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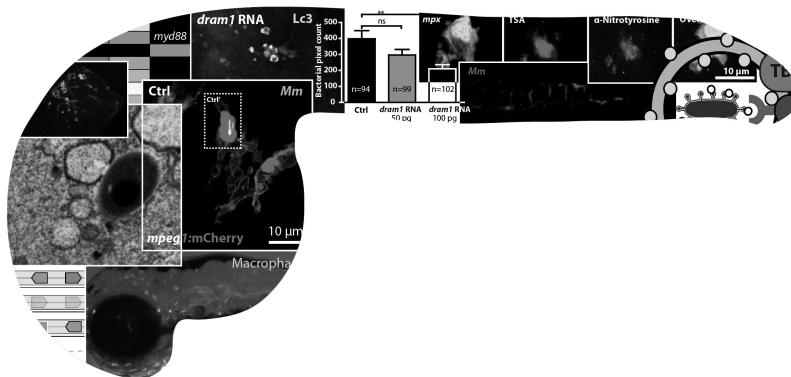
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Innate host defense against intracellular pathogens

Michiel van der Vaart



Cover: A macrophage (magenta) has phagocytosed *Mycobacterium marinum* bacteria (black)

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Innate host defense against intracellular pathogens

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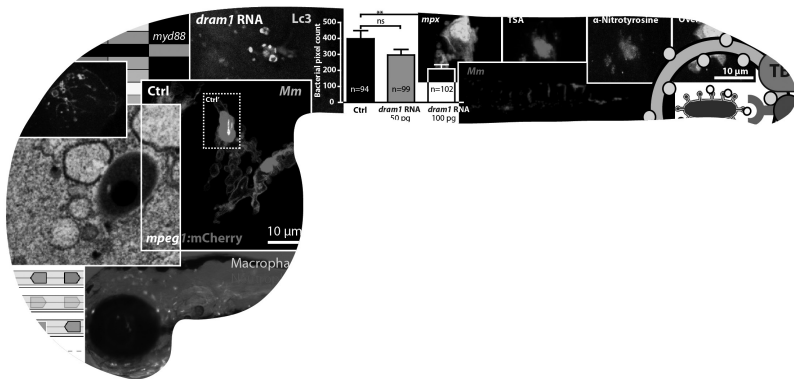
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***Aan mijn familie,
Bedankt!***

Chapter 1

1

Outline of the thesis



Humans rely on their immune system for defense against invading microorganisms, such as pathogenic bacteria, fungi, parasites, and viruses. The defense against pathogens can be divided into two general types of host responses: (1) the innate immune response, and (2) the adaptive immune response. The innate immune response consists of cells and proteins that form the first line of defense against invading pathogens. Their function is to quickly arrive at the site of infection and fight off microbes that have breached physical barriers such as the skin or mucosal surfaces. The adaptive immune system is called into action when the innate immune response alone is not sufficient to kill the pathogen. Adaptive immunity involves the production of antibodies that bind and neutralize the pathogen, and the activation of T-cells that directly recognize and kill the pathogen. The innate immune system is the most ancient defense mechanism and many of its components are shared between invertebrate and vertebrate species. The adaptive immune system is unique to vertebrates and arose during the evolution of fishes. Nowadays the zebrafish is one of the most powerful model organisms for the study of the immune system.

This thesis focuses on the recognition of pathogenic bacteria and the defense mechanisms that are activated during the innate immune response. Innate immune detection of pathogens depends on a number of pattern recognition receptors (PRRs) capable of detecting the presence of evolutionary conserved structures on microbes, called pathogen-associated molecular patterns (PAMPs). In **chapter 2**, we introduce the different classes of PRRs available to the innate immune system and the defense mechanisms which they can activate upon detection of a pathogen. We also review the conservation of these receptors and mechanisms in zebrafish, and discuss recent studies using zebrafish infection models that have advanced our general understanding of the innate immune system.

The most extensively studied class of PRRs is the Toll-like receptor (TLR) family, which signals via adaptor molecules to initiate gene expression and activate the appropriate response upon recognition of a pathogen. In **chapter 3**, we present a zebrafish mutant line for the TLR signaling adaptor myeloid differentiation factor 88 (MyD88), which is central to the innate immune processes underlying infectious diseases, inflammatory disorders, and cancer. Zebrafish *myd88* mutant embryos were more susceptible to infection with the acute bacterial pathogens *Salmonella typhimurium* and *Edwardsiella tarda*, as well as the chronic bacterial pathogen *Mycobacterium marinum*. Further characterization revealed that gene expression of transcription factors and defense proteins important to innate immunity depends on MyD88 during these bacterial infections.

Mycobacterium marinum is a close relative of the human pathogen *Mycobacterium tuberculosis*, and causes tuberculosis disease in its natural zebrafish host. A potent innate defense mechanism against intracellular pathogens such as *Mycobacterium marinum* is the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). We studied the MyD88-dependent production of RNS in zebrafish innate

immune cells in **chapter 4**, and found that *Mycobacterium marinum* could attenuate this response and thereby avoid the cytostatic and cytotoxic effects of RNS.

Autophagy is a cellular homeostasis process that acts as a quality control system for eukaryotic cells. It has a central role in programmed cell death and survival by providing energy and nutrients and ridding the cytoplasm of unwanted components. Selective autophagy has recently emerged as defense mechanism activated by PRRs against tuberculosis disease, although the responsible molecular signaling pathway remained unclear. In **chapter 5**, we used zebrafish embryos and human macrophages to demonstrate that anti-mycobacterial autophagy is orchestrated by TLR-MYD88-NFκB signaling via DNA-damage regulated autophagy modulator 1 (DRAM1). Furthermore, *in vivo* overexpression of this autophagy regulator decreased mycobacterial infection by increased autophagosomal targeting of mycobacteria, presenting a potential novel therapeutic strategy for tuberculosis disease. Finally, the findings presented in this thesis are put into perspective of current knowledge of the innate immune system in **chapter 6**.

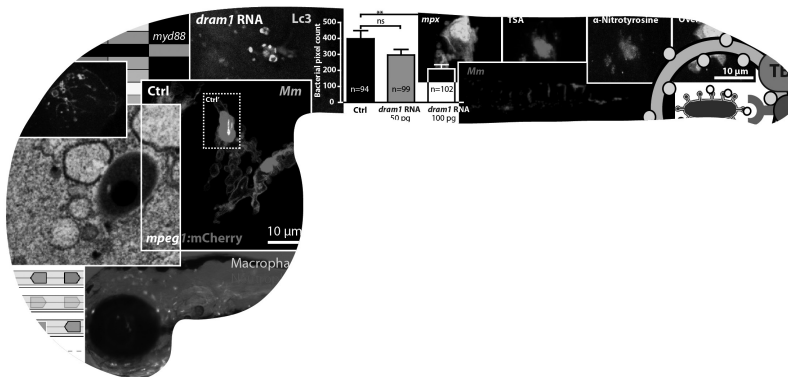
Chapter 2

2

Pathogen recognition and activation of the innate immune response in zebrafish

Michiel van der Vaart, Herman P. Spaink, and Annemarie H. Meijer

Advances in Hematology, 2012



Abstract

2 The zebrafish has proven itself as an excellent model to study vertebrate innate immunity. It presents us with possibilities for *in vivo* imaging of host-pathogen interactions which are unparalleled in mammalian model systems. In addition, its suitability for genetic approaches is providing new insights on the mechanisms underlying the innate immune response. Here, we review the pattern recognition receptors that identify invading microbes, as well as the innate immune effector mechanisms that they activate in zebrafish embryos. We compare the current knowledge about these processes in mammalian models and zebrafish, and discuss recent studies using zebrafish infection models that have advanced our general understanding of the innate immune system. Furthermore, we use transcriptome analysis of zebrafish infected with *E. tarda*, *S. typhimurium*, and *M. marinum* to visualize the gene expression profiles resulting from these infections. Our data illustrate that the two acute disease causing pathogens, *E. tarda* and *S. typhimurium*, elicit a highly similar pro-inflammatory gene induction profile, while the chronic disease causing pathogen, *M. marinum*, induces a weaker and delayed innate immune response.

1. Introduction

The use of adult zebrafish (*Danio rerio*) and their transparent offspring as hosts to model infectious diseases caused by human pathogens, or closely related animal pathogens, has recently provided novel insights into pathogenesis, which in many cases could not have been achieved using mammalian models¹⁻⁶. The power of the zebrafish model lies in its suitability for genetic approaches, high-throughput screening, and live imaging studies. Fluorophore-marked transgenic lines are now available that allow unprecedented visualization of pathogen interactions with macrophages and neutrophils, the major phagocytic innate immune cell types of zebrafish larvae⁷⁻¹¹. As early as one day post fertilization (dpf), zebrafish embryos display phagocytic activity towards microbial infections¹² and are able to mount an innate immune response with a transcriptional signature that resembles responses in mammalian or cell culture systems¹³. Adaptive immunity becomes active after approximately three weeks of development¹⁴. Therefore innate immunity can be studied during the earlier zebrafish embryonic and larval stages in the absence of T- and B-cell responses. In this review we focus on signaling pathways involved in pathogen recognition and activation of the innate immune response in zebrafish embryos and larvae. We compare the knowledge of the zebrafish innate immune system with that of human and mammalian models, and discuss results from transcriptomic analyses that show clear specificity in responses to different bacterial pathogens, such as Salmonella and Mycobacteria species.

2. Pattern recognition receptors

The innate immune system is the host's first line of defense against infection, therefore its main role is to recognize invading pathogens early and trigger an appropriate proinflammatory response¹⁵. The innate immune system utilizes a limited number of germ line-encoded pattern recognition receptors (PRRs) to recognize evolutionary conserved structures on pathogens, named pathogen-associated molecular patterns (PAMPs)¹⁵. PRRs are also capable of indirectly sensing the presence of pathogens^{16, 17}. This occurs when infection, inflammation, or other cellular stresses cause host factors to be present in aberrant locations, or to form abnormal molecular complexes, so called danger-associated molecular patterns (DAMPs)¹⁷. PRRs located on the cell surface are scouting the extracellular environment for the presence of microbes. PRRs located on endosomes identify microbes that have entered the phagolysosomal degradation pathway, and cytoplasmic PRRs recognize intracellular cytosolic pathogens or components of internalized microbes¹⁸. Upon PAMP recognition, PRRs signal the presence of infection and initiate proinflammatory and antimicrobial responses by activating several intracellular signaling pathways¹⁹, ultimately leading to activation of gene expression and synthesis of a broad range of molecules. These include proinflammatory and chemotactic cytokines, and antimicrobial peptides²⁰. The different families of PRRs present in both humans and zebrafish and their downstream signaling pathways are summarized in Figure 1 and will be discussed below.

2.1 Toll-like receptors

The most extensively studied class of PRRs are the Toll-like receptors (TLRs), a family of 10 proteins in human. TLRs are named after the *Drosophila* Toll protein, which functions in dorso-ventral patterning and antifungal responses²¹. TLRs are integral glycoproteins which possess an extracellular or luminal, ligand-binding domain with leucine-rich repeat (LRR) motifs and a cytoplasmic signaling Toll/Interleukin-1 (IL-1) receptor homology (TIR) domain^{20, 22}. In mammals, the main cell types expressing TLRs are antigen presenting cells (APCs), including macrophages and dendritic cells, and B lymphocytes¹⁸. However, most cell types are capable of expressing TLRs, for instance in response to a localized infection²³. In mammals, TLR4 recognizes Gram-negative bacteria via the lipid A portion of lipopolysaccharide (LPS), while TLR2 recognizes Gram-positive bacteria via lipoteichoic acid (LTA), lipoproteins, and peptidoglycan, and TLR5 recognizes the motility apparatus protein flagellin, which can be present on both Gram types¹⁸. Other TLRs are specialized in recognizing nuclear acids in endosomal and phagosomal compartments. TLR3 can detect viral replication by binding to double-stranded RNA (dsRNA), TLR7 and TLR8 specifically recognize single-stranded RNA (ssRNA) of RNA viruses, and unmethylated CpG DNA present in the genomes of viruses and bacteria is detected by TLR9¹⁸. Ligand binding by a TLR will induce it to form homomeric or heteromeric oligomers, which triggers intracellular signal transduction via their TIR domains¹⁸. The mammalian TLR signaling pathway uses five different TIR domain containing adaptor

molecules: MYD88, MAL/TIRAP, TRIF/TICAM1, TRAM/TICAM2, and SARM^{19, 22}. Among these, MYD88 is the most universal adaptor, since it is used for downstream signaling by all TLRs, with the exception of TLR3²⁴. Downstream signaling via central intermediate molecules such as TRAF6, will eventually lead to the activation of transcription factors, mostly members of the ATF, NFκB, AP-1, IRF, and STAT families, regulating the expression of a battery of antimicrobial and proinflammatory genes²⁴.

Putative orthologs of mammalian TLRs have been identified in zebrafish, in addition to some fish-specific family members^{25, 26}. A genome duplication during the evolution of teleost fish most likely explains why zebrafish have two counterparts for some of the mammalian TLRs (e.g. *tlr4ba/tlr4bb* for *TLR4* and *tlr5a/tlr5b* for *TLR5*), but it is still unknown whether this increase in the number of receptors is associated with diversification in PAMP recognition⁴. Only some of the zebrafish TLR ligands are currently known²⁷. The specificity of TLR2, TLR3, and TLR5 is conserved between mammals and fish, recognizing lipopeptides, dsRNA, and flagellin respectively^{13, 28, 29}. Additionally, the fish-specific TLR22 has been shown to recognize dsRNA and PolyI:C²⁹. However, zebrafish TLR4 cannot be stimulated by LPS, illustrating that not all ligand specificities are conserved between mammals and zebrafish^{30, 31}. Signaling intermediates in the pathway downstream of mammalian TLRs have also been identified in zebrafish, including homologs of four of the adaptor proteins, Myd88, Mal/Tirap, Trif/Ticam1, and Sarm, and the central intermediate Traf6³². Among these, Myd88 and Traf6 have been functionally studied by knockdown analysis in zebrafish embryos, showing their requirement for a proinflammatory innate immune response to microbial presence^{13, 33-35}. Furthermore, triggering of the innate immune response in zebrafish embryos also leads to induction of members of the ATF, NFκB, AP-1, IRF, and STAT families of transcription factors^{13, 33, 36}.

2.2 NOD-like receptors

Pathogens that escape the surveillance of cell surface and endosomal PRRs may end up in the cytosol, where nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) detect their presence by intracellular PAMPs and DAMPs³⁷. The NLRs constitute a family of 23 proteins in humans. Their defining features are the presence of a centrally located NOD domain responsible for oligomerization, a C-terminal LRR capable of ligand-binding, and an N-terminal protein-protein interaction domain, such as the caspase recruitment domain (CARD), pyrin (PYD), or baculovirus inhibitor repeat (BIR) domain³⁸. Two of the NLRs, NOD1 and NOD2, can sense bacterial presence by directly or indirectly detecting molecules produced during synthesis or breakdown of peptidoglycan³⁸. NOD1 recognizes g-D-glutamyl-meso-diaminopimelic acid (iE-DAP), a dipeptide produced mostly by Gram-negative bacteria, whilst NOD2 can recognize both Gram types, since it is activated upon binding to muramyl dipeptide (MDP), a more common component of peptidoglycan^{39, 40}. Interestingly, both NOD1 and NOD2 have recently been implicated in detection of parasites lacking peptidoglycan, indicating

that these receptors can recognize a broader range of pathogens than was originally assumed^{41, 42}. Upon ligand-binding, NOD1 and NOD2 recruit the serine/threonine kinase RIPK2 (also known as RIP2) via CARD-CARD interactions, eventually leading to the activation of NF κ B^{43, 44}. In addition, NOD1/2 stimulation also induces MAP kinase signaling⁴⁵. Synergistically, with TLR activation, NOD1/2 signaling cascades induce the expression of cytokines and chemokines, such as TNF, IL6, IL8, IL10, and IL12, as well as the production of anti-microbial peptides^{44, 46, 47}.

Other NLRs, such as IPAF, NALP1, and NALP3 mainly function to create a multiprotein complex known as the inflammasome, in which they associate with an adaptor called ASC (apoptosis-associated speck-like protein containing a CARD) and with pro-caspase 1⁴⁸. Oligomerization of the proteins in an inflammasome via CARD-CARD interactions ultimately leads to the cleavage of pro-caspase 1 into its active form, caspase 1, which is then available to catalyze the cleavage of accumulated pro-IL1 β and pro-IL18 into their secreted forms, biological active IL1 β and IL18³⁸. The NLR family member incorporated into these complexes determines which PAMPs and DAMPs are recognized by the inflammasome. A role for NALP3 has been established in the recognition of ATP⁴⁹, uric acid crystals⁵⁰, viral RNA⁵¹, and bacterial DNA⁵². Both NALP1 and NALP3 share NOD2's ability to respond to MDP⁵³. Furthermore, NALP1 can associate with NOD2 (Hsu 2008), showing a role for NOD2 in MDP-triggered IL1 β activation, separate from its role as an inducer of proinflammatory gene expression.

Although the function of NLR family members in zebrafish is not widely studied, it is known that the canonical members of the mammalian NLR family, NOD1, NOD2, and NOD3 (or Nlrc3), are conserved. Additionally, a subfamily of NLRs resembling the mammalian NALPs and a unique teleost NLR subfamily are present^{32, 54}. Confirmation of the antibacterial role of NOD1 and NOD2 in zebrafish was achieved by gene knockdown, resulting in higher bacterial burdens and decreased survival of embryos following *Salmonella enterica* infection⁵⁵. Moreover, *nod1/2* depletion significantly decreased expression of dual oxidase (DUOX), required for production of reactive oxygen species (ROS)⁵⁵. These findings illustrate that the family of Nod-like receptors and their downstream signaling pathways are important for antibacterial innate immunity, both in mammals and in zebrafish.

2.3 RIG-I like receptors

Another family of cytosolic PRRs, the RIG-I like receptors (RLRs), consists of three members: RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation associated factor 5), and LGP2 (laboratory of genetics and physiology 2). All three members are DExD/H box RNA helicases that can detect the presence of RNA from a broad range of viruses⁵⁶. While expressed at low levels in most tissues, their expression is greatly increased upon viral infections or interferon (IFN) exposure^{57, 58}. The RNA helicase domain of RLRs has the capacity to hydrolyze ATP and bind to RNA⁵⁹. Furthermore, RIG-I and MDA5 contain a tandem of CARDs, which facilitate protein-protein interactions⁵⁸.

LGP2 lacks the two CARDs and is thought to function as a negative regulator of RIG-I and MDA5 signaling⁶⁰. Following recognition of viral RNA, the CARDs of RIG-I and MDA5 become available for binding to a common mitochondrial signaling adaptor, IPS-1 or MAVS⁶¹. The subsequent signaling cascade culminates in the induction of transcription factors like interferon regulatory factor 3 (IRF3), IRF7, and NFκB⁶². Activation of these transcription factors leads to the production of type I IFN, which binds to the IFN receptor to initiate expression of interferon-stimulated genes (ISGs)⁶³. Amongst these ISGs are antiviral proteins, immune-proteasome components, all three RLRs, members of the TLR family, transcription factors like IRF7, and various proinflammatory cytokines and chemokines⁶³. As such, the RLR-induced pathway works cooperatively with TLR signaling to prepare the cell for elimination of viral infections⁵⁶.

