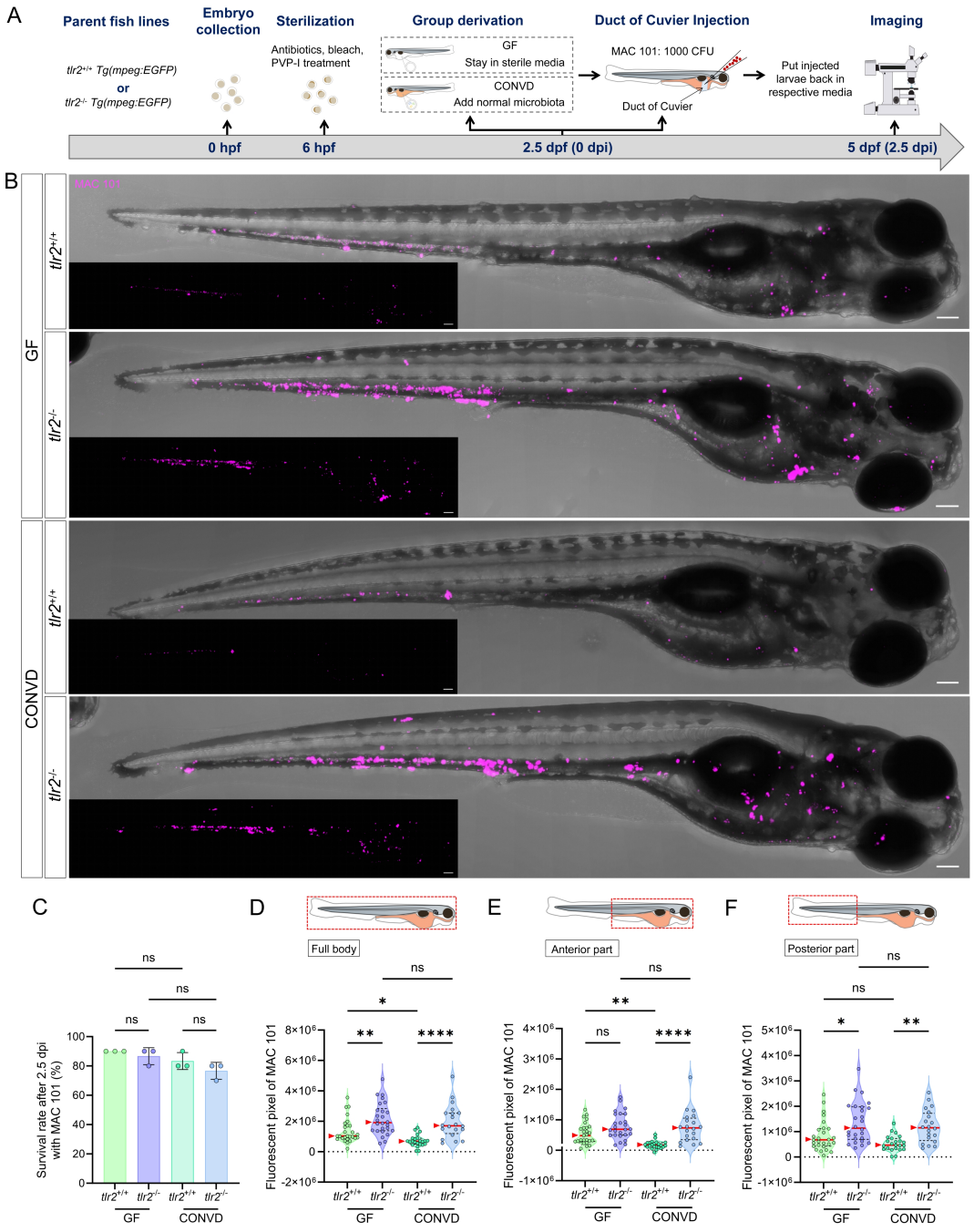

Figure 1. Absence of TLR2 as well as the microbiome facilitate the proliferation of MAC 101 after systemic infection. (A) Schematic of the experimental workflow. Germ-free (GF) and conventionalized (CONVD) zebrafish larvae derived from *tlr2* wild-type and mutant lines were injected with mCherry-labeled *M. avium complex* (MAC 101) bacteria into Duct of Cuvier and put back to respective media. Confocal imaging was performed at 2.5 days post injection. (B) Representative fluorescence images showing MAC 101 dissemination to anterior and posterior regions. Bacteria are shown in magenta. Scale bar: 100 µm. (C) Survival rates for all groups after MAC 101 infection showing no significant differences between genotypes or microbial conditions. The results are based on 3 independent experiments. Error bars represent mean ± SD. (D-F) Quantification of full body (D), anterior region (E) and posterior region (F) bacterial burden (fluorescent pixel