Zebrafish homologs of RIG-I, MDA5, and DXH58 were identified in a genome search⁶⁴. However, *in silico* analysis of the predicted proteins revealed that the domain distribution differs between humans and zebrafish⁶⁴. For instance, whilst human RIG-I contains two CARDs, one DExD/H domain, and a Helicase C domain, zebrafish RIG-I consists of a single CARD and a DExD/H domain⁶⁴. Whilst functional studies of the RLR pathway are scarce, it is clear that zebrafish and other teleosts possess a strong antiviral IFN system, which shares a common evolutionary origin with mammals^{65,66}. The mitochondrial RLR adaptor, IPS-1/MAVS, was recently cloned from salmon and zebrafish, and overexpression in fish cells led to a constitutive induction of ISGs⁶⁶. Furthermore, MITA, another adaptor functioning downstream of IPS-1/MAVS and upstream of Tank-binding kinase 1 (TBK1), was cloned from crucian carp (*Carassius auratus*) and shown to activate zebrafish IFN promoter gene constructs, dependent on IRF3 or IRF7⁶⁷.

2.4 Scavenger receptors

Scavenger receptors are a large family of transmembrane cell surface receptors, present on macrophages, dendritic cells, mast cells⁶⁸ and some endothelial and epithelial cell types⁶⁹. Although originally defined for their role in uptake of low-density lipoproteins (LDL), they are now known to act as PRRs for a wide variety of PAMPs, like LPS, LTA, CpG DNA, yeast zymozan, and microbial surface proteins⁷⁰. Commonly, PAMP binding to a scavenger receptor will induce the cell to directly phagocytose the pathogen⁷¹. Upregulation of scavenger receptor expression via TLR signaling can be a mechanism to increase phagocytic activity⁷². Moreover, scavenger receptors can also contribute to cytokine production as co-receptors for TLRs^{73,74}. Some of the C-type lectins, discussed below, also display scavenger receptor activity.

Based upon their multidomain structure, scavenger receptors are divided into eight subclasses (A-H) (Murphy 2005). Subclasses A and B are the most extensively studied, but members from other subclasses have also been shown to recognize bacterial PAMPs⁷⁰. SR-A, the founding member of subclass A, functions as a phagocytic receptor for bacterial pathogens like *Staphylococcus aureus*, *Neisseria meningitides*, *Streptococcus*

pneumonia and *Escherichia coli*⁷⁵⁻⁷⁷. Macrophage receptor with collagenous structure (MARCO), another subclass A member with established PRR activity⁷⁸, functions as a phagocytic receptor for *S. pneumonia*⁷⁹ and *N. meningitidis*⁸⁰. MARCO was shown to cooperate with TLR2 to trigger macrophage cytokine responses to the mycobacterial cell wall glycolipid trehalose dimycolate (TDM) and *Mycobacterium tuberculosis*⁸¹. CD36, the most prominent member of subclass B, is a sensor for LTA and diacylated lipopeptide (MALP-2) and also acts as a co-receptor for TLR2⁷³. CD36-mediated phagocytosis of *S. aureus* was shown to be required for initiation of TLR2/6 signaling⁸². SR-BI (or CLA-1), also in subclass B, can bind to LPS and was implicated in phagocytosis of both Gram-negative and Gram-positive bacteria⁸³. As well as their anti-bacterial roles, CD36 and SR-BI are also known for increasing the pathogenesis of malaria and hepatitis C virus (HCV). CD36 can function as a receptor for erythrocytes that have been parasitized by *Plasmodium falciparum*, adhering these cells to the venular endothelium of various organs⁷⁰. Furthermore, SR-BI is used by *Plasmodium* sporozoites and HCV as an entry site into hepatocytes⁷⁰.

Many homologs of the mammalian scavenger receptor family can be identified in the zebrafish genome, but a systematic analysis is still awaited. A zebrafish homolog of human MARCO was identified as a specific marker for macrophages and dendritic cells from adult zebrafish⁸⁴, and this gene is also myeloid-specific in zebrafish embryos⁸⁵. Expression of the *cd36* gene was up-regulated after exposing zebrafish to haemorrhagic septicemia rhabdovirus⁸⁶. In contrast, *cd36* expression was down-regulated by *Mycobacterium marinum* infection in adult zebrafish and larvae⁸⁷.

2.5 C-type lectins

The C-type lectin receptors (CLRs) are a large family of carbohydrate-binding proteins that are highly conserved amongst mammals⁸⁸. The diversity of the CLR family is illustrated by the fact that up to 17 groups are present in vertebrates, with some consisting of soluble serum proteins, whilst others consist of transmembrane proteins. These are mainly expressed in myeloid cells (macrophages and dendritic cells) but also in natural killer cells^{89, 90}. The best known CLR in serum is mannose-binding lectin (MBL), a member of the collectin class, which binds to a variety of sugar moieties present on viruses, bacteria, fungi, and protozoa, and activates the complement system⁹¹. In terms of their function as PRRs, the transmembrane CLRs that are expressed on myeloid cells are the most interesting. Transmembrane CLRs can be divided into two groups: the mannose receptor family and the asialoglycoprotein receptor family⁹². CLRs recognize pathogens mainly via ligand-binding to mannose, fucose, and glucan carbohydrate structures, which means that together they are capable of recognizing most classes of human pathogens⁹². Like scavenger receptors, CLRs can act as phagocytic receptors for non-opsonized bacteria, leading to their destruction in acidified phagolysosomes⁷¹. The best-studied member of the asialoglycoprotein receptor family is Dectin-1, which mediates phagocytosis of yeast and the yeast-derived protein zymozan⁹³. Phagocytosis

induced by CLRs like Dectin-1 is not only important for the lysosomal breakdown of pathogens, but also for antigen presentation^{94, 95}. Besides their role in phagocytosis, CLRs can directly induce gene expression upon carbohydrate recognition. PAMP recognition by Dectin-1, Dectin-2 and macrophage-inducible C-type lectin (Mincle) ultimately leads to activation of NFκB⁹⁶⁻⁹⁸. Where Dectin-1 associates with the kinase Syk to activate NFκB⁹⁹, Dectin-2 and Mincle are dependent on Fc receptor Y-chain as an adaptor molecule^{97, 98}. Other CLRs, for example DC-specific ICAM3-grabbing non-integrin (DC-SIGN), induce specific gene expression profiles upon pathogen recognition by modulating TLR signalling⁹². When DC-SIGN recognizes mannose or fucose moieties on pathogens such as *Mycobacteria*, HIV-1, measles virus, and *Candida albicans*, it activates a Raf-1-dependent signaling pathway that modulates TLR-induced NFκB activation, increasing the production of IL8 and anti-inflammatory IL10 production¹⁰⁰.

Only a few homologs of CLRs have been described in zebrafish. A homolog of the complement activating mannose-binding lectin (MBL) was associated with resistance against *Listonella anguillarum*¹⁰¹. Expression of another soluble lectin, *Igals91l*, is enriched in zebrafish embryonic myeloid cells and is dependent on the Spi1/Pu.1 transcription factor that plays a crucial role in myeloid cell development in vertebrates⁸⁵. A membrane type collectin, CL-P1 (collectin placenta 1), was shown to be involved in vasculogenesis during zebrafish embryogenesis¹⁰². In humans, CL-P1 is mainly expressed on vascular endothelial cells and has been shown to act as a scavenger receptor mediating the phagocytosis of bacteria and yeast¹⁰³. A putative homolog for DC-SIGN has recently been proposed, and is upregulated in immune-related tissues following infection by *Aeromonas anguillarum*¹⁰⁴. Finally, putative homologs for the mammalian C-type lectin NK cell receptors have been identified in zebrafish and are differentially expressed on cells from the myeloid and lymphoid lineages¹⁰⁵.

3. Effector mechanisms of the innate immune response in zebrafish

While the adaptive immune system requires several days before reacting to invading microbes, the innate immune system consists mostly of defenses that are constitutively present and activated immediately upon infection (Figure 1). The general inflammatory response is a crucial innate defense mechanism. A state of inflammation is necessary for proper function of host defenses, since it focuses circulating immune cells and antimicrobial components of the plasma at the site of infection. Below, we focus on the effector mechanisms involved in the cell-mediated part of the innate immune response. In addition, soluble serum proteins, including complement factors and other acute-phase proteins, make an important contribution to the innate defenses, and strong induction of their encoding genes has been observed in adult and embryonic zebrafish infection models^{13, 34, 36, 106-108}.

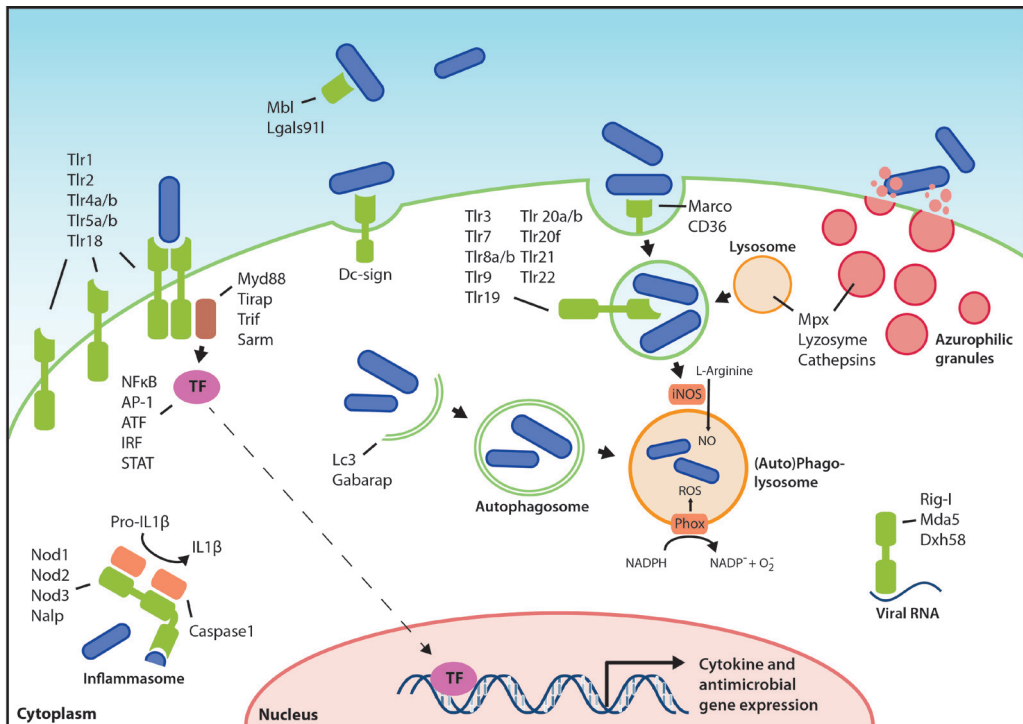


Figure 1: Pattern recognition receptors and effector mechanisms of the innate immune system. The localization of Tlrs on the cell surface or on endosomes is hypothetical and based on the known or proposed functions of their homologs in other fish or mammals. The ability of PRRs (depicted in green) to recognize PAMPs present on various types of microorganisms, like bacteria, viruses, and fungi, has been simplified here by depicting microorganisms as rod-like bacteria (in blue). PAMP recognition by PRRs leads to activation of transcription factors (TF), which translocate to the nucleus and initiate transcription of cytokine genes, antimicrobial genes, and other immune related genes. Defense mechanisms such as autophagy, ROS and NO production, and degranulation can be immediately activated upon microbial recognition, without de novo gene transcription.

3.1 Secreted peptides and lipid mediators of the innate immune response

Cytokines, including interleukins, chemokines, and interferons, are small secreted proteins that steer the host's immune system into a cytotoxic, humoral, cell-mediated, or allergic response¹⁰⁹. Since this review focuses on innate immunity, we will mainly discuss the cytokines produced by or acting on phagocytic cells. A distinction can be made between cytokines that promote a state of inflammation and cytokines that are anti-inflammatory. The main pro-inflammatory cytokines produced by phagocytes are TNFα, IL1α, IL1β, IL6, and IL8¹⁰⁹. TNF-α is processed as a membrane-bound protein and, when required, the active soluble factor is cleaved off by the TNF-α converting enzyme (TACE)¹¹⁰. Similarly, IL1α and IL1β are synthesized as inactive precursors that are only secreted as active cytokines after inflammasome-mediated cleavage by caspase 1¹¹¹. The most potent anti-inflammatory cytokine in humans is IL10, which deactivates the

pro-inflammatory cytokine production by macrophages and T-cells¹¹². The IL10/IL12 balance, maintained by cells of the innate immune system, determines whether adaptive immunity polarizes towards a Th1 (promoted by IL12) or Th2 response. A Th1 response, which activates the bactericidal activities of macrophages, is the most important for controlling intracellular pathogens. The single type II IFN, IFN γ , is also required for activating macrophage bactericidal functions, while type I IFNs (IFN α and IFN β) and type III IFN (IFN λ) function in mounting anti-viral responses. Finally, eicosanoid lipid mediators also promote (e.g. prostaglandins and leukotrienes) or inhibit (e.g. lipoxins) inflammation, thus synergizing with or antagonizing cytokine functions.

Many of the cytokine subfamilies are conserved between zebrafish and mammals³². However, there has been extensive expansion and diversification of members of the chemokine gene family in zebrafish and their specific functions are yet to be determined¹¹³. Several of the main cytokines, like IL1 β , IL6, and IL10, have been cloned and characterized¹¹⁴⁻¹¹⁶. Furthermore, the zebrafish homolog of Interleukin 10 receptor 1 (IL10R1) has recently been identified and seems to contain all the protein domains that are required for its function in anti-inflammatory signaling¹¹⁷. The pro-inflammatory chemokine IL8 (CXCL8) and its receptors, CXCR1 and CXCR2, are also conserved between mammals and zebrafish¹¹⁸. In addition, a second IL8/CXCL8 lineage has been identified in both zebrafish and common carp (*Cyprinus carpio*), and the chemotactic properties of carp IL8/CXCL8 molecules of both lineages were demonstrated by *in vitro* chemotaxis assays using carp leukocytes¹¹⁹. Both pro- and anti-inflammatory cytokines are upregulated upon infection of zebrafish embryos with pathogens such as *S. typhimurium*¹³, *P. aeruginosa*¹²⁰, and *E. tarda*^{121, 122}.

The role of TNF during *Mycobacterium marinum* infection of zebrafish embryos was studied by knockdown analysis of the TNF receptor (*tnfrsf1a*), which revealed that intracellular bacterial burdens, granuloma formation, and necrotic death of macrophages are increased in the absence of TNF signaling¹²³. The importance of TNF signaling during *M. marinum* infection was further illustrated when the same model was used to show that a strict balance between pro-inflammatory TNF and anti-inflammatory lipoxins is vital for control of mycobacterial infections, with either too much or too little TNF expression leading to a more severe outcome of the disease¹. Another study using the zebrafish model indicates that TNF- α is a potent activator of endothelial cells, leading to the production of chemokines, whilst it has little effect on the activation status of phagocytes¹²⁴. This suggests that fish TNF- α mainly functions in the recruitment of leukocytes to the site of infection, rather than activating them.

The three IFN groups present in humans are not conserved unambiguously in zebrafish and other fish species. The type II group of IFNs in zebrafish consists of IFN γ 1 and IFN γ 2¹²⁵. Expression levels of the corresponding genes did not change upon infection of zebrafish embryos with *E. coli* or *Y. ruckeri*, but was increased by *M. marinum* infection^{125, 126}. Viral infection induced their expression in adult zebrafish but not in embryos¹²⁵.

IFN γ 1 and IFN γ 2 were shown to bind to different receptor complexes and Janus kinase 2a (Jak2a), but not Jak2b, was shown to be required for intracellular transmission of the IFN γ signal. Two groups of antiviral IFNs, named IFN ϕ 1 and IFN ϕ 2 exist in zebrafish, and structural analysis showed that these are evolutionarily closer to type I than to type III human IFNs^{32, 65, 127}. IFN ϕ 1 and IFN ϕ 2 signal via distinct receptor complexes^{65, 128}. All zebrafish IFN ϕ genes induce the expression of genes that are predicted to be involved in antiviral activities⁶⁵.

3.2 Phagocytosis, autophagy, and lysosomal destruction

Internalization of microorganisms is triggered when they are recognized by phagocytic receptors, mainly by scavenger receptors discussed above. This type of direct phagocytosis is termed non-opsonic phagocytosis, while opsonic phagocytosis relies on host-derived proteins that coat the surface of the microbe thereby enhancing phagocytosis efficiency. Opsonins include complement fragments, most notably C3b, which are recognized by complement receptors¹²⁹. Mannose binding lectin, which can initiate C3b formation, and antibodies that bind to Fc receptors (IgG) or that activate complement (IgM) are also considered opsonins. Regardless of which receptor initiates the process, phagocytosis requires the activation of kinases and Rab GTPases that control alterations in the phospholipid membrane and remodeling of the actin cytoskeleton¹³⁰. In macrophages, fusion of the resulting vesicle with early and late endosomes will decrease the pH of the immature phagosome and alter the proteins present on its membrane. Ultimately, maturing phagosomes turn into phagolysosomes when lysosomes fuse with them, mixing their contents¹³¹. Lysosomes are highly acidic endocytic vesicles (pH<5.5), containing active proteases and lipases, and hydrolytic enzymes such as cathepsin D¹³². In addition, phagolysosomes also contain bactericidal peptides (defensins) and have the ability to generate toxic oxidative compounds that help microbial degradation¹³³. Most of our knowledge about phagosome maturation comes from studies of phagocytosis in macrophages, much less is known about phagosome maturation in neutrophils. While macrophage phagosomes fuse with endosomes and lysosomes, neutrophil phagosomes obtain their bactericidal properties by fusing with secretory vesicles and granules^{134, 135}. In contrast to phagosome maturation in macrophages, neutrophil phagosomes do not acidify in order to become microbicidal^{134, 135}.

Many intracellular pathogens, like *M. tuberculosis*, *S. typhimurium*, and *Legionella pneumophila* have evolved the ability to prevent phagosome maturation in macrophages and survive inside these vesicles¹³⁶. To a certain extent such pathogens can also withstand the hostile environment of the (phago)lysosome. Other pathogens like *Listeria monocytogenes*, *Francisella tularensis*, and many viruses can escape the phagosome and enter the cytosol¹³⁷. *Mycobacterium marinum*, a pathogen studied extensively in zebrafish to model human tuberculosis, can survive inside phagosomes, but also escape into the cytosol and spread to neighbouring cells by actin-based motility^{138, 139}. Phagosomal escape has also been observed for the human pathogen

M. tuberculosis and is dependent on a virulence factor, the ESX-/RD1 secretion system, shared by all pathogenic mycobacteria¹⁴⁰. Together, these data indicate that host cells face numerous pathogens that have developed multiple strategies to avoid the pathway of phagolysosomal degradation. To counter such threats, cells may use autophagy to clear microbes and microbe containing vesicles from the cytosol. Autophagy is well-known as a metabolic process that recycles nutrients by degrading intracellular organelles and proteins. Only recently it has been recognized that autophagy also plays an important role in the innate immune response against intracellular pathogens¹⁴¹. Autophagy is initiated when an autophagosomal isolation membrane is formed around its target, enclosing it entirely in a double membrane vesicle. This process relies on Class III phosphatidylinositol 3-kinase (PI3-kinase) and autophagy-related genes (Atgs), such as Atg6 (or Beclin-1)¹⁴². The hallmark of autophagosomes is the presence of Atg8 (or LC3) in their membranes, which is essential for membrane elongation¹⁴³. Similar to a maturing phagosome, the autophagosome also fuses with lysosomes to achieve its degradative properties¹⁴⁴. In addition, autolysosomes acquire unique antimicrobial properties due to the function of autophagic adaptor protein p62, which delivers cytosolic components to autolysosomes where they are processed into potent antimicrobial peptides¹⁴⁵. As reviewed elsewhere¹⁴⁶, pathogen-targeted autophagy can be induced by several TLRs and NLRs, TNF- α , NF κ B, and many other immune-related signalling molecules.