count) reveals higher MAC 101 load in *tlr2* mutants compared to the wild type under both GF and CONVD conditions. the data from GF *tlr2*$^{+/+}$ (n = 27) group, GF *tlr2*$^{-/-}$ (n = 26) group, CONVD *tlr2*$^{+/+}$ (n = 23) group and CONVD *tlr2*$^{-/-}$ (n = 20) group are based on three independent experiments Statistical significant difference was determined by one-way ANOVA, red arrows point to the median, ns, non-significant, *, $P < 0.05$, **, $P < 0.01$, ****, $P < 0.0001$.

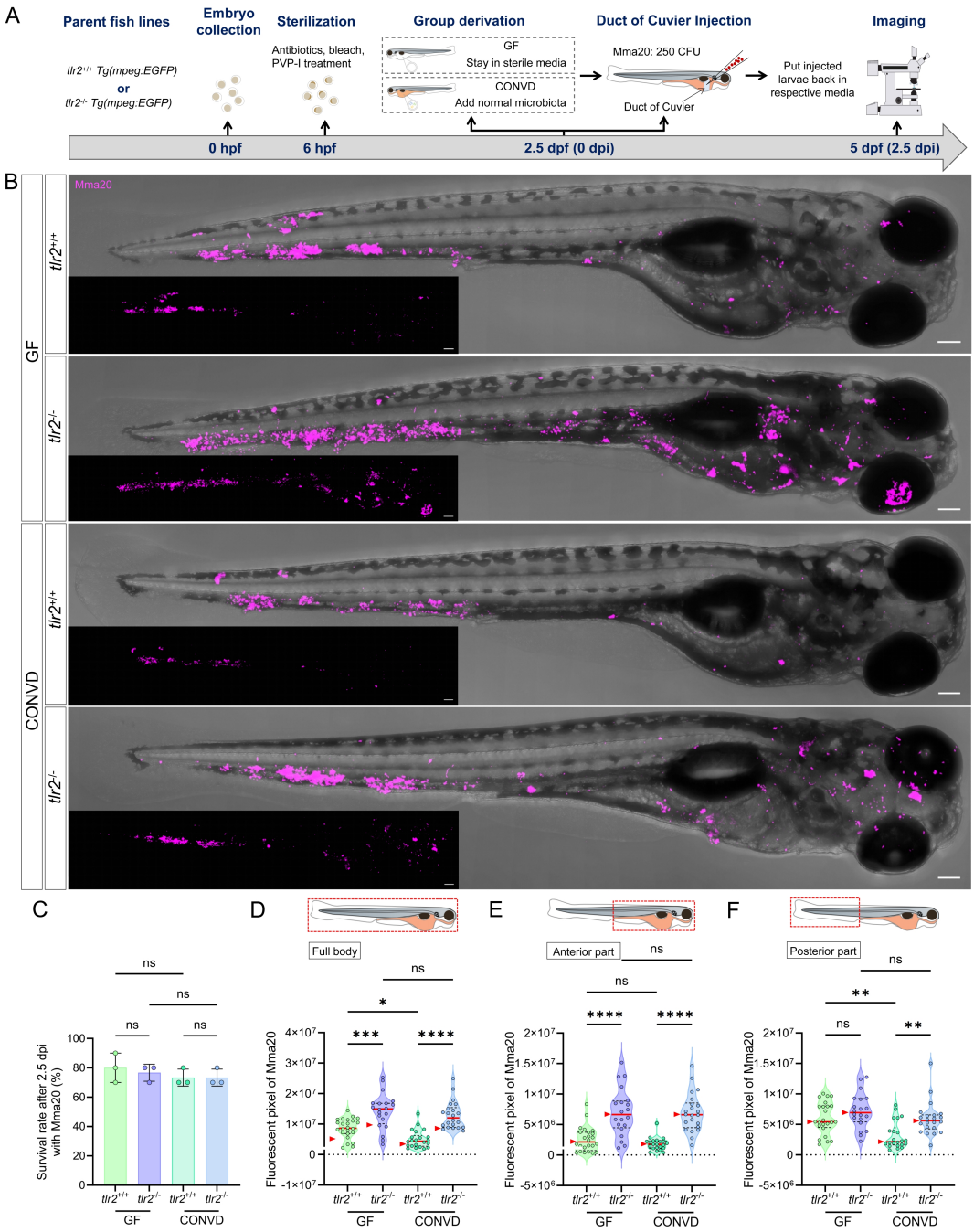

Figure 2. Absence of TLR2 as well as the microbiome facilitate the proliferation of Mma20 after systemic infection. (A) Schematic of the experimental workflow. Germ-free (GF) and conventionalized (CONVD) zebrafish larvae derived from *tlr2* wild-type and mutant lines were injected with DsRed-labeled *M.*

marinum (Mma20) bacteria into Duct of Cuvier and put back to respective media. Confocal imaging was performed at 2.5 days post injection. (B) Representative fluorescence images showing Mma20 dissemination to anterior and posterior regions. Bacteria are shown in magenta. Scale bar: 100 μm. (C) Survival rates for all groups after Mma20 infection showing no significant differences between genotypes or microbial conditions. The results are based on 3 independent experiments. Error bars represent mean ± SD. (D-F) Quantification of full body (D), anterior region (E) and posterior region (F) bacterial burden (fluorescent pixel count) reveals higher Mma20 load in *tlr2* mutants compared to the wild type under both GF and CONVD conditions. the data from GF $tlr2^{+/+}$ (n = 24) group, GF $tlr2^{-/-}$ (n = 22) group, CONVD $tlr2^{+/+}$ (n = 20) group and CONVD $tlr2^{-/-}$ (n = 22) group are based on three independent experiments Statistical significant difference was determined by one-way ANOVA, red arrows point to the median, ns, non-significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.

6. Potential translational role of TLR2 and the microbiome in the mycobacterial gut disease