The transparency of zebrafish embryos and availability of fluorescent macrophage and neutrophil reporter lines allows for study of the process of phagocytosis in great detail^{7, 147-149}. It was recently shown that zebrafish embryonic macrophages efficiently engulf *E. coli* bacteria from blood- and fluid-filled cavities, while neutrophils are hardly capable of phagocytosing bacteria present in fluids¹⁴⁹. However, neutrophils did prove to be highly phagocytic when moving over bacteria present on tissue surfaces. This shows that the type of immune cell that clears an infection not only depends on the PAMPs present on the invading microbe, but also on the characteristics of the infection site. An *in vivo* phagocytosis assay was used to show that functions of Wasp1, Wasp2, Abi2 and cofilin regulator 14-3-3 ζ (Ywab) in bacterial phagocytosis are conserved in zebrafish¹⁵⁰. The recent generation of a transgenic zebrafish line with GFP-tagged LC3 has enabled *in vivo* visualization of the interactions between microbes and this core component of the autophagy machinery¹⁵¹. The importance of autophagy in the innate immune response of zebrafish remains to be studied, but we have shown that LC3-labeled structures accumulate around *M. marinum* infection sites in zebrafish embryos (Figure 2). Furthermore, autophagy-related genes were induced in adult zebrafish infected with *Citrobacter freundii* and zebrafish embryos infected with *S. typhimurium*^{35, 152}.

3.3 Oxidative defenses in leukocytes

In several systems it has been shown that neutrophils are the first immune cells to arrive at the site of infection or wounding. They facilitate their migration by exocytosing granules that contain metalloproteinases and other enzymes that degrade the extracellular

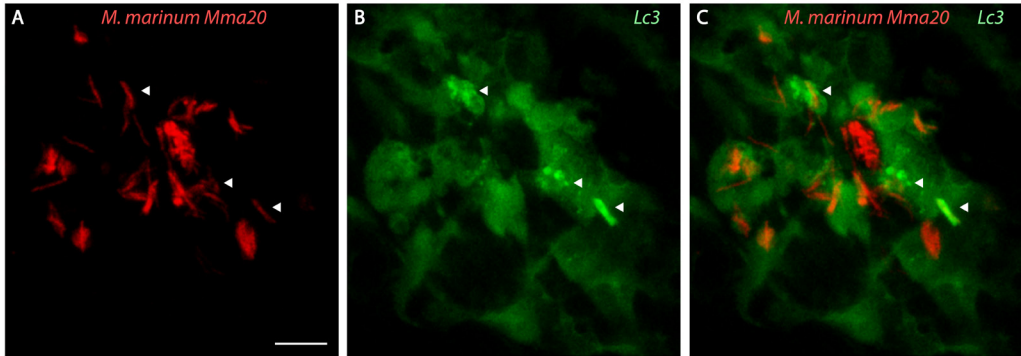


Figure 2: *In situ* detection of autophagy by Lc3 accumulation. CMV::LC3-GFP transgenic (He et al. 2009) zebrafish embryos (28 hpf) were injected into the caudal vein with 200 colony forming units (CFU) of *M. marinum* Mma20 expressing a pMST3::mCherry vector. Confocal images were taken of a tail region of the developing larva at 3 days post infection (3 dpi), a point at which the *M. marinum* infection (A) has been established. Low levels of Lc3-GFP signal (B) can be observed throughout the cells, whilst brighter regions (indicated by arrowheads) are only observed upon Lc3 accumulation and formation of autophagic membranes associated with bacteria (C). Scale bar: 10 μ m.

matrix¹⁵³. Upon recognition of pathogens, neutrophils release their antimicrobial granules, called azurophilic granules, into phagosomes or the extracellular environment^{154, 155}. Azurophilic granules are packed with acidic hydrolases and antimicrobial proteins, such as lysozyme, cathepsins, and myeloperoxidase (MPO)¹⁵⁶. The primary function of MPO is to react with hydrogen peroxide (H_2O_2), which subsequently oxidizes chloride, tyrosine, and nitrite to form hypochlorous acid (HOCl), tyrosine radicals, and reactive nitrogen intermediates¹⁵⁷. These highly reactive chemicals attack the surface membranes of microbes. Additionally, microbes can be bound by neutrophil extracellular traps (NETs), which are fibrous networks of granule proteins and chromatin released by neutrophils¹⁵⁸.

While MPO is mostly produced in neutrophils, all professional phagocytes produce high levels of reactive oxygen species (ROS), including superoxide, H_2O_2 , and hydroxyl radicals, produced by the enzymes NADPH oxidase (NOX) and dual oxidase (DUOX)¹⁵⁹. The NOX of phagocytes (Phox) is only activated upon exposure to microorganisms or other pro-inflammatory stimuli¹⁶⁰. When active, Phox is located in the phagosomal membrane and catalyzes the respiratory burst, which consists of the large scale production of ROS that helps degrade phagocytosed microbes by non-specifically oxidizing protein, DNA, lipid, and carbohydrate¹⁶¹. H_2O_2 produced during the respiratory burst can also function as a substrate for MPO activity. The oxidative enzyme DUOX may even combine the two functions, by generating H_2O_2 as a substrate for its own peroxidase domain¹⁵⁹.

Nitric oxide (NO) is produced from the amino acid L-arginine by nitric oxide synthase (NOS) enzymes, and functions as a signaling molecule in numerous biological processes as well as having antimicrobial activity¹⁶². There are two constitutively expressed NOS

enzymes, neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3), and one inducible NOS (iNOS or NOS2) that is important in innate immunity. Regulation of NOS2 plays an important role in the inflammatory response and many cells of the immune system are capable of producing NO^{163, 164}. NO has cytostatic and cytotoxic antimicrobial effects when high amounts are excreted by immune cells into mammalian tissues, most likely via reactive nitrogen species (RNS) which are generated when NO interacts with O₂¹⁶⁵. These RNS subsequently lead to lipid peroxidation, DNA damage, oxidation of thiols, and nitration of tyrosine residues¹⁶⁶. It has recently been shown that Nos2a, the zebrafish homolog of NOS2, is also required for the expansion of hematopoietic stem cells and progenitor cells during infection, leading to increased numbers of the required immune cells¹⁶⁷. This discovery further adds to the importance of NOS2 in the inflammatory response.

The oxidative defense mechanisms need to be tightly controlled, since high levels of reactive chemicals like ROS and RNS cause tissue damage at sites of infection. Therefore, the resolution phase of inflammation is critical in order to restore the tissue to its normal state and prevent chronic inflammation. The molecules produced during oxidative defenses are often self-limiting and help initiate resolution of inflammation by inducing neutrophil apoptosis^{159, 168}. Furthermore, iNOS-induced NO production can be countered by activation of arginase (ARG), which depletes the substrate for iNOS by converting L-arginine to the harmless compounds urea and L-ornithine, thus creating conditions more favorable for wound healing^{162, 169}.

The zebrafish homolog of MPO, officially named MPX, is specifically expressed in neutrophils during embryonic development. Transgenic reporter lines driven by the *mpx* promoter have made the zebrafish a highly suitable model organism to study neutrophilic inflammation^{8, 170}. In fact, using one of these lines it was demonstrated for the first time that H₂O₂ produced in the context of wounding not only functions as an antiseptic compound, but also forms a gradient that is required for rapid attraction of leukocytes¹⁷¹. However, this H₂O₂ gradient is only generated at wounds and does not occur at infected tissues¹⁷². The formation of this H₂O₂ gradient was shown to be dependent on the oxidase activity of Duox. The Src family kinase Lyn has been identified as the redox sensor that mediates neutrophil migration towards the wound¹⁷³. The innate immune function of Duox and the importance of ROS in zebrafish was further established by studies showing that knockdown of Duox impaired the ability of zebrafish larvae to control enteric *Salmonella* infections¹⁷⁴. It has also been shown that zebrafish Phox is important in controlling the *in vivo* growth of the pathogenic fungus *Candida albicans*¹⁷⁵. A 5,5-dimethyl-1-pyrroline N-oxide (DMPO) based immuno-spin trap technique has been adopted for *in situ* detection of ROS production in zebrafish embryos¹⁷⁶. DMPO is a chemical substrate that binds to reactive oxygen, which can later be detected with an anti-DMPO antibody. This protocol detects the build-up of the conjugated product, thereby showing a cumulative ROS production. Furthermore, a respiratory burst assay has been developed for zebrafish embryos, which was used

to demonstrate that macrophages and neutrophils are the ROS producing cells in zebrafish¹⁷⁷. A similar method is available to image the production of NO in zebrafish embryos, using a diamino-fluorescein probe that only becomes fluorescent in the presence of NO¹⁷⁸. As mentioned before, nitration of tyrosine residues is a hallmark of NO production. Forlenza et al. (2008) used an anti-nitro tyrosine antibody on common carp tissue to visualize the tissue nitration that occurs at sites of *Trypanoplasma borreli* infection¹⁷⁹. We used the same antibody for immunohistochemistry on zebrafish embryos to visualize the production of RNS in response to *M. marinum* infection (Figure 3). This technique also visualizes the nitrosative stress that the host tissue suffers upon release of RNS. The resolution of inflammation that should prevent tissue damage following such stresses has also been studied in zebrafish. This has led to new insights on the mechanisms underlying resolution, including apoptosis and retrograde chemotaxis of neutrophils, with the oxygen-sensing transcription factor hypoxia-inducible factor-1 α (Hif-1 α) playing a role in the control of these mechanisms^{170, 180}.

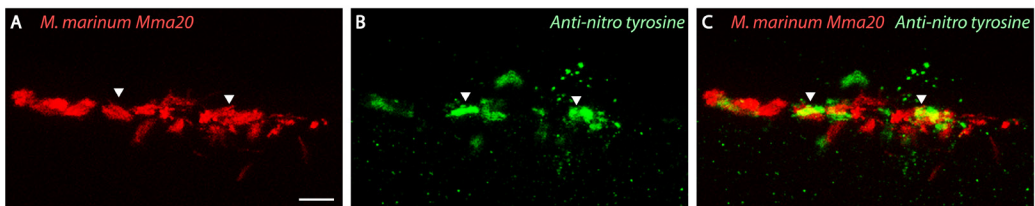


Figure 3: *In situ* detection of reactive nitrogen species. Wild type zebrafish embryos (Albino; 28 hpf) were injected into the caudal vein with 200 colony forming units (CFU) of *M. marinum* Mma20 expressing a pMST3::mCherry vector. Confocal images were taken of a tail region of the developing larva at 3 days post infection (3 dpi), a point at which the *M. marinum* infection (A) has been established. Embryos were fixed in 4% paraformaldehyde at 3 dpi and immunohistochemistry was performed, using an anti-nitrotyrosine antibody that detects tissue nitration (B) [179]. Co-localization (C) between bacteria and extensive tissue nitration can be observed at this time point. Scale bar: 10 μ m.

4. Gene expression programs reflecting innate immune responses

4.1 Genome-wide expression profiling

The availability of the zebrafish genome sequence facilitates the use of microarray and deep sequencing techniques for genome-wide expression profiling. Zebrafish embryos and larvae are useful for *in vivo* analysis of gene expression profiles upon infection, since large numbers can be pooled to level out individual variation. However, pooling should be done with caution and it is advisable to verify conclusions by analysis at the single embryo level¹²². A protocol has been developed for single embryo RNA isolation that gives sufficient RNA for microarray or RNA sequencing¹⁸¹. Expression profiling can be done either at whole organism level or on FACS sorted immune cells from transgenic lines. The latter approach was used to determine the transcriptional signature of early myeloid cells⁸⁵. Microarray analysis of zebrafish adults and embryos infected with

various pathogens has provided insights into the transcriptome during infection, and has provided leads for further functional studies (Table 1). The transcriptional response of both zebrafish embryos and adults showed clear conservation with host responses detected in other vertebrate models and human cells. Genes that were induced upon infection included receptors involved in pathogen recognition, signaling intermediates, their downstream transcription factors (like NFκB and AP-1), and inflammatory mediators. Furthermore, these studies led to the identification of novel immune responsive genes and infection markers, for example the DNA-damage regulated autophagy modulator 1 gene (*dram1*), which was identified in a knockdown study of Traf6, a central intermediate in TLR and TNF receptor signaling³⁵.

4.2 Comparison of gene expression profiles induced by different bacterial pathogens

Table 1. Transcriptome profiling studies on infection models in adult and embryonic zebrafish

Bacterial species	Strain	Infection model	Reference
<i>Mycobacterium marinum</i>	M; E11	Adult (IP)	Meijer et al. (2005)*
<i>Mycobacterium marinum</i>	Mma20; E11	28hpf (CV); Adult (IP)	Van der Sar et al. (2009)
<i>Mycobacterium marinum</i>	M; E11	Adult (IP)	Hegedus et al. (2009)*
<i>Salmonella enterica</i> serovar Typhimurium (<i>Salmonella typhimurium</i>)	SL1027; LPS derivative SF1592 (Ra),	28hpf (CV)	Stockhammer et al. (2009)**
<i>Streptococcus suis</i>	HA9801	Adult (IP)	Wu et al. (2010)
<i>Salmonella enterica</i> serovar Typhimurium (<i>Salmonella typhimurium</i>)	SL1027; LPS derivative SF1592 (Ra),	28hpf (CV)	Ordas et al. (2011)**
<i>Edwardsiella tarda</i>	FL6-60	28hpf (CV)	Van Soest et al. (2011)
<i>Citrobacter freundii</i>	Not specified	Adult (IM)	Lü et al. (2012)

* and **: these studies used the same samples, but applied microarray analysis and deep sequencing respectively
(IP): Intraperitoneal; (CV): Caudal vein; (IM): Immersion.

To illustrate the similarities and differences in the innate immune response against different bacterial pathogens, Figure 4 shows a comparison of the gene expression profiles of zebrafish infected with *Edwardsiella tarda*, *S. typhimurium*, and *M. marinum*. *E. tarda* is a Gram-negative, naturally occurring fish pathogen that belongs to the Enterobacteriaceae family. Inside its host, *E. tarda* is able to resist complement activity and can survive inside macrophages¹⁸². It causes a progressive disease when injected into the caudal vein of 28 hours post fertilization (hpf) embryos, leading to mortality within 2 days post infection (dpi)¹²². *S. typhimurium* (short for *S. enterica* serovar Typhimurium), also belonging to the Gram-negative Enterobacteriaceae family, causes salmonellosis in a broad range of hosts. *S. typhimurium* is a facultative intracellular species that can survive within phagocytic and non-phagocytic cells. Following internalization, it survives and replicates in a modified phagosome, known as the *Salmonella*-containing vacuole. Like *E. tarda*, injection of *S. typhimurium* into the caudal vein at 28 hpf leads to

a progressive disease which leads to mortality of the embryo during the first 30 hours post infection (hpi)^{13, 183}. In contrast, *M. marinum* injection at the same stage leads to a chronic infection that persists during larval development. *M. marinum* is a natural pathogen of teleost fish and a close relative of *M. tuberculosis*, the causative agent of tuberculosis in humans. *Mycobacteria* have a thick, waxy, acid-fast staining cell wall containing characteristic lipids that are important for virulence. Both *M. marinum* and *M. tuberculosis* have the ability to replicate inside macrophages, eventually causing them to undergo apoptosis. Dependent on secreted virulence factors that are conserved between *M. marinum* and *M. tuberculosis*, other macrophages are attracted to the initial infection site. These become infected by phagocytosing the apoptotic remains, which ultimately leads to the formation of a granuloma¹⁸⁴. Using the zebrafish embryo model, Ramakrishnan et al. have provided new insights demonstrating the importance of the innate immune system to control *M. marinum* infection during early stages of pathogenesis^{1, 2, 123, 185, 186}.

Complementary to previously reported transcriptome data (Table 1), here we present new data comparing the gene expression profiles induced by *E. tarda*, *S. typhimurium*, and *M. marinum* under similar conditions (Figure 4). We injected 200 colony forming units (CFUs) of each pathogen into the caudal vein of 28 hpf zebrafish embryos and analyzed the response at 8 hpi. Since *M. marinum* develops a chronic infection, we also sampled at 4 dpi, a time point at which granulomas are present. Finally, we compared the transcriptome profile of the embryonic samples with data from a previous study, in which adult zebrafish were infected with the same strain of *M. marinum*⁸⁷.

The two progressive Gram-negative pathogens, *E. tarda* and *S. typhimurium*, induced a strong early immune response at 8 hpi, while the chronic *M. marinum* infection hardly induced any response at this time point. At 4 dpi, the transcriptome profile of *M. marinum* infected embryos did show an immune response, although it was still weaker than the response to *E. tarda* or *S. typhimurium* infection at 8 hpi. In adults, the immune response to *M. marinum* infection has been shown to develop in a similar manner, with hardly any induction of proinflammatory genes at 1 dpi and a stronger response at 6 dpi, when the fish began to show symptoms of disease⁸⁷. Infections with *E. tarda* and *S. typhimurium* resulted in a remarkably similar transcriptome. Nevertheless, subtle differences were observed, like the up-regulation of Tlr3 that was specific to *E. tarda* infection in this data set, and the variation in the panel of cytokines expressed upon these infections.

Interestingly, various PRRs, for example Tlr5a and 5b, showed increased expression upon infection, most likely indicating an elevated state of awareness needed to identify the invading pathogens. In contrast, the fish specific Tlr18, the scavenger receptors CD36, scarb1 and scarb2, and the C-type lectin Mbl were down-regulated in some conditions. In many cases, signaling intermediates downstream of PRRs were up-regulated, relaying and possibly amplifying the activating signals they receive from their respective

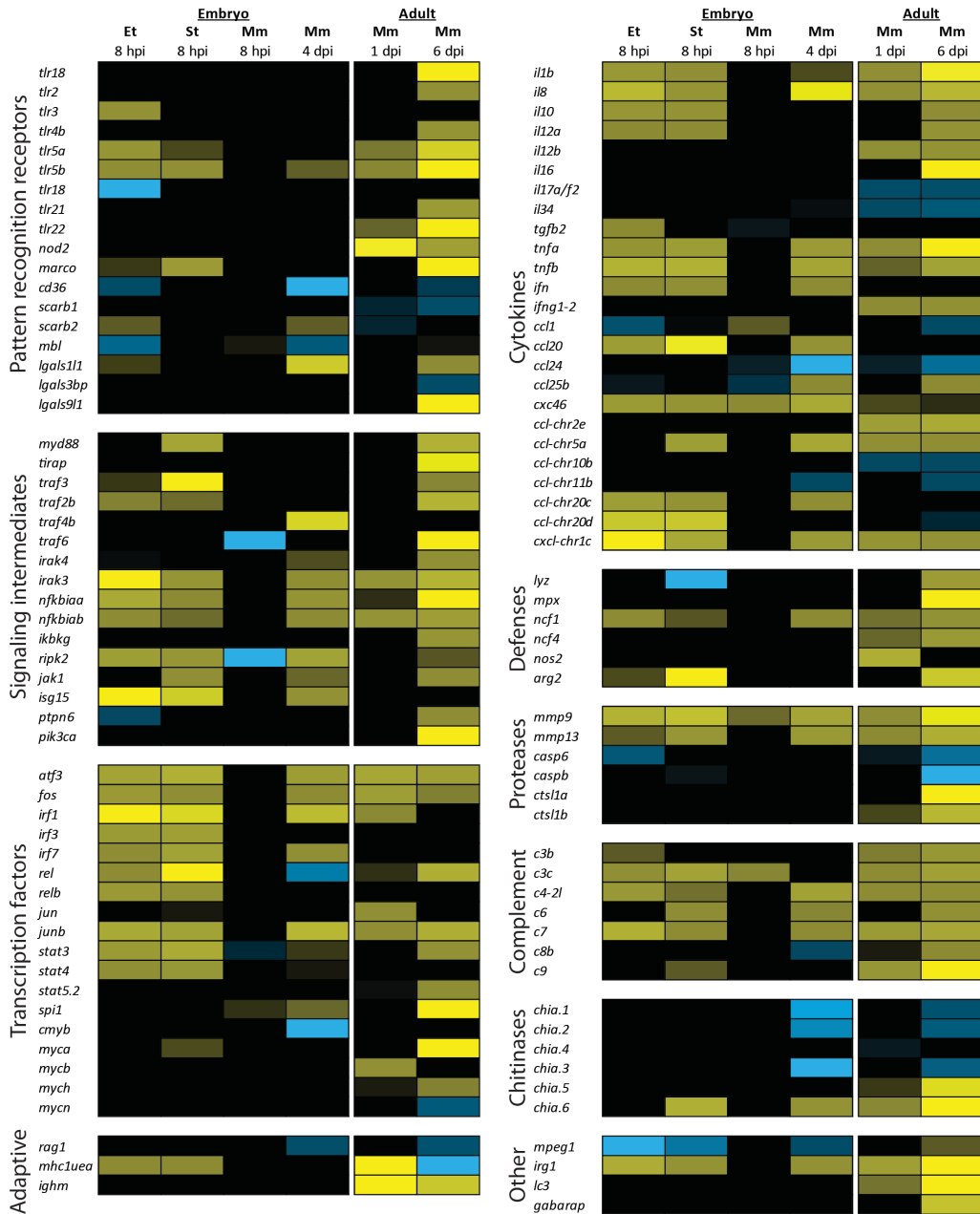


Figure 4: Comparison of the zebrafish innate immune response to different bacterial pathogens. Gene expression profiles of zebrafish embryos and adults infected with *E. tarda* FL6-60 (Et), *S. typhimurium* SL1027 (St), and *M. marinum* Mma20 (Mm) are depicted in a heat map. Embryos were infected with 200 CFU of each pathogen into the caudal vein at 28 hpf and snap frozen individually at 8 hpi for *E. tarda* and *S. typhimurium*, and at 8 hpi and 4 dpi for *M. marinum*. Triplicate samples for each infection condition were compared with samples from control embryos (injected with PBS) using a common reference microarray design.

Figure 4 continued: The raw data were deposited in the Gene Expression Omnibus database under accession number GSE35474. The data derived from embryonic infections were compared with data from a study in which adult zebrafish were infected intraperitoneally with *M. marinum* *Mma20*, after which RNA samples were taken at 1 dpi and 6 dpi [87]. The dose of the *Mma20* strain used in the adult infection study was lethal within days after the final sampling point at 6 dpi. Only genes relevant to this review were included in the heatmap. All selected genes are represented by a minimum of two probes that showed significant up- or down-regulation (Significance cut-offs for the ratios of infected vs control groups were set at 2-fold with $p < 10^{-5}$). Up-regulation is indicated by increasingly bright shades of yellow and down-regulation is indicated by increasingly bright shades of blue. It should be noted that the genes listed in this figure are named according to sequence homology with mammalian counterparts and in most cases have not yet been confirmed functionally.

receptors. A wide range of transcription factors with well-established functions in immunity (e.g. Atf3, Jun and Fos, Rel, and the IRF and Stat family members) were significantly up-regulated under all conditions tested, except for the 8 hpi time point of *M. marinum* infection), whereas we observed up-regulation of transcription factors of the oncogenic Myc family mainly in adult fish. The hematopoietic transcription factor Spi1 (Pu.1) was upregulated in *M. marinum* infection of embryos and adults. Genes for the key pro-inflammatory cytokines, like TNF α (two genes in zebrafish: *tnfa* and *tnfb*), IL1 β , and IL8, and for the anti-inflammatory cytokine IL10 were induced by infection with any of the three pathogens. Other cytokines appeared to be more specific for certain pathogens, or might not be expressed at the specific time-point of infection that we sampled.

We also observed increased expression of genes involved in effector mechanisms. However, upregulation of the genes encoding lysozyme, myeloperoxidase, and iNos was detectable only in adult zebrafish infected with *M. marinum*. Infection with any of the three pathogens led to increased gene expression of *ncf1*, a subunit of the neutrophil NADPH oxidase complex. Proteases are an important part of the innate immune response, functioning in reorganizing the extracellular matrix to allow leukocyte migration, in degradation of microbes, and in processing of cytokines. In adult zebrafish infected with *M. marinum* we observed up-regulation of cathepsin-like 1a and 1b (*ctsl1a* and *ctsl1b*), members of lysosomal cathepsin family that aids in the destruction of microbes. Expression levels of *casp6* and *caspb*, members of the cysteine-aspartic acid protease (caspase) family involved in apoptosis, were down-regulated at different stages of infection in adults and embryos. The matrix metalloproteinase (mmp) genes 9 and *mmp13* proved to be excellent markers for infection, since their gene expression was induced by *E. tarda*, *S. typhimurium* and *M. marinum*.

Our data further suggest that complement activation plays an important role during the early innate immune response, since a large number of complement factor genes show increased expression upon infection. Up-regulated expression of the autophagy marker genes *lc3* and *gabapap* in adults infected with *M. marinum* hints towards a role

for autophagy in the control of this infection. Intriguingly, a macrophage expressed gene with unknown function in immunity, *mpeg1*¹⁸⁵, is down-regulated during the embryonic immune response against all three pathogens. The mouse homolog of this gene encodes a perforin-like protein that is expressed in mature macrophages and prion-infected brain cells¹⁸⁷. We have also observed specific up-regulation of genes with as of yet unknown function in immunity, like immunoresponsive gene 1 (*irg1*). This gene is highly conserved in vertebrates and has high homology to bacterial methylcitrate dehydrogenase¹⁸⁸. We also included some genes involved in adaptive immunity in our comparison, the lymphocyte marker *rag1*, the immunoglobulin heavy chain gene *ighm*, and the antigen presenting major histocompatibility complex class I UEA gene (*mhc1uea*). Even though no cells of the adaptive immune system are present yet, embryos infected with *E. tarda* or *S. typhimurium* increase the expression of the MHC I gene. Finally, upon infection with *S. typhimurium* and *M. marimum* we observe up- and down-regulation of chitinases, a family of genes which has been attributed a role during the host-microbial interactions involved in the development of acute and chronic inflammatory conditions¹⁸⁹.

5. Discussion

Zebrafish infectious disease models have started to make an important contribution to the understanding of host-pathogen interaction mechanisms. A good example is the discovery of the mechanism whereby a mycobacterial virulence factor (ESAT6) induces *mmp9* expression in host epithelial cells neighboring infected macrophages, which enhances macrophage recruitment and formation of granuloma-like aggregates that provide a replication niche for mycobacteria². The combination of genetics and *in vivo* imaging in zebrafish embryos is unparalleled in other vertebrate models. Furthermore, zebrafish embryos provide an ideal model for high-throughput *in vivo* screening of antimicrobial drug candidates or novel vaccine candidates^{190, 191}. Knowledge of the zebrafish immune system is also important in high throughput screening for cancer in zebrafish embryos¹⁹². However, many aspects of zebrafish immunity still require further characterization and validation.

Currently available transgenic lines clearly distinguish macrophages (marked by *csf1r/fms* and *mpeg1*) from neutrophils (marked by *mpx* and *lyz*) in embryos and larvae, but there is insufficient knowledge of surface markers to identify different macrophage and neutrophil subpopulations. Similar to mammals, there is evidence of the existence of subpopulations of classically activated macrophages (M1: high producers of proinflammatory mediators, ROS, and NO) and alternatively activated macrophages (M2: high producers of anti-inflammatory mediators) in fish¹⁹³. The polarization of macrophages towards these subtypes plays a critical role in the pathology of both infectious diseases and cancer¹⁹⁴. Furthermore, different subpopulations of mammalian neutrophils (N1 and N2) have been recently described that display pro- and anti-tumorigenic properties¹⁹⁵, and that probably will also turn out to have distinctive

functions during infectious disease pathology. Tumor implants in zebrafish embryos were shown to attract a heterogeneous population of leukocytes, including cells that express arginase, a marker of alternatively activated macrophages¹⁷⁶. In addition, the neutrophil markers *mpx*, *mych* and *lyz* do not show complete overlap^{176, 196}, and markers such as *cxc3.2* and *ptpn6*, which are macrophage-specific in one-day old embryos, also label a subset of neutrophils at later stages⁸⁵. Future development of transgenic lines that can distinguish these multiple myeloid subsets would further strengthen the use of zebrafish models for innate immunity and infectious disease studies.

As detailed in this review, counterparts of the major vertebrate PRRs and downstream signaling components have been identified in zebrafish, but relatively few have thus far been functionally studied in infectious disease models. Recently, new PRRs have been described in mammals, like the INF-inducible dsRNA-activated protein kinase R (PKR)¹⁹⁷, the cytosolic DNA sensor DNA-dependent activator of IFN-regulatory factors (DAI)¹⁹⁸, and a cytosolic DNA receptor named AIM2 (absent in melanoma 2)¹⁹⁹. Thus far only the zebrafish homolog for PKR has been identified. Furthermore, autophagic adaptors known as sequestosome 1/p62-like receptors (SLRs), conserved between zebrafish and human, have recently been suggested as a new category of PRRs, since they have the ability to recognize and capture targets for immune-related autophagy²⁰⁰.

Various datasets derived from transcriptome analyses have shown the specificity of immune responses to different pathogens. In future studies, the analysis of these responses can be refined by FACS-sorting of immune cell populations from infected embryos, using labeled pathogens in combination with transgenic lines for different immune cell types. For example, it now comes within reach to aim at dissecting the differences in gene expression between *M. marinum*-infected macrophages inside a granuloma and recently attracted uninfected macrophages. In addition, simultaneous profiling of pathogen and host genes will be a challenging approach to help unravel the complex mechanisms underlying host-pathogen interactions. Transcriptome analysis only reveals altered RNA levels upon infection and therefore the application of proteomic and epigenetic analyses are needed to study the regulation of immune responses on different levels. Transcriptome studies have revealed infection-responsiveness of many genes that have not yet been well studied (for example *dram1*, *mpeg1*, *irg1*, and *irg1l*, mentioned above), and an emerging immune function for several chitinase-like proteins during infection^{13, 35, 122}. Many zebrafish infection models have been described here and in other recent reviews^{4, 201, 202} that can be used to investigate the functions of these genes in different pathogenic interactions, either using morpholino knockdown in embryos or using stable knockout lines which nowadays can be identified very efficiently by high-throughput resequencing of mutant libraries or by targeted knock-down approaches using technologies such as zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs)²⁰³.

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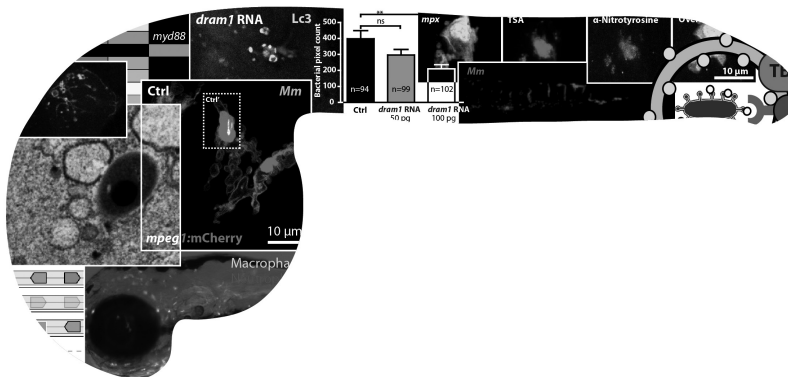
Chapter 3

Functional analysis of a zebrafish *myd88* mutant identifies key transcriptional components of the innate immune system

3

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Abstract

Toll-like receptors (TLRs) are an important class of pattern recognition receptors (PRRs) that recognize microbial and danger signals. Their downstream signaling upon ligand-binding is vital for initiation of the innate immune response. In human and mammalian models, myeloid differentiation factor 88 (MYD88) is known for its central role as an adaptor molecule in interleukin 1 receptor (IL-1R) and TLR-signalling. The zebrafish is increasingly used as a complementary model system for disease research and drug screening. Here, we describe a line with a truncated version of Myd88 as the first zebrafish mutant for a TLR-signaling component. We show that this immune-compromised mutant has a lower survival rate under standard rearing conditions and is more susceptible to challenge with the acute bacterial pathogens *Edwardsiella tarda* and *Salmonella typhimurium*. Microarray and quantitative PCR (qPCR) analysis revealed that expression of genes for transcription factors central to innate immunity, including NFκB and AP-1, and the pro-inflammatory cytokine Il1b, is dependent on Myd88-signalling during these bacterial infections. Nevertheless, expression of immune genes independent of Myd88 in the *myd88* mutant line was sufficient to limit growth of an attenuated *S. typhimurium* strain. In the case of infection with the chronic bacterial pathogen *Mycobacterium marinum*, we show that Myd88-signaling has an important protective role during early pathogenesis. During mycobacterial infection, the *myd88* mutant shows accelerated formation of granuloma-like aggregates and increased bacterial burden, with associated lower induction of genes central to innate immunity. This zebrafish *myd88* mutant will be a valuable tool to further study the role of IL1R- and TLR-signaling in innate immunity processes underlying infectious diseases, inflammatory disorders, and cancer.

Introduction

The host innate immune response is the first line of defense against invading microbes. Its function is to recognize invading pathogens at the first stage of infection and initiate an appropriate immune response¹. Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are recognized by pattern recognition receptors (PRRs), of which the Toll-like receptor (TLR) family has been studied most extensively^{1, 2}. Myeloid differentiation factor 88 (MYD88) is an important adaptor protein in the TLR signaling pathway, since it is used by all TLRs except for TLR3³. Its C-terminal TIR-domain enables interaction with TLRs, while the N-terminal death domain enables interaction with IL-1 receptor associated kinase 4 (IRAK4), which in turn recruits IRAK1 or IRAK2 to form the 'Myddosome' signalling complex, activating NF-κB (nuclear factor κB) and MAP-kinase (mitogen activated protein) signaling⁴⁻⁸. The Myddosome also functions downstream of the receptors for interleukin 1 (IL-1), IL-18 and IL-33, and it has been associated with IFN-γ receptor signaling, adding to its central role in inflammation and host defense^{5, 9, 10}.

Patients with a deficiency in MYD88 suffer from a primary immunodeficiency syndrome^{11, 12}. This syndrome is characterized by an increased susceptibility to pyogenic bacteria, like *Streptococcus pneumonia*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella* species¹³. Mice deficient in Myd88 were shown to be hyporesponsive to lipopolysaccharide (LPS) II-1 stimulation and resistant to endotoxic shock^{9, 14, 15}. As a consequence of their reduced ability to recognize PAMPs, Myd88-deficient mice are more susceptible to infection by *Toxoplasma gondii* parasites¹⁶ and several bacterial pathogens, including *S. aureus*¹⁷, *Listeria monocytogenes*¹⁸, *Chlamydia pneumonia*¹⁹, and *Mycobacterium tuberculosis*²⁰.

The zebrafish (*Danio rerio*) is an excellent model to study the innate immune system that can complement research in mouse models and human cell lines²¹. Major advantages are its suitability for genetic approaches, high-throughput screening, and live imaging studies that make use of the many available fluorophore-marked transgenic lines. As early as one day post fertilization (dpf), zebrafish embryos are capable of mounting an effective innate immune response against microbial infections²². This immune response has a transcriptional signature similar to responses observed in mammalian and cell culture systems²³. Furthermore, the major PRRs, their downstream signaling pathways, and innate effector mechanisms are conserved between humans and zebrafish²⁴. Expression of *myd88* in zebrafish leukocytes was confirmed using a transgenic reporter line²⁵. Maturation of the adaptive immune system is not complete until approximately three weeks post fertilization²⁶, providing a window of at least two weeks in which the innate immunity can be studied in the absence of T- and B-cell responses.

The use of zebrafish as a model for infectious diseases, inflammatory disorders, cancer, and other immune related diseases is growing rapidly²⁷. Therefore, zebrafish mutants for important components of the immune system are eagerly awaited. As an alternative to mutant lines, morpholino antisense oligonucleotides may be used for blocking of protein production, but knockdown by morpholinos is only temporal and may also cause off-target effects²⁸. Knockdown of Myd88 by morpholino in zebrafish embryos affected their ability to combat infection with the attenuated *Salmonella enterica* serovar *Typhimurium* (*S. typhimurium*) Ra strain²⁹. It was also shown that expression of the pro-inflammatory cytokine gene *il1b* was dependent on Myd88, both during *Salmonella* infection and when embryos were stimulated with TLR ligands^{23, 30}. Knockdown analysis also demonstrated a role for Myd88 in LPS- or trinitrobenzene sulfonic acid (TNBS)-induced intestinal inflammation and microbial-dependent intestinal epithelial cell proliferation in zebrafish larvae³¹⁻³³.

Here we characterize the first *myd88* mutant zebrafish line (*myd88*^{-/-}) and compare its immune response to that of wild types when infected with the bacterial pathogens *Edwardsiella tarda*, *Salmonella typhimurium*, and *Mycobacterium marinum*. The mutant allele contains a premature stopcodon, resulting in a truncated protein that lacks part of its death domain and the complete TIR-domain, required for interaction

with IRAK4 and TLRs respectively. As expected, the homozygous mutant showed an immune compromised phenotype. We show that *myd88*^{-/-} zebrafish embryos are more susceptible to infection by all three pathogens tested, but have sufficient remaining innate immunity to limit infection with attenuated *S. typhimurium* Ra bacteria. Finally, we compare the gene expression profile of *myd88*^{-/-} embryos with that of wild type embryos to distinguish between Myd88-dependent and -independent gene expression upon infection with different pathogens. This analysis revealed that gene expression of transcription factors central to innate immunity, including NFκB and AP-1, and many innate immunity signalling and effector genes is dependent on Myd88-signalling during bacterial infections. Using this mutant as a model for mycobacterial pathogenesis, we demonstrated that Myd88-signaling has an important protective role during early stages of tuberculosis.

Results

Reduced survival of homozygous *myd88* mutants

Sequencing of an ENU-mutagenized zebrafish library resulted in the identification of a *myd88* mutant allele, *myd88*^{hu3568}, which carries a T to A point mutation creating a premature stopcodon (figure 1A). The mutation is located in the N-terminal death domain, leading to a truncated protein, lacking part of its death domain required for interaction with Irak4. The truncated protein is also missing the complete C-terminal TIR-domain required for interaction with the cytoplasmic TIR-domain of TLRs. In addition, we observed that the mutant transcript is less stable than the wild type transcript (supplementary figure 1). Zebrafish embryos homozygous for the mutation were found at Mendelian frequencies after crossing of heterozygous parents. They showed no developmental differences with their wild type siblings and could only be distinguished by genotyping. Two groups of *myd88*^{-/-} and *myd88*^{+/-} embryos were compared under normal embryo rearing conditions to test for differences in unchallenged survival during development. Up to 8 dpf there was no difference in survival between mutants and wild types (figure 1B). After this period *myd88*^{-/-} larvae showed a significantly decreased survival rate compared to wild type larvae (figure 1B). The steady decline of *myd88*^{-/-} larvae ceased at around 20 dpf. Thus, under our rearing conditions, 8 – 20 dpf was a critical period for rearing *myd88*^{-/-} larvae, but subsequent development was normal and adults were capable of breeding. We have experienced that the success rate of rearing *myd88*^{-/-} families is highly variable. Furthermore, *myd88*^{-/-} adults display an increased mortality rate compared to wild type individuals and heterozygotes, often showing pathological features linked with infection (data not shown). To conclude, we observe a reduced survival in embryonic and adult *myd88*^{-/-} zebrafish, most likely caused by a primary immune deficiency due to the lack of functional Myd88.