Our results establish TLR2 as a critical regulator of macrophage function during intestinal mycobacterial infection. In zebrafish, *tlr2* deficiency impaired macrophage phagocytosis and migration, leading to increased bacterial burden, consistent with its protective role described in pulmonary infection models [27-30]. These findings support the broader view of TLR2 as a potential therapeutic target for mycobacterial disease, with most previous work focusing on the lung. We further demonstrate that the microbiome strongly influences the outcome of gut mycobacterial infection. Germ free larvae showed increased bacterial colonization, reduced macrophage motility and altered host transcriptional responses compared to conventionalized larvae, effects that were dependent on TLR2 signaling. This identifies a TLR2-microbiome axis that regulates immune defense in the intestine, extending earlier gnotobiotic zebrafish studies that linked TLR2 to systemic inflammatory control in the absence of pathogens [4]. The clinical relevance of these findings is supported by parallels to Johne's disease in ruminants and Crohn's disease in humans [31, 32]. TLR2 polymorphisms have been associated with susceptibility to Johne's disease, while Crohn's disease is marked by TLR2 dysregulation, microbiome imbalance, and chronic inflammation [33-36]. Our zebrafish model provides a powerful platform to dissect these connections, offering high-throughput infection assays, live imaging, and genetic manipulation. Importantly, the observation that the TLR2 inhibitor C29 phenocopies *tlr2* mutants underscores the potential of chemical screening in this system to identify therapeutic strategies that limit early dissemination of mycobacteria in the gut.

7. Towards future understanding of TLR2 signaling

The work presented in this thesis identifies TLR2 as a central regulator at the intersection of metabolism, inflammation, microbiome interactions, and infection. Despite significant progress, many mechanistic questions remain unresolved. The following sections outline potential directions for future research that could expand the current understanding of TLR2-mediated immune and metabolic regulation.