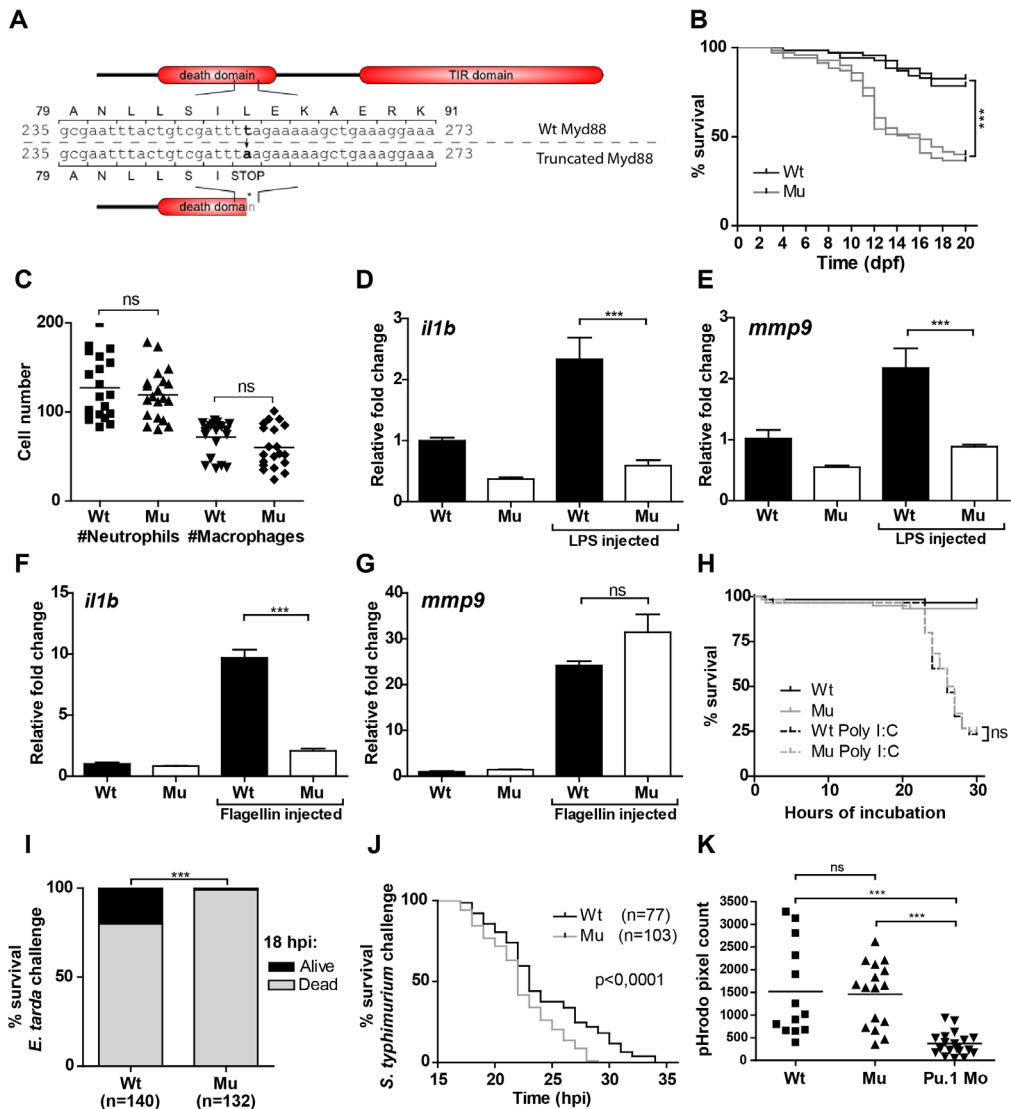


Figure 1. Characterization of *myd88* mutant zebrafish and survival following infection with *E. tarda* and *S. typhimurium*. (A) Mutant sequence and protein structure. A point mutation (T to A) in the death domain sequence of zebrafish *myd88* introduces a premature stopcodon. The truncated protein lacks the TIR domain and part of the death domain. 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 per group) of *myd88*^{-/-} mutants (Mu, grey lines) and *myd88*^{+/+} wild type (Wt, black lines), grown in two individual experiments. The groups are the F1 offspring of *myd88*^{-/-} and *myd88*^{+/+} siblings born from heterozygous parents. At 20 dpf, 83% and 79% of wild types survived, compared to 37% and 40% of mutants. The asterisks indicate the significant difference between wild type and mutant survival ($p < 0.0001$), tested with a logrank test. (C). Quantification of leukocyte numbers. Total numbers of GFP-labeled neutrophils in 3 dpf *myd88*^{-/-}-*mpx::egfp* and *myd88*^{+/+}-*mpx::egfp* embryos (n=20 per group) were counted under a

fluorescence stereo microscope. Total numbers of macrophages were determined by performing whole mount L-plastin immunohistochemistry and deducting the number of *mpx::egfp*-positive neutrophils from the number of L-plastin-positive total leukocytes per embryo. Each data point represents an individual embryo and lines indicate the mean value. No significant difference (ns) in numbers of neutrophils or macrophages was observed with a t-test. (D-G). *Myd88*^{-/-} and wild type embryos were injected with purified LPS (100 µg/ml), flagellin (100 µg/ml), or PBS as a control. Expression of *il1b* and *mmp9* at 2 hours post injection was analyzed by qPCR. Data are combined from three biological replicates (n=20 per group) and statistical significance was determined by one-way ANOVA with Tukey's Multiple Comparison method as a post-hoc test (***, p<0.001 and **, p<0.01). (H). Poly I:C exposure. *Myd88*^{-/-} and wild type embryos were exposed to poly I:C (500 µg/ml eggwater) starting at 5 dpf. Survival curves are based on data pooled from two individual experiments (n=60 per group in total). Statistical significance (ns) was determined by a logrank test. (I) *E. tarda* infection. At 28 hpf, *myd88*^{-/-} and wild type embryos were infected with approximately 150 CFU *E. tarda* FL6-60 by injection into the blood island. The percentages of embryos surviving infection at 18 hpi are shown for *myd88*^{-/-} (Mu, n=132, 1% surviving) and wild type (Wt, n=140, 20% surviving). Data is pooled from two individual experiments and statistical significance (***, p<0.001) was tested with a contingency test. (J) *S. typhimurium* infection. Embryos were injected into the blood island at 28 hpf with approximately 150 CFU of *S. typhimurium* strain SL1027. Survival curves for mutant (n=103, median survival 22 hpi) and wild type embryos (n=77, median survival 23 hpi) are based on data pooled from two individual experiments. Statistical significance (p<0.001) was determined by a logrank test. (K) Phagocytosis assay. Mutant and wild type embryos were injected at 28 hpf into the blood island with *E. coli* cell wall particles labeled with a pH-dependent fluorogenic dye, pHrodo. After 2 hours, stereo fluorescence images were taken for fluorescent pixel quantification. Pu.1 morpholino-injected embryos (Pu.1 Mo), deficient in phagocytic leukocytes, were included as a control. Each data point represents an individual embryo and lines indicate the mean value. No significant difference (ns) was observed between wild type and mutant embryos, but both groups were significantly different (***, p<0.001) from Pu.1 morpholino-injected controls by one-way ANOVA with Tukey's Multiple Comparison method as a post-hoc test.

Myd88 deficiency has no overt effect on leukocyte development

To test whether *myd88*^{-/-} embryos have normal leukocyte development, we crossed *myd88*^{-/-} adults with fish carrying the *mpx::egfp* transgene that specifically marks neutrophils³⁴. Leukocyte differentiation was assessed at 3 dpf by GFP fluorescence of neutrophils in combination with fluorescent immunohistochemistry for the pan-leukocytic marker L-plastin (leukocyte-plastin). We observed no differences between mutant and wild type larvae in the total numbers of the two major subtypes of leukocytes, neutrophils (*mpx::GFP*/L-plastin double fluorescent cells) and macrophages (L-plastin positive and *mpx::GFP* negative) (figure 1C). Therefore, we conclude that early leukocyte hematopoiesis in zebrafish embryos is not overtly affected by *Myd88* deficiency and does not account for the reduced survival observed when rearing *myd88*^{-/-} families.

Myd88-dependent signaling is required for recognition of LPS and flagellin, but not Poly I:C

It is expected that *myd88*^{-/-} embryos are affected in their ability to sense and respond to microbial PAMPs. To test this hypothesis, we injected LPS purified from *S. typhimurium*

into the blood island of 28 hpf *myd88*^{-/-} and wild type embryos. We isolated RNA 2 hours post injection (hpi) and determined the response to LPS by analyzing the expression of *il1b* and *mmp9* by quantitative PCR (qPCR). It has been reported that, unlike in humans, recognition of LPS in fish does not occur via Tlr4^{35, 36}. Nevertheless, we show here that recognition of LPS in zebrafish embryos does require Myd88, since expression of both *il1b* and *mmp9* after injection with LPS was significantly lower in mutants compared to wild types (figure 1D, E). We also injected groups of embryos with flagellin, a known ligand for the Tlr5-Myd88 pathway in zebrafish²³. The observed expression levels of *il1b* following injection confirmed the Myd88-dependency for flagellin recognition (fig 1F). Interestingly, flagellin-induced *mmp9* expression occurred independent of Myd88. As a negative control, we also tested poly I:C, a ligand of Tlr3, the only TLR that is known to signal completely independent of MyD88. Poly I:C injection did not lead to a reproducible induction of immune related genes in 1 dpf embryos (data not shown). We therefore determined the sensitivity to poly I:C exposure by incubation starting at 5 dpf. We observed a sharp decrease in survival after approximately 24 hours of incubation, which was similar between *myd88*^{-/-} and wild type embryos, showing that poly I:C recognition and toxicity occurs Myd88-independently, in contrast to the MyD88-dependent recognition of LPS and flagellin.

Increased susceptibility of *myd88*^{-/-} embryos to acute bacterial pathogens is not caused by a general defect in phagocytosis

To test the ability of *myd88*^{-/-} embryos to mount a successful defense response, we infected 28 hpf *myd88*^{-/-} and wild type embryos by blood island injection with two acute bacterial pathogens: *E. tarda* and *S. typhimurium*. These two pathogens are known to cause progressive and fatal infection in wild type zebrafish embryos^{37, 38}. At 18 hpi with *E. tarda*, the percentage of surviving *myd88*^{-/-} embryos (1%) was significantly lower than the percentage of wild type embryos (20%) that had not yet succumbed to the infection (figure 1I). Lethality of *S. typhimurium* infection also occurred significantly faster in mutant than in wild type embryos (figure 1J). These results demonstrate that *myd88*^{-/-} embryos are affected in their response towards invading pathogens. To exclude the possibility that the impaired immune response might be due to a defect in phagocytosis of bacteria in *myd88*^{-/-} embryos, we injected both mutants and wild types with *Escherichia coli* cell wall particles labelled with the pH-dependent fluorogenic dye, pHrodo. This provides a quantitative measure for phagocytosis, since pHrodo only becomes fluorescent in acidic environments, like those encountered in the phagolysosomal pathway²⁵. The results showed similar levels of phagocytosis in *myd88*^{-/-} and wild type embryos at 2 hours post injection (figure 1K). A control group, where a Pu.1 morpholino was injected to severely deplete leukocyte populations³⁹, showed low levels of phagocytosis. These results demonstrate that the increased susceptibility of *myd88*^{-/-} embryos to acute bacterial pathogens is not the result of a defect in phagocytosing bacterial components, but that *myd88*^{-/-} embryos are not capable of mounting a wild type innate immune response towards these bacteria at a stage after primary phagocytosis.

The expression of genes that function in immunity are affected in the *myd88*^{-/-} mutant after infection with *E. tarda* and *S. typhimurium*

To study the effect of Myd88 deficiency on gene expression during infection, we analyzed the transcriptome profile of *myd88*^{-/-} and *myd88*^{+/-} embryos infected with *E. tarda* and *S. typhimurium*. Embryos from a heterozygous incross (*myd88*^{+/-}) were infected by injecting 150 CFU of either pathogen into the blood island at 28 hpf. Embryos injected with the carrier solution alone were taken along as a control. At 8 hpi, RNA was isolated from three *myd88*^{-/-} and three wild type individuals per condition. Transcriptome analysis of uninfected *myd88*^{-/-} and wild type embryos showed that no immune related genes were differentially expressed in the absence of Myd88, except for *myd88* itself, which was consistently downregulated in Myd88-deficient embryos ($p < 0.00001$). Eight hours after infection with *E. tarda* or *S. typhimurium*, a large number of probes (619 and 1214, respectively) were upregulated in wild type embryos (figure 2A). In comparison, the numbers of upregulated probes were approximately 3-fold lower in *myd88*^{-/-} embryos (190 for *E. tarda* and 339 for *S. typhimurium*) (figure 2A). At the same time point, the smaller number of downregulated probes was comparable between wild types (199 for *E. tarda* and 315 for *S. typhimurium*) and *myd88*^{-/-} embryos (117 for *E. tarda* and 196 for *S. typhimurium*) (figure 2B). Gene ontology (GO) analysis revealed that the GO term 'Immune system process' was significantly enriched both in wild type and in *myd88*^{-/-} embryos infected with *E. tarda* or *S. typhimurium* (figure 2C). However, while all GO terms belonging to the 'Immune system process' subclass were enriched for infected wild type embryos, only 1 or 3 out of 5 were significantly enriched for *myd88*^{-/-} embryos infected with *E. tarda* or *S. typhimurium*, respectively. GO term-analysis of the probe sets that were up- or down-regulated by the different infections in *myd88*^{-/-} mutants did not reveal a specific Myd88-independent signaling pathway that has been activated to compensate for the loss of Myd88 function.

For a more in-depth analysis of gene regulation in *myd88*^{-/-} and wild type embryos infected with *E. tarda* or *S. typhimurium*, we selected all immune related genes with significantly altered expression in mutants, wild types, or both (a fold change of at least 2 with $P < 0.00001$ for at least two probes). A heatmap was created to visualize the expression differences of these genes between individual embryos. In agreement with genotyping of the embryos, *myd88* itself was expressed at low levels in all *myd88*^{-/-} embryos. In general, infection with either bacterial strain resulted in a large scale upregulation of immune related genes in wild type embryos, while *myd88*^{-/-} embryos showed a much weaker transcriptional response (figure 3). Surprisingly, however, in one of the *myd88*^{-/-} embryos infected with *S. typhimurium* the response of several of the selected genes was similar to the wild type level. The most consistent differences in gene expression between *myd88*^{-/-} and wild type embryos were observed for transcription factors and genes involved in signal transduction. The number of upregulated genes in these categories was lower in *myd88*^{-/-} embryos, but also the expression level of genes that did respond was lower compared to wild type. The residual induction of immune-related transcription factors in *myd88*^{-/-} embryos was reflected in the number

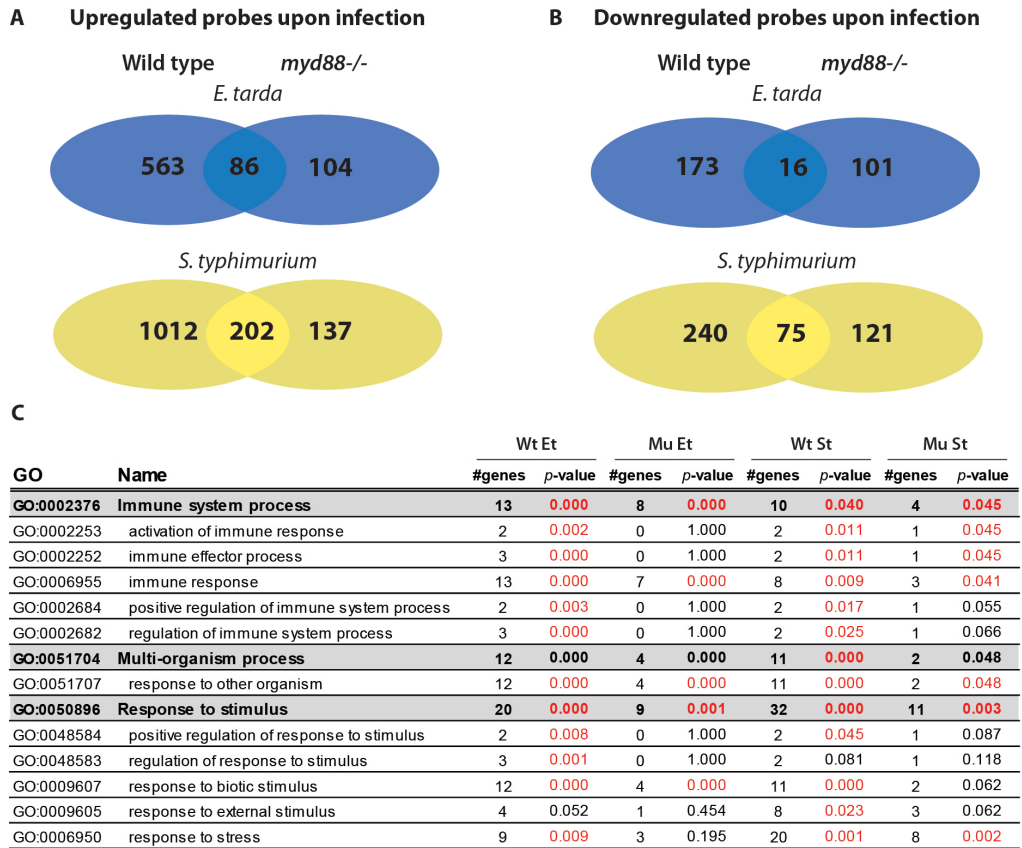


Figure 2. Transcriptome analysis of *myd88*^{-/-} and wild type embryos following infection with *E. tarda* and *S. typhimurium*. (A,B) Venn-diagrams showing the overlap and differences between mutant (Mu) and wild type embryos in numbers of probes up-regulated (A) or down-regulated (B) by *E. tarda* FL6-60 (Et) or *S. typhimurium* SL1027 (St) infection. Embryos were infected with approximately 150 CFU of either pathogen into the caudal vein at 28 hpf and snap frozen individually at 8 hpi. Triplicate samples for each infection condition were compared groupwise with samples from control embryos (injected with PBS) using a common reference microarray design. Significance cut-offs for the ratios of infected vs control groups were set at 2-fold with $p < 10^{-5}$. (C) Gene ontology (GO) analysis. The table displays the enrichment of immune-related GO terms following infection of *myd88*^{-/-} (Mu) and wild type (Wt) embryos with *E. tarda* (Et) or *S. typhimurium* (St). Go-term enrichment in the gene lists up-regulated by infection was determined by master-target statistical testing using eGOn (Beisvag et al., 2006) with the *Danio rerio* UniGene build #124 as background. The numbers of genes associated with each GO-term and the p -values for enrichment are indicated. We note that GO analysis is limited by the number of Unigene clusters annotated for zebrafish and therefore not all upregulated probes could be included in the analysis.

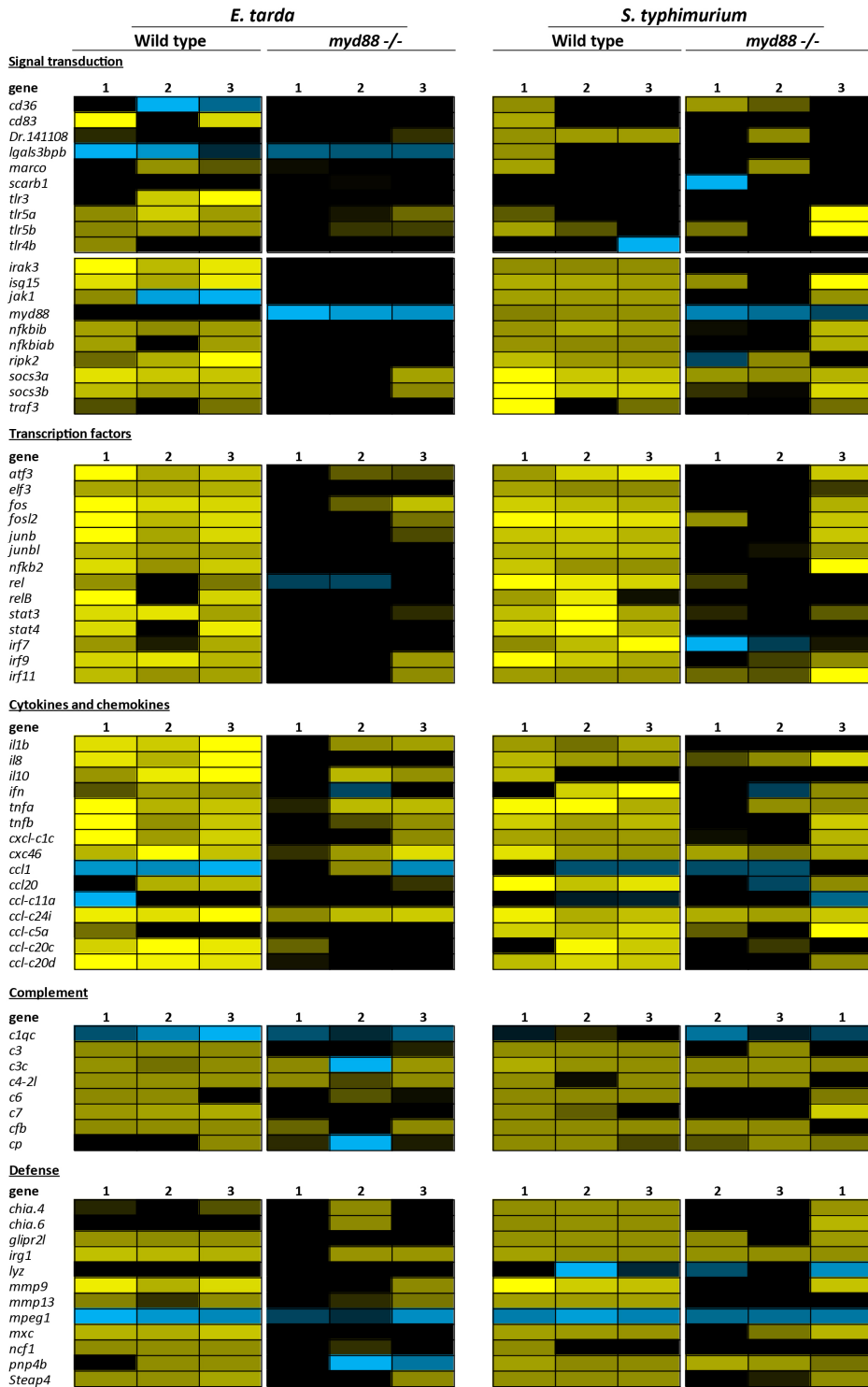


Figure 3: Comparison of the innate immune response of *myd88*^{-/-} and wild type embryos to *E. tarda* and *S. typhimurium*. Gene expression profiles of *myd88*^{-/-} and wild type embryos infected with *E. tarda* FL6-60 or *S. typhimurium* SL1027 are depicted in a heat map. Embryos were infected with 150 CFU of either pathogen into the caudal vein at 28 hpf and snap frozen individually at 8 hpi. Triplicate samples for each infection condition were compared with samples from control embryos (injected with PBS) using a common reference microarray design. Immune-related genes in the heat map are ordered in functional groups. All genes included in the heat map are represented by a minimum of two probes that showed significant up- or down-regulation (Significance cut-offs for the ratios of infected vs control groups were set at 2-fold with $p < 10^{-5}$). Up-regulation and down-regulation is indicated by increasingly bright shades of yellow and blue, respectively. All genes listed in this figure are named according to sequence homology with mammalian counterparts and in most cases have not yet been confirmed functionally.

of cytokine genes that were regulated upon infection, since those individuals that had higher expression of transcription factors also showed more significant upregulation of cytokine probes. This indicates a role for Myd88-independent signaling in the activation of these cytokine genes. Moreover, the induction of certain cytokine genes (*cxc46* and *ccl-c24i*) and components of the complement system (like *c3c*, *c4-2l*, and *cp*) did not seem to be changed in the *myd88*^{-/-} embryos.

The transcription factor genes that were affected in the infected *myd88*^{-/-} embryos included members of the known transcription factor families activated by TLR-MYD88 and cytokine signaling in other vertebrates: NFκB (*nfkb2*, *rel*, and *relb*), AP-1 (*fos*, *fos2l*, *junb*, and *junbl*), ATF (*atf3*) and STAT (*stat3* and *stat4*). Expression of the IRF family members, *irf7*, *irf9* and *irf11*, was dependent on Myd88 during an *E. tarda* infection, while *irf9* and *irf11* appeared Myd88-independent in the presence of *S. typhimurium*. Among the downstream cytokine genes, upregulation of *il1b* in response to *S. typhimurium* infection was strictly dependent on Myd88 in the microarray analysis. During *E. tarda* infection *il1b* was upregulated at low levels in *myd88*^{-/-} embryos, with significant reduction compared to wild type embryos. The other major pro-inflammatory cytokine gene, *tnfa*, could still be upregulated in the absence of Myd88 during infections with both pathogens. Expression of the gene encoding the chemotactic cytokine IL8 was clearly dependent on Myd88 in embryos infected with *E. tarda*, while it was expressed at wild type levels in *myd88*^{-/-} embryos infected with *S. typhimurium*.

In conclusion, analysis of the microarray data indicated that a large proportion of the genes which are regulated during an innate immune response against *E. tarda* or *S. typhimurium* rely on proper TLR-Myd88 or IL1R-Myd88 signaling. Without Myd88, important transcription factors, cytokines, and other genes involved in defense were upregulated at a lower level or not at all. Interestingly, we also identified genes whose upregulation was completely independent of Myd88-signaling or appeared to be pathogen specific.

Expression levels of Myd88-dependent and -independent genes are highly variable during infection

Our transcriptome analysis of *myd88*^{-/-} and wild type embryos infected with *E. tarda* and *S. typhimurium* showed that Myd88-signaling is required for a large part of the gene expression involved in the innate immune response. However, performing microarray analysis on three embryos per condition revealed variation between individuals in the infection-induced expression levels of important components of the immune response. To further investigate the Myd88-dependency of a subset of these genes, we isolated RNA from a larger number (n=10) of *myd88*^{-/-} and wild type embryos infected with *E. tarda* or *S. typhimurium* and performed qPCR analysis. In agreement with the microarray data, wild type embryos showed highly variable infection-induced expression levels of *il1b* (11 to 35 fold change difference compared to controls during *E. tarda* infection; 4 to 8 fold change difference in *S. typhimurium* infection) (figure 4). The *il1b* induction levels of infected *myd88*^{-/-} embryos were also distributed over a broad range, but were significantly lower than in wild type embryos (2 to 20 fold change difference during *E. tarda* infection; no difference to 4 fold change difference for *S. typhimurium* infection). The expression levels of *tnfa* were spread over a similar range in wild type and *myd88*^{-/-} embryos. This gene was strongly induced by *S. typhimurium* (2 to 17 fold in wild type and *myd88*^{-/-}), while *E. tarda* induction of *tnfa* in wild types and mutants was low and not significant. These data demonstrate that expression of *il1b* was dependent on Myd88 during infection with *E. tarda* and *S. typhimurium*, while *tnfa* expression occurred via Myd88-independent pathways during infection with *S. typhimurium*.

We used the same approach to analyze the expression of the matrix metalloproteinase gene *mmp9*, the chemotactic cytokine genes *il8* and *cxcl-c1c*, and the antiviral cytokine gene *ifnphi1*, in mutants and wild types infected with *E. tarda* and *S. typhimurium* (figure 4). We found that expression of *mmp9* and *cxcl-c1c* was dependent on Myd88 during infection with either pathogen, similar to *il1b*. Expression of *il8* was significantly lower in *myd88*^{-/-} embryos infected with *E. tarda* compared to infected wild types. In *S. typhimurium* infection, the average induction of *il8* in *myd88*^{-/-} embryos was also lower than in wild type embryos but did not result in a significant difference. Infections with *E. tarda* did not significantly induce expression levels of *ifnphi1*. Expression of *ifnphi1* was slightly upregulated in wild type embryos infected with *S. typhimurium*, but not in infected *myd88*^{-/-} embryos. Interestingly, only in the case of *mmp9* expression during *E. tarda* infection, the induction was completely inhibited by Myd88 deficiency. All other genes that showed significant Myd88 dependency could still be induced to low levels in mutant embryos. Therefore, the important conclusion from these experiments is that pro-inflammatory gene expression in *myd88*^{-/-} embryos is reduced but not completely absent.

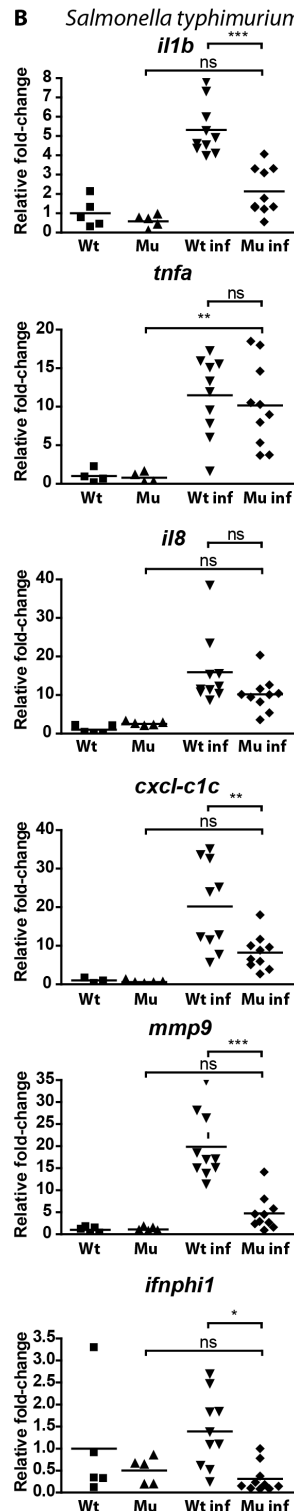
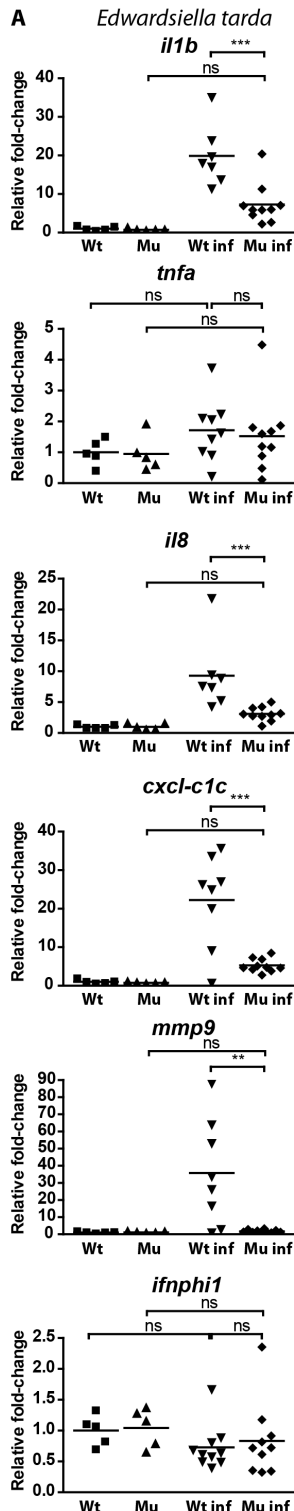


Figure 4: qPCR analysis of the response of *myd88*^{-/-} and wild type embryos to *E. tarda* or *S. typhimurium* infection. Expression of *il1b*, *tnfa*, *il8*, *cxcl-c1c*, *mmp9*, and *ifnphi1* was analyzed by qPCR in *E. tarda* (A) or *S. typhimurium* (B) infected wild type (Wt) and mutant (Mu) embryos. RNA samples from infected embryos (inf) and uninfected controls were taken 8 hpi. Each data point represents an individual embryo and lines indicate the mean relative expression level, with uninfected wild type set at 1. Significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) were calculated by one-way ANOVA with Tukey's Multiple Comparison method as a post-hoc test.

Myd88-deficient embryos can limit growth of the attenuated *S. typhimurium* Ra strain

Based on the partial induction of cytokine genes in *myd88*^{-/-} embryos, we hypothesized that these embryos are not completely immune-compromised. To test this hypothesis, we infected *myd88*^{-/-} and wild type embryos with a fluorescently labeled *S. typhimurium* LPS mutant (*S. typhimurium* Ra) that has previously been shown to induce attenuated infection in zebrafish embryos³⁸. Four hours after injecting the bacteria into the bloodstream, we observed clustered fluorescent bacteria, indicative of phagocytosis, in both *myd88*^{-/-} and wild type embryos (figure 5A and C). In contrast, clustering was greatly decreased in a Pu.1 morpholino-injected control group deficient in leukocytes (figure 5E). One day post infection, we noted increased infection in *myd88*^{-/-} embryos compared to wild type, which was quantifiable by bacterial pixel counting (figure 5B, D, and G). At the same timepoint, embryos in the Pu.1 morpholino-injected control group were completely overgrown with infection, with the bacteria spreading throughout the entire vasculature (figure 5F). The bacterial pixel count in both *myd88*^{-/-} and wild type embryos gradually declined over the next four days. At the 5dpi timepoint, 58% of the wild types had already cleared the infection, while wild types with remaining infection showed greatly decreased numbers of bacteria (figure 5H). At the same time, we observed a broad range of infection states in *myd88*^{-/-} embryos, varying from complete clearance of the bacteria (28%) to highly infected (30%), and lethally infected (4%). Thus, embryos developed more severe infection levels under conditions of Myd88 deficiency, but still were capable of limiting the growth of the attenuated *S. typhimurium* strain. In one of the two experimental repeats, we isolated RNA from all individual infected embryos for qPCR analysis. Although *il1b* expression was lower in *myd88*^{-/-} embryos compared to wild type, the difference was not significant (figure 5I). Induction of *mmp9* was also lower in *myd88*^{-/-} and significantly different from the wild type embryos (figure 5J). Together, these data confirm the partial induction of pro-inflammatory genes in *myd88*^{-/-} embryos and show that growth of attenuated *S. typhimurium* Ra bacteria in zebrafish embryos can be limited in a Myd88-independent manner.

***Myd88*^{-/-} embryos are more susceptible to chronic infection by *Mycobacterium marinum* strains**

In contrast to the acute infections with *E. tarda* and *S. typhimurium*, *M. marinum* injection into the blood island at 28 hpf leads to a chronic infection that persists during larval development in granuloma-like aggregates of immune cells⁴⁰. *M. marinum* is a natural pathogen of teleost fish and a close relative of *M. tuberculosis*, the causative agent of tuberculosis in humans. We aimed to address the importance of TLR-signaling during mycobacterial disease by infecting *myd88*^{-/-} and wild type embryos with two different *M. marinum* strains: Mma20 and E11. Bacterial pixel counting was performed at 3 dpi for embryos infected with *M. marinum* Mma20 and at 5 dpi for embryos infected with the less virulent E11 strain. The formation of granuloma-like structures and bacterial burden in *myd88*^{-/-} embryos infected with either *M. marinum* strain far exceeded that of wild types (figure 6G and H). Furthermore, we compared the effect of the *myd88* mutant during mycobacterial infection to that of a previously described

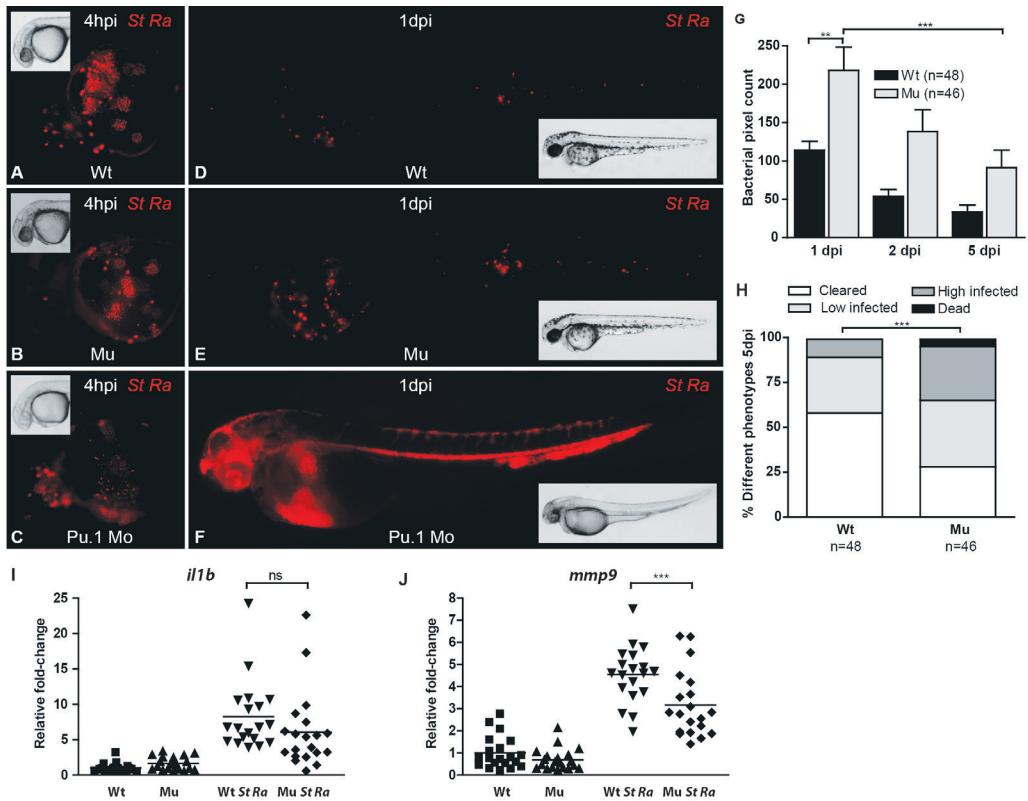


Figure 5: Bacterial burdens and qPCR analysis of gene expression in *myd88*^{-/-} and wild type embryos infected with the attenuated *S. typhimurium* Ra strain. (A-F) Representative stereo fluorescence images of infected embryos. At 28 hpf, embryos were infected by injection into the blood island using approximately 150 CFU of DsRed-labeled *Salmonella typhimurium* Ra. Pu.1 morpholino-injected embryos (Pu.1 Mo: C,F), deficient in phagocytic leukocytes, were included for comparison with wild type (Wt: A,D) and *myd88*^{-/-} (Mu: B,E) embryos. Dispersal of infected leukocytes over the yolk sac at 4 hpi (A-C) and the progression of infection is shown at 1 dpi (D-F). (G). Quantification of bacterial burden. Stereo fluorescence images of infected embryos at 1, 2, and 5 dpi were used for quantification of bacterial fluorescent pixels. Data is accumulated from two individual experiments. Significant differences of (**, $p < 0.01$; ***, $p < 0.001$) were determined by one-way ANOVA with Tukey's Multiple Comparison method as a post-hoc test. (H) Variation in phenotypes at 5 dpi. Embryos were categorized according to infection levels as cleared (no remaining bacterial fluorescent pixels or pixel count below 10), low infected (pixel count between 10 and 100), high infected (pixel count above 100), or dead. The distribution over categories was significantly different (***, $p < 0.001$) based on a contingency test. (I,J) qPCR analysis of pro-inflammatory genes. RNA samples from infected embryos (St Ra) and their controls were taken at 5 dpi to determine differences between *myd88*^{-/-} (Mu) and wild type (Wt) in the expression levels of *il1b* (I) and *mmp9* (J). Each data point represents an individual embryo and lines indicate the mean relative expression level, with uninfected wild type set at 1. Statistical analysis performed by one-way ANOVA with Tukey's Multiple Comparison method as a post-hoc test. The mean expression levels of both genes were lower in infected mutants than in infected wild types, but the difference for *il1b* was not significant (ns) while the difference for *mmp9* was significant with $p < 0.001$ (***).