7.1 TLR2 signaling in metabolic regulation

The mechanisms linking TLR2 activation to glucose and lipid metabolism under basal physiological conditions remain to be fully elucidated. While our data demonstrate metabolic alterations in *tlr2* mutants, the precise endogenous or microbiota-derived ligands that engage TLR2 and modulate host metabolism are yet to be identified. Future studies should employ targeted metabolite profiling and metabolomic-transcriptomic integration to pinpoint such ligands and signaling intermediates. Furthermore, coupling metabolic flux analysis with single-cell transcriptomics could uncover how TLR2 orchestrates cell type-specific metabolic programs and coordinates systemic energy balance. These approaches will help delineate whether TLR2 functions primarily as a metabolic sensor or as a downstream effector of immune-metabolic cross-talk.

7.2 Adaptor-specific control of inflammation

Our findings show that adaptor-specific signaling through TIRAP and MyD88 leads to distinct transcriptional, metabolic, and inflammatory outcomes. This adaptor specificity raises key questions about how different downstream pathways determine the quality and magnitude of inflammation. Future research should apply high-resolution multi-omics, including single-cell RNAseq, proteomics, and spatial imaging, to dissect the cell type and tissue-specific contributions of these adaptors *in vivo*. Particular attention should be given to the ribosomal stress and calcium-dependent signaling signatures observed upon TIRAP deficiency, as they may represent unconventional immune-regulatory axes linking stress responses to inflammation.

7.3 TLR2 and the microbiome interface

The zebrafish model established in this work provides a tractable system to explore how TLR2 shapes host-microbiome symbiosis. Future investigations should move beyond mono-association studies and explore complex microbial communities using gnotobiotic recolonization and defined bacterial consortia. Integrating single-cell host transcriptomics with bacterial metatranscriptomics will enable a spatially resolved understanding of host-microbe communication at the mucosal barrier. Such approaches may identify specific microbial taxa, metabolites, or pattern-recognition signatures that drive tolerance versus inflammation through TLR2-dependent mechanisms.

7.4 TLR2 in mycobacterial infection and translational relevance

Our zebrafish infection models revealed that the TLR2-microbiome axis influences mycobacterial dissemination and immune control in the gut. Extending these findings to different Mycobacterium species, and to co-infection or antibiotic-perturbed microbiota contexts, will clarify the generality of these mechanisms. From a translational perspective, TLR2 and its adaptor network represent promising therapeutic targets for nontuberculous mycobacterial infections and inflammatory bowel diseases. Future research combining host genetic modulation with microbiome-based interventions such as probiotic consortia or metabolite supplementation may pave the way toward precision immunometabolic therapies that restore mucosal balance without compromising antimicrobial defense.

Overall, the translational relevance of TLR2 signaling should be further explored in mammalian models and human systems. The parallels between our zebrafish findings and pathological processes in Johne’s disease and Crohn’s disease suggest that TLR2 modulation could be harnessed for therapeutic benefit. Chemical screening in zebrafish, as demonstrated with the TLR2 inhibitor C29, could provide a pipeline for identifying novel host-directed therapies against metabolic disorders, chronic inflammation, and mycobacterial infections. Future research should aim to integrate molecular, cellular, and ecological perspectives to build a systems-level understanding of TLR2 signaling. Such integrative approaches will not only deepen our knowledge of host physiology but also advance the development of targeted therapies that exploit the TLR2-microbiome-metabolism axis to improve human health**.**

8. Concluding remarks

The work presented in this thesis establishes TLR2 as a central coordinator of vertebrate physiology, linking metabolic regulation, inflammatory signaling, infection control, and host-microbiome interactions. We show that TLR2 and its adaptor protein TIRAP maintain glucose homeostasis under unchallenged conditions, couple metabolic state to leukocyte migration during wounding, and especially TLR2 regulates macrophage behavior and dissemination dynamics during nontuberculous mycobacterial infection. Extending beyond immunity, TLR2 also shapes the diversity and functional potential of the gut microbiome across developmental stages, highlighting its role as a molecular interface between host metabolism, microbial communities, and immune defense.

By integrating microbial sensing with metabolic regulation, leukocyte dynamics, and microbiome control, the TLR2 signaling axis emerges as a central hub in vertebrate immunometabolism. Using zebrafish transcriptomic, metabolomic, and gnotobiotic models, this work demonstrates that TLR2 modulates both immune responses and microbial communities, underscoring its potential as a therapeutic target in metabolic disorders, inflammatory diseases, and mycobacterial infections in humans.

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