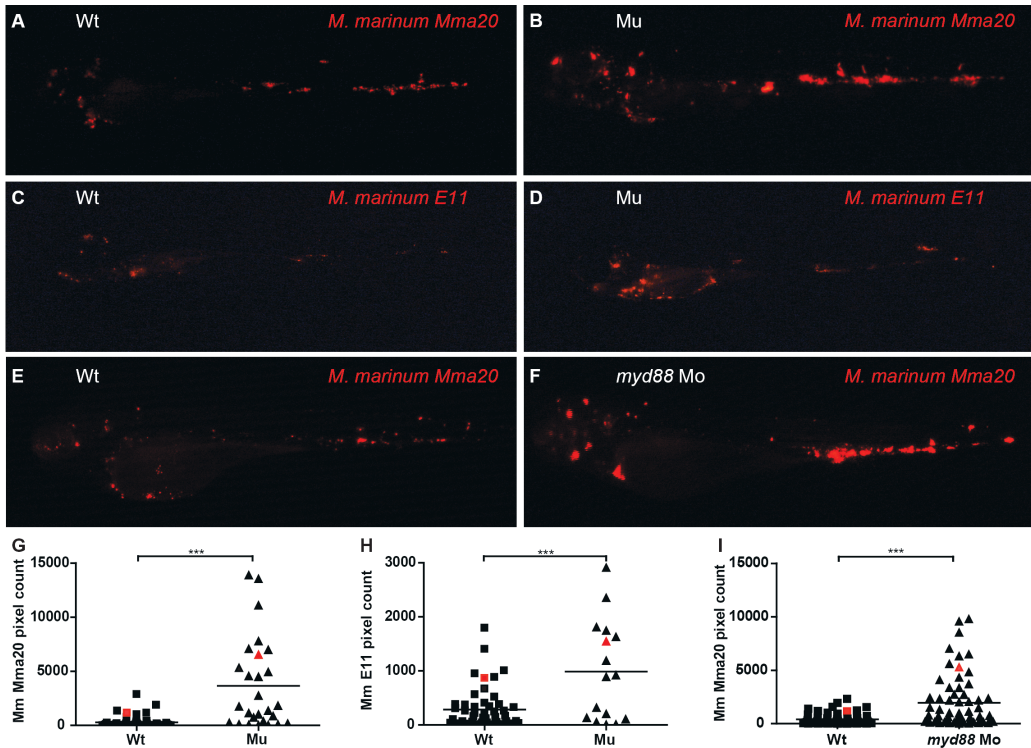


Figure 6: Bacterial burdens of *myd88*^{-/-} and wild type embryos infected with *M. marinum*. (A-F) Representative stereo fluorescence images of infected embryos. At 28 hpf, *myd88*^{-/-} (Mu) and wild type (Wt) embryos were infected with approximately 200 CFU of mCherry-labeled *M. marinum* strain Mma 20 (A,B) or strain E11 (C,D) by injection into the blood island and stereo fluorescence images were taken at 3 (Mma20 strain) or 5 dpi (E11 strain). For comparison, wild type and Myd88 morpholino-injected embryos were infected with the same dose of *M. marinum* Mma 20 (E,F-). (G-H) Quantification of bacterial burden. Red symbols indicate which infected individuals are shown as representative images (A-F). Bacterial pixel counts were determined based on stereo fluorescence images. Significant differences (***, $p < 0.001$) were determined by one-way ANOVA with Tukey's Multiple Comparison method as a post-hoc test.

splice-morpholino against *myd88*³¹. We observed similar differences between *myd88*^{-/-} and wild type embryos as for *myd88* morpholino injected embryos versus wild type (figure 6G and I). To further validate that the truncated version of Myd88 described here is inactive, we injected an AUG-morpholino targeting the *myd88* transcript in both wild type and mutant embryos²⁹. The AUG-morpholino increased infection in wild type individuals compared to mismatch morpholino injected wild types, but did not increase infection in *myd88*^{-/-} compared to mismatch-control injected mutants (supplementary figure 2). These results support that *myd88*^{hu3568} is a null mutant allele and demonstrate that Myd88-dependent innate immune signaling is required for the control of *M. marinum* infection in zebrafish embryos.

Myd88-signaling is required for induction of key components of the innate immune system during mycobacterial infection

Recently, the zebrafish has been successfully used to unravel the role of dysregulated Tnf levels during mycobacterial disease, showing that either too little or too much Tnf leads to aggravated infection^{41,42}. We performed qPCR analysis on RNA from individual *myd88*^{-/-} and wild type embryos infected with *M. marinum* Mma20 at a time point with clear granuloma formation (4 dpi). Both *il1b* and *tnfa* were expressed at significantly lower levels in infected *myd88*^{-/-} embryos (figure 7A and B). Using a morpholino knockdown approach, it was previously shown that the mycobacterial virulence factor ESAT-6 can induce *mmp9* expression in host epithelial cells independent of Myd88⁴³. Consistent with this result, we found that part of the host's *mmp9* induction occurred independent of Myd88 during infection with *M. marinum* (figure 7C). However, the overall level of *mmp9* expression was significantly decreased in infected *myd88*^{-/-} compared to wild type embryos. Although the average expression of the chemotactic cytokines *il8* and *cxcl-c1c* was lower in infected *myd88*^{-/-} compared to infected wild type, the difference was not significant (figure 7D and E). Finally, the anti-viral cytokine interferon phi1 (*ifnphi1*) was induced independently of Myd88 in both infected groups (figure 7F). In conclusion, like during *E. tarda* and *S. typhimurium* infection, Myd88-signaling is required for induction of key components of the innate immune system during mycobacterial infection.

Discussion

myd88^{hu3568} carries a non-functional allele of *myd88*

In this report we describe a zebrafish mutant for Myd88, a central component in TLR/IL1R-signaling. A premature stop codon in the mutant allele deletes the complete sequence of the domain important for interaction with TLRs (TIR domain) and disrupts the domain required for interaction with IRAKs (death domain) by truncating it before the location of a critical residue (K95) for signaling⁴⁴. In addition to disruption of the coding sequence, *myd88* mutant mRNA was expressed at a significantly lower level than mRNA of the wild type allele. This may be explained by a lower stability of *myd88*^{hu3568} mRNA and/or by a possible feed-back mechanism by which wild type Myd88 regulates its own expression level. RT-PCR analysis of *myd88* transcripts in heterozygous individuals demonstrated that the mutant transcript indeed has a lower stability (supplementary figure 1). Innate immune responses to stimulation with known Myd88-dependent TLR ligands, LPS and flagellin, and bacterial challenge were significantly affected in *myd88* mutants. The non-functional role of the mutant allele is further supported by the fact that the phenotype of *myd88*^{-/-} embryos infected with *M. marinum* is phenocopied by morpholino knockdown of *myd88* expression and that morpholino injection could not further enhance *M. marinum* infection levels in *myd88* mutants (figure 6, supplementary figure 2).

Although early leukocyte hematopoiesis was not affected in these mutants, we observed a striking increase in mortality during larval development between 8 and 20 dpf.

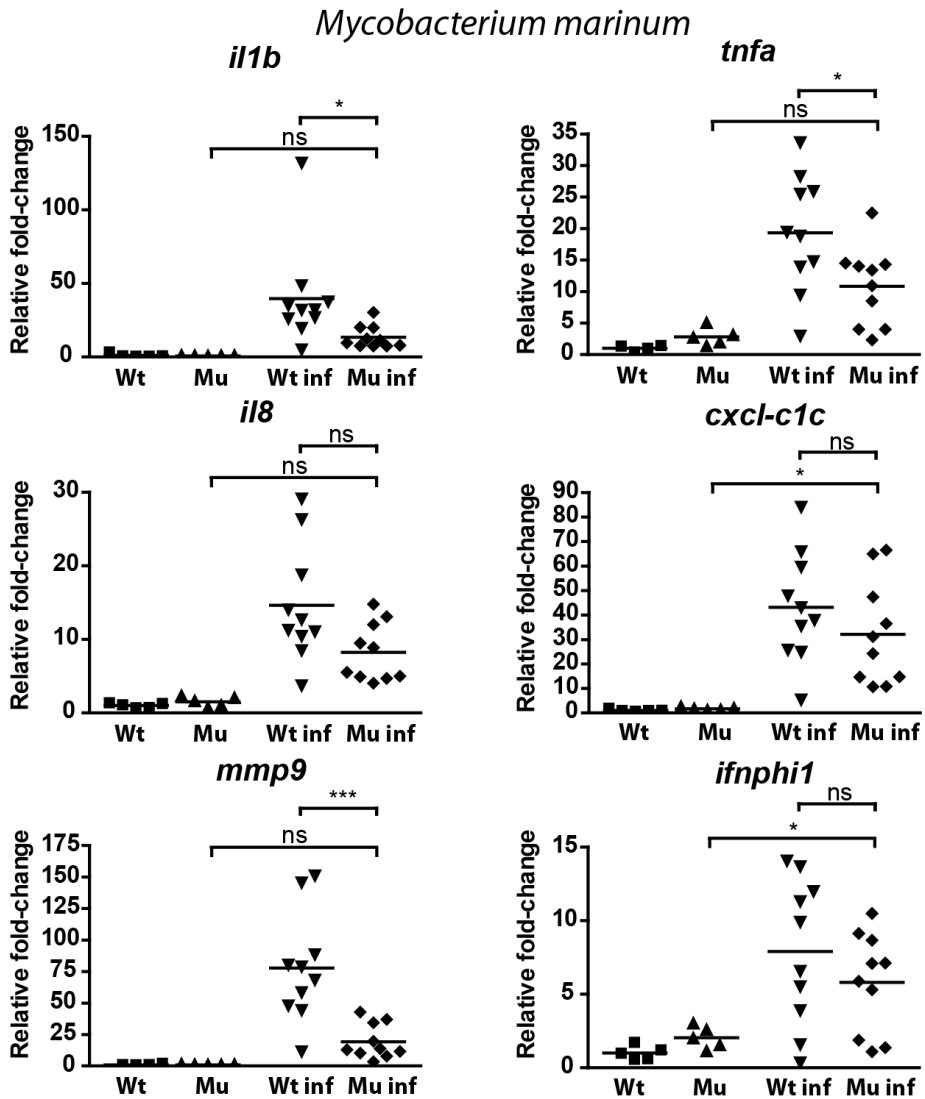


Figure 7: qPCR analysis of the response of *myd88*^{-/-} and wild type embryos to *M. marinum* infection. Expression of *il1b*, *tnfa*, *il8*, *cxcl-c1c*, *mmp9*, and *ifnphi1* was analyzed by qPCR in *M. marinum* Mma20-infected wild type (Wt) and mutant (Mu) embryos. RNA samples from *M. marinum* infected embryos (inf) and uninfected controls were taken at 4 dpi. Each data point represents an individual embryo and lines indicate the mean relative expression level, with uninfected wild type set at 1. Significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) were calculated by one-way ANOVA with Tukey's Multiple Comparison method as a post-hoc test.

The mortality rate of developing *myd88*^{-/-} larvae decreased after this period, which might be correlated with the onset of active adaptive immunity. Nevertheless, adult *myd88*^{-/-} zebrafish still display increased mortality compared to wild types. For instance, fin clipping of adult *myd88*^{-/-} for genotyping purposes is followed by a higher incidence of death in the next days, possibly caused by infections associated with the wounded tailfin or by increased stress sensitivity (data not shown). This is similar to what was observed for *rag1*^{-/-} zebrafish lacking functional T- and B-cells⁴⁵. Interestingly, humans with defective MYD88 suffer from a primary immunodeficiency, with life-threatening infections occurring during early infancy⁴⁶. Children with this deficiency have a cumulative mortality of 30-40%⁴⁶, while adult patients with this immune deficiency had no major infections⁴⁷. This difference was ascribed to the development of proficient adaptive immune responses later in life that may compensate for the defects in the inflammatory reaction¹³, a hypothesis that is supported by our observations on the survival of *myd88*^{-/-} zebrafish. With its temporal separation between active innate and adaptive immunity, the *myd88*^{-/-} zebrafish will be an ideal tool to further investigate this phenomenon.

***myd88*^{-/-} mutant as a research model for immunity, cancer, and development**

The *myd88* mutant presented here is a valuable model to determine the role of TLR/IL1R signaling and innate immunity in numerous important processes. Not only could it be used to study host-pathogen interactions in zebrafish²¹, but also to study the role of TLR signaling in the tumor microenvironment⁴⁸, as well as the role of Myd88 in the establishment of normal intestinal microbiota and maturation of the immune system during development⁴⁹. The fact that these processes require a certain progression of larval development in order to be studied makes a mutant line more useful than a morpholino knock down approach. In this report, we used the *myd88* mutant to determine the role of TLR-Myd88 signaling during bacterial infections in zebrafish embryos and larvae. Migration of leukocytes towards bacteria⁵⁰ and phagocytosis of bacteria are not affected by the absence of Myd88 (figure 1F and 5B). Nevertheless, we found that *myd88*^{-/-} embryos are more susceptible to infection by acute bacterial pathogens (*E. tarda* and *S. typhimurium*), chronic bacterial pathogens (*M. marinum* strains), and even non-pathogenic mutant bacteria (*S. typhimurium* Ra). The increased susceptibility to bacterial infections is a result of the inability of *myd88*^{-/-} embryos to mount an appropriate innate immune response towards these invading microbes. Our transcriptome analysis revealed that important pro-inflammatory regulators are expressed at lower levels in infected *myd88*^{-/-} embryos, including the genes encoding the components of the transcription factors NFκB and AP-1, and the cytokine Il1b (figure 3). These differences could not have been caused by variations in the injection dose, since we visually controlled for this by using fluorescently labeled bacteria. Since the embryos used for microarray analysis of gene expression were offspring from heterozygous parents, we could not exclude the possibility that maternally deposited Myd88 influenced our observations. Therefore, we validated our results using larger groups of single embryos from homozygous wild type or mutant parents, also demonstrating

that the induction levels of innate immune response genes were in significantly lower ranges in mutant than in wild type embryos (figures 5 and 8).

An important finding is that we identified genes, including the chemotactic cytokine *il8*, of which the expression was strongly dependent on Myd88-signaling during infection with one pathogen, but Myd88-independent for another pathogen. When analyzed on a larger group of *myd88*^{-/-} and wild type embryos infected with *E. tarda* and *S. typhimurium*, we demonstrated that the level of *il8* expression in infected *myd88*^{-/-} was lower for both pathogens, but the difference with wild type was only significant during *E. tarda* infection. The natural fish pathogen *E. tarda* may have evolved more specialized methods of manipulating or evading the immune system of their aquatic hosts, compared to the natural mammalian pathogen *S. typhimurium*. This possibility is also reflected in the total numbers of significantly up- or downregulated microarray probes during infection with these two pathogens, which was almost two-fold higher in *S. typhimurium* infection compared with *E. tarda* infection (figure 2A and B). A similar situation occurs when the transcriptome of zebrafish embryos exposed to *E. tarda* is compared to embryos exposed to *Pseudomonas aeruginosa*, a broad host range pathogen, capable of infecting plants, invertebrates, and vertebrates³⁷.

The *myd88*-dependency of *E. tarda* and *S. typhimurium* induced *il1b* and *mmp9* expression was consistent with our earlier observations in *myd88* morpholino knockdown studies, where the induction of these genes by *S. typhimurium* infection was also found to be *myd88*-dependent²³. However, in the previous studies it was not possible to discriminate between complete or partial *myd88*-dependency because the morpholino knockdown effect itself may be incomplete. With the use of the mutant line we could now establish that the *E. tarda* or *S. typhimurium*-induced *il1b* expression at 8 hpi is lower but not completely absent in *myd88*^{-/-} embryos, and that *mmp9* induction at this time point is completely inhibited by Myd88 deficiency during *E. tarda* infection, while partial induction occurs during *S. typhimurium* infection. Furthermore, a partial induction of *tnfa* was observed in *myd88* mutants during *M. marinum* infection, while *tnfa* induction during *S. typhimurium* infection was not affected by Myd88 deficiency. The (partial) Myd88 dependency of several interleukin and chemokine genes was in agreement with studies of cells and organs from *Myd88*-deficient mice^{9, 14, 17, 51-53}. The gene for *lfnphi1*, which belongs to the type I interferon group⁵⁴, was dependent on Myd88 for its induction during *S. typhimurium* infection, as was described for mouse *IFN1* when macrophages lacking MYD88 were stimulated with LPS⁵¹.

Myd88-independent limitation of the growth of non-pathogenic bacteria

When *myd88*^{-/-} embryos were infected with non-pathogenic *S. typhimurium* Ra bacteria, we observed large variation in *il1b* expression levels (figure 5I). Approximately half of the infected *myd88*^{-/-} larvae showed *il1b* expression levels comparable to uninfected controls, while the other half displayed *il1b* expression at a similar level as infected wild types. This variation was also reflected in the outcome of infection observed by

microscopy, ranging from complete clearance of the bacteria to lethality of the embryos (figure 5H). The data clearly shows that a subset of *myd88*^{-/-} embryos were capable of inducing pro-inflammatory gene expression and even clear the infection in the absence of Myd88. Potential mechanisms that could be responsible for recognizing and clearing *S. typhimurium* Ra bacteria in the absence of Tlr-Myd88 signaling are other PRRs, like NOD-like receptors or C-type lectins, or activation of the complement system. It is known that *S. typhimurium* Ra are more susceptible to complement lysis, due to a defect in the synthesis of the LPS O-antigen⁵⁵. Transcriptome analysis of infected *myd88*^{-/-} embryos revealed that expression of genes involved in complement is for a large part independent of Myd88 (figure 3). It has also been shown that complement component C5a is a potent endogenous pro-inflammatory peptide, capable of triggering production of Il1b upon infection⁵⁶. TLRs and complement can independently induce pro-inflammatory responses, but their synergistic interaction results in amplified responses⁵⁷. This may explain why *il1b* induction in *myd88*^{-/-} embryos infected with pathogenic bacteria was significantly lower, but not completely absent (figure 4). Finally, some TLRs, like TLR4, can signal via both Myd88-dependent and -independent routes. It is possible that loss of one route can be (partially) compensated by the other route. The presence of compensatory signaling routes is also suggested by the observed gene expression following flagellin injection, which led to a completely abrogated *il1b* response in *myd88* mutant embryos, while *mmp9* induction was similar to wild type level.

Myd88-signaling has a protective role during early mycobacterial pathogenesis

Although it has been shown that the innate immune system can control early mycobacterial infections⁵⁸, the role of MYD88 in mycobacterial disease remains controversial. Mice deficient in MYD88 are more susceptible to infection by *Mycobacterium tuberculosis*²⁰, but it remains unclear whether this is due to the function of MYD88 in innate responses or in adaptive immunity. More recently, MYD88 polymorphisms in a Columbian population were not associated with increased susceptibility to *Mycobacterium tuberculosis*⁵⁹. We used the *myd88* mutant zebrafish line to study the function of this gene during mycobacterial infection in zebrafish larvae that do not yet possess a functional adaptive immune system. We demonstrated that Myd88 is important for early control of these infections, since homozygous *myd88* mutant and Myd88 morpholino injected larvae showed accelerated formation of granulomas and at least 5-fold higher bacterial burdens compared to their wild type siblings when infected with *M. marinum* strains. Morpholino knockdown of the Tnf receptor and Mmp9 has also been shown to increase *M. marinum* granuloma formation and bacterial burden^{43,60}. In agreement, the impaired control of *M. marinum* in the absence of functional Myd88 is most likely caused by a marked reduced induction of *mmp9* and pro-inflammatory cytokines, like *il1b* and *tnfa* (figure 7). These data point towards a central role for Myd88 in innate immunity during the early stages of mycobacterial pathogenesis. Interestingly, expression of most immune related genes at the time point of granuloma formation was not completely abolished in *myd88*^{-/-} embryos, demonstrating that other pathways also contribute to

attract and activate immune cells at this point. These results from application of the *myd88* mutant in a zebrafish model for tuberculosis illustrate how this mutant could serve as a valuable tool for studying innate immune responses in many other zebrafish models for human infectious diseases, inflammatory disorders, and cancer, which have been developed in recent years.

Materials and methods

Zebrafish husbandry

Zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (www.zfin.org). Embryos were grown at 28.5–30°C in egg water (60 µg/ml Ocean Salts). For the duration of bacterial injections embryos were kept under anaesthesia in egg water containing 0.02% buffered 3-aminobenzoic acid ethyl ester (Tricaine).

Myd88 mutant, genotyping, and morpholino injection

The *myd88*^{hu3568} mutant allele was identified by sequencing of an ENU-mutagenized zebrafish library. The mutant line was obtained from the Hubrecht Laboratory and the Sanger Institute Zebrafish Mutation Resource. Heterozygous carriers of the mutation were outcrossed twice against wild type (AB strain), and were subsequently incrossed twice. Heterozygous fish of the resulting family were used to produce embryos for the microarray and *M. marinum* infection experiments. In all other experiments, we used embryos from *myd88*^{-/-}/*mpx::egfp* or *myd88*^{+/-}/*mpx::egfp*GFP parents, obtained by incrossing the heterozygous offspring of *myd88*^{+/-} fish outcrossed against *Tg(mpx::egfp)*^{i114 34}. For genotyping, genomic DNA was amplified using forward primer 5'-GAGGCGATTCCAGTAACAGC-3' and reverse primer 5'-GAAGCGAACAAAGAAAAGCAA-3' and the product of this reaction was digested with MseI. The mutant allele can be distinguished from the wild type allele by the presence of an extra MseI site that cuts a fragment of approximately 300 bp into 200 and 100 bp products. Determining the stability of the mutant transcript versus the wild type transcript was done with a RT-PCR using forward primer 5'-GAGGCGATTCCAGTAACAGC-3' and reverse primer 5'-GAAAGCATCAAAGGTCTCAGGTG-3' and the product of this reaction was digested with MseI. Knockdown of *myd88* by splice morpholino was performed as previously described³¹. Knockdown of *myd88* by AUG-morpholino was previously described by Van der Sar et al. (2006).

Immunohistochemistry

Immunolabeling with L-plastin antibody⁶¹ and Alexa568-conjugated secondary antibody was as described⁶².

Injection conditions

Edwardsiella tarda strain FL6-60 labeled with mCherry⁶³; *Salmonella typhimurium* wild type strain SL1027 and its isogenic LPS derivative SF1592 (Ra), both containing the DsRed expression vector pGMDs3²³; and *Mycobacterium marinum* strains Mma20

and E11 labeled with mCherry⁶⁴ were used for the infection of zebrafish embryos. Bacteria were washed and subsequently suspended in PBS (phosphate-buffered saline), amended with 2% polyvinylpyrrolidone (PVP) for *E. tarda* and *M. marinum*. Embryos were manually dechorionated at 24 hpf. Approximately 150-200 CFUs were injected into the blood island after the onset of blood flow at 28 hpf, or PBS (2% PVP for *E. tarda* and *M. marinum*) was injected as a control. After injection, embryos were kept at 28°C. Purified TLR ligands: LPS from *S. enterica* serovar *typhimurium* (Sigma, #L6511), poly I:C high molecular weight (Invivogen, #tirl-pic), flagellin from *S. enterica* serovar *typhimurium* (Invivogen, #fla-st ultrapure).

Phagocytosis assay

E. coli pHrodo particles (Life Technologies Europe BV, Bleijswijk, Netherlands) were injected into the blood island at 28 hpf (1nl of a 0.5 mg/ml solution). Fluorescent pixel quantification was performed at 2 hpi.

DNA and RNA isolation

The single embryo RNA isolation procedure using TRI reagent (Life Technologies Europe BV, Bleijswijk, the Netherlands) was performed as previously described by De Jong et al⁶⁵. DNA for genotyping was isolated from the organic phase and was done according to the alternate DNA isolation protocol of the TRI reagent DNA/protein isolation protocol, using glycogen as a co-precipitant. RNA from the aqueous phase was purified using the Rneasy MinElute Cleanup kit (QIAGEN Benelux B.V., Venlo, Netherlands).

Microarray analysis

Microarray was performed using our custom-designed Agilent 44k platform GPL10042, as previously described³⁷. The raw data were submitted to the Gene Expression Omnibus database under accession no. GSE39274. To compare wild type control, mutant control, wild type infected and mutant infected samples, a re-ratio experiment was performed using the Rosetta built-in re-ratio with common reference application. Data were analyzed at the level of probes with significant cut-offs for the ratios set at 2-fold change at $p < 10^{-5}$. For the heat map construction the fold change values of significant probes representing the same gene were averaged. Gene ontology (GO) analysis was performed using the GeneTools eGOn v2.0 web-based gene ontology analysis software⁶⁶.

cDNA synthesis and quantitative reverse transcriptase PCR

cDNA synthesis reactions and qPCR were performed as previously described and normalized against the expression of *ppial* as a housekeeping gene³⁷. Sequences for forward and reverse primers are described in supplementary table 1.

Microscopy and fluorescent pixel quantification

Embryos injected with fluorescently labeled bacteria or pHrodo-labeled *E. coli* cell wall particles were imaged using a (Leica MZ16FA stereo fluorescence microscope with Leica DFC420C camera. Total fluorescent pixels per fish were determined using dedicated software⁶⁷.

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Competing interests statement

The authors have nothing to declare.

Author contributions

All authors conceived and designed the experiments. MV and JS performed the experiments and analyzed the data under supervision of AM. MV wrote the manuscript and the final version was read and approved by all authors.

Translational impact box

Background

Toll-like receptors (TLRs) are an important class of receptors that can detect microbial and danger signals during infection and inflammation. As an adaptor molecule, myeloid differentiation factor 88 (MYD88) is central in TLR-signaling and innate immunity, which is illustrated by the fact that patients with MYD88 deficiency suffer from a primary immunodeficiency syndrome. The use of zebrafish as a model for infectious diseases, inflammatory disorders, cancer, and other immune related disease is growing rapidly, owing to many advantages for intravital imaging, genetic screening, and high-throughput drug discovery. Therefore, zebrafish mutants for important components of the immune system are eagerly awaited.

Results

In this study, we have characterized a zebrafish line with a premature stopcodon in the *myd88* gene, which is the first zebrafish mutant for a TLR/IL1R-signaling component. The mutant line appeared to be immune-compromised and had a higher susceptibility to infection by acute (*Edwardsiella tarda* and *Salmonella typhimurium*) and chronic (*Mycobacterium marinum*) bacterial pathogens. Microarray and quantitative PCR (qPCR) analysis of gene expression revealed that expression of transcription factors central to

innate immunity, like NF κ B and AP-1, and pro-inflammatory genes like *il1b* and *mmp9* are dependent on Myd88-signaling during these bacterial infections. Nevertheless, expression of immune genes independent of Myd88 in these mutants was sufficient to limit bacterial burdens of an attenuated *S. typhimurium* strain.

Implications and future directions

The zebrafish *myd88* mutant is a valuable addition to mammalian knockout models, especially when combined with transgenic lines with fluorescently marked immune cells that facilitate intravital imaging. During zebrafish development, innate immunity is active from day 1 onwards, whereas adaptive immunity is not fully functional during the first weeks. Zebrafish mutant models therefore can be used not only to study the interplay between innate and adaptive immunity (at juvenile and adult stages), but also provide the possibility to study functions of the innate immune system with minimal interference of adaptive immunity (at embryo and larval stages). In our study we exploited this possibility using a zebrafish model for tuberculosis and demonstrated that Myd88 has a protective role during early mycobacterial pathogenesis in zebrafish larvae when only innate immunity is functional. The zebrafish *myd88* mutant model will also be a valuable tool for studying innate immune responses in many other zebrafish models for human infectious diseases that have emerged in the recent years. Furthermore, it can make an important contribution to zebrafish models for inflammatory disorders and cancer.

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Chapter 4

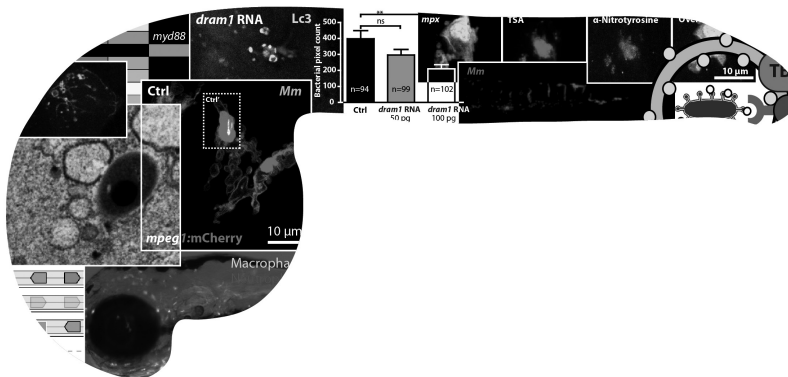
Mycobacteria counteract a nitrosative host defense mechanism initiated upon TLR/IL1R-MyD88 signaling

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Manuscript in preparation

4



Abstract

Pulmonary tuberculosis (TB), caused by the intracellular bacterial pathogen *Mycobacterium tuberculosis* (*Mtb*), is a major world health problem. The production of reactive nitrogen species (RNS) is a potent cytostatic and cytotoxic defense mechanism against intracellular pathogens. Nevertheless, the protective role of RNS during *Mtb* infection remains controversial. Here we use a nitrotyrosylation readout for nitrosative stress to study RNS production by the zebrafish host during early mycobacterial pathogenesis. We found that recognition of *Mycobacterium marinum*, a close relative of *Mtb*, was sufficient to induce a nitrosative defense mechanism in a TLR/IL1R-MyD88 dependent fashion. However, this host response was attenuated by mycobacteria via a virulence mechanism independent of the well-characterized RD1 virulence locus. Our results indicate a mechanism of pathogenic mycobacteria to circumvent host defense *in vivo*. Shifting the balance of host-pathogen interactions in favor of the host by targeting this virulence mechanism may help overcome the problem of *Mtb* strains that are resistant to multiple drug treatments.

4

Introduction

Pulmonary tuberculosis (TB) is a common and often lethal infectious disease that represents a major global health threat. The pathogenic bacterium *Mycobacterium tuberculosis* (*Mtb*) is the causative agent of TB and the high incidence and associated mortality worldwide is partly explained by the increasing occurrence of *Mtb* strains with resistance to multiple drug treatments. This makes TB a key priority for infectious disease research, since understanding of the host-pathogen interactions during *Mtb* pathogenesis is necessary to develop novel therapeutic strategies^{1, 2}. Mycobacteria phagocytosed by macrophages evade the majority of leukocyte bacterial killing mechanisms and create a niche for themselves in which they can survive and proliferate³. Recruitment of macrophages, neutrophils, and T-cells to the primary infected cells leads to the formation of highly organised structures known as granulomas^{4, 5}. During latent TB infection, a condition estimated to affect one-third of the human population, *Mtb* can persist for many years within granulomas before its reactivation

The combined efforts of the innate and adaptive arms of the immune system are relatively efficient in containing and killing *Mtb* in immunocompetent individuals. It is estimated that only 5-10 out of a 100 individuals newly infected with *Mtb* develop active TB during their lifetime⁷. One of the most effective host-defense mechanisms against intracellular pathogens is the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS)^{8, 9}. The production of ROS, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical ($OH^\bullet$), is catalyzed by the NADPH-oxidase complex during respiratory burst⁸. In vertebrates nitric oxide (NO) is formed when the guanidine nitrogen of L-arginine is oxidized by nitric oxide synthases (NOS). There are two constitutively expressed NOS enzymes, neuronal NOS (nNOS or NOS1)

and endothelial NOS (eNOS or NOS3), and one inducible NOS enzyme (iNOS or NOS2). Regulation of *iNOS* plays an important role in the inflammatory response and many cells of the immune system are capable of producing NO upon infection¹⁰.

Although a mutation in the components of the NADPH-complex increased susceptibility to *Mtb*¹¹⁻¹³, ROS production itself was found to be relatively ineffective in killing *Mtb*¹⁴. In contrast, exposure to low concentrations of NO is cytotoxic to mycobacteria, killing more than 99% of *Mtb* in culture¹⁵. However, a significant part of the cytostatic and cytotoxic antimicrobial effects of NO *in vivo* are attributed to RNS, the various reaction products of NO[•]¹⁶. The reaction of NO radicals with O₂ radicals produced during the respiratory burst results in the generation of peroxynitrite (ONOO⁻), a potent RNS causing lipid peroxidation, DNA damage, oxidation of thiols, and nitration of tyrosine residues^{17, 18}. The addition of a nitrogen-group to tyrosine residues is also catalyzed by the neutrophil specific enzyme myeloperoxidase (Mpx), using H₂O₂ and NO₂⁻ as substrates¹⁹. Tyrosine nitration can lead to disruption of tyrosine phosphorylation-dependent signaling pathways²⁰. The bactericidal activity of peroxynitrite has been demonstrated against *Salmonella enterica* serovar typhimurium²¹, and *Escherichia coli*²². Peroxynitrite is also required for the fungicidal activity of macrophages infected with *Candida albicans*²³.

The murine TB model was used to demonstrate an essential role for NO in the killing of *Mtb* by mononuclear phagocytes^{24, 25}, and iNOS deficient mice lacking inducible NO production were highly susceptible to *Mtb* infection^{12, 26, 27}. In contrast, the cytostatic and cytotoxic role of NO during *Mtb* infection in humans is debated, since early mycobacteriostatic activity of human macrophages was found to be independent of NO^{20, 28}. Nevertheless, there is an increasing amount of evidence that NO produced by macrophages and epithelial cells also provides protection against *Mtb* in humans^{27 29-32 33}. In agreement, polymorphisms in the gene for iNOS have been associated with susceptibility to TB in different populations³⁴. The conflicting results on the role of NO and iNOS in defense against mycobacterial infection may, in part, be explained by the range of mechanisms utilized by *Mtb* to counteract ROS and RNS produced by the host³⁵. Oxidative and nitrosative stresses induce a transcriptional response in *Mtb* that can lead to direct scavenging of reactive species and repair and protection of proteins and DNA^{35, 36}.

The natural fish pathogen *Mycobacterium marinum* (*Mm*) is a close relative of human *Mtb* and displays similar pathogenesis, including the formation of caseating granulomas and development of latency^{37, 38}. The zebrafish embryo model, which allows for detailed observation of host-pathogen interactions, is well-established for the *in vivo* study of host signaling and mycobacterial virulence determinants during early stages of TB disease^{39, 40}. In this study we used an antibody against nitration of tyrosine residues as readout for the oxidative and nitrosative response of the zebrafish host to infection with *Mm*⁴¹⁻⁴³. We found that mycobacterial infection led to an increase of nitrosative stress in leukocytes which was dependent on functional IL1R/TLR-Myd88 signaling. During the course of infection, this host response was attenuated by live mycobacteria

via a virulence mechanism independent of the well-characterized mycobacterial RD1 virulence locus³. Our results shed light on the intriguing host-pathogen interactions that occur during the onset of mycobacterial disease and can help understand the relevance of RNS as anti-mycobacterial defense mechanisms *in vivo*.

Results

Tyrosine nitration levels are detectable in neutrophils and macrophages

4 Tyrosine nitration is a hallmark of nitrosative stress caused by ROS and RNS produced by macrophages and neutrophils⁴¹. An antibody against nitrated tyrosine residues has been successfully used in common carp (*Cyprinus carpio*) to study the differential contribution of macrophages and neutrophils to nitrosative stress in response to a parasitic infection⁴¹. We have previously used a similar approach as readout for nitrosative stress induced by *Mm* infection in zebrafish embryos^{42, 43}. In the absence of infection, tyrosine nitration was mainly detectable in a cellular pattern in the caudal hematopoietic tissue (figure 1A). Neutrophils display high basal levels of tyrosine nitration, since the neutrophilic peroxidase Mpx (myeloperoxidase) catalyzes tissue nitration without peroxynitrite present^{41, 42}. Indeed, levels of anti-nitrotyrosine staining were detectable in *mpx:GFP*-labelled neutrophils of uninfected zebrafish embryos (figure 1B), but not or in rare cases in *mpeg1:GFP*-macrophages (figure 1D)⁴². Mpx activity can be visualized in zebrafish embryos using an immunohistochemical peroxidase detection protocol⁴⁴. Mpx activity co-localized with anti-nitrotyrosine staining in neutrophils of wild type embryos, while both the TSA and anti-nitrotyrosine staining were undetectable in neutrophils of embryos mutated in Mpx (Figure 1B and C). These data confirm our previous observation that the basal levels of tyrosine nitration in neutrophils were independent of iNOS activity⁴². Nitration of tyrosine residues was occasionally observed in *mpeg1:EGFP*-labelled macrophages when embryos were infected with *Mm* (Figure 1D and E). Furthermore, a significant increase in anti-nitrotyrosine staining was observed in neutrophils surrounding infected macrophages, confirming the infection inducible nature of NO production by iNOS (figure 2)⁴².

RNS production is an early host response to detection of live, heat-killed, or Δ RD1 mutant mycobacteria

Mycobacteria are rapidly phagocytosed by macrophages when injected into the bloodstream of zebrafish embryos⁴⁵. Tyrosine nitration and iNOS levels were greatly increased in leukocytes, predominantly neutrophils, one day post infection (dpi) with *Mm* and the increased NO output of these immune cells was dependent on iNOS⁴². To better understand the dynamics of this process, we looked at nitrosative stress levels in leukocytes at early time points after infection with *Mm*. At 4 and 8 hours post infection (hpi) we could not detect increased levels of anti-nitrotyrosine staining compared to uninfected controls, but the levels of tyrosine nitration in neutrophils were significantly higher at 12 hpi (figure 2A and B). The lag between the onset of infection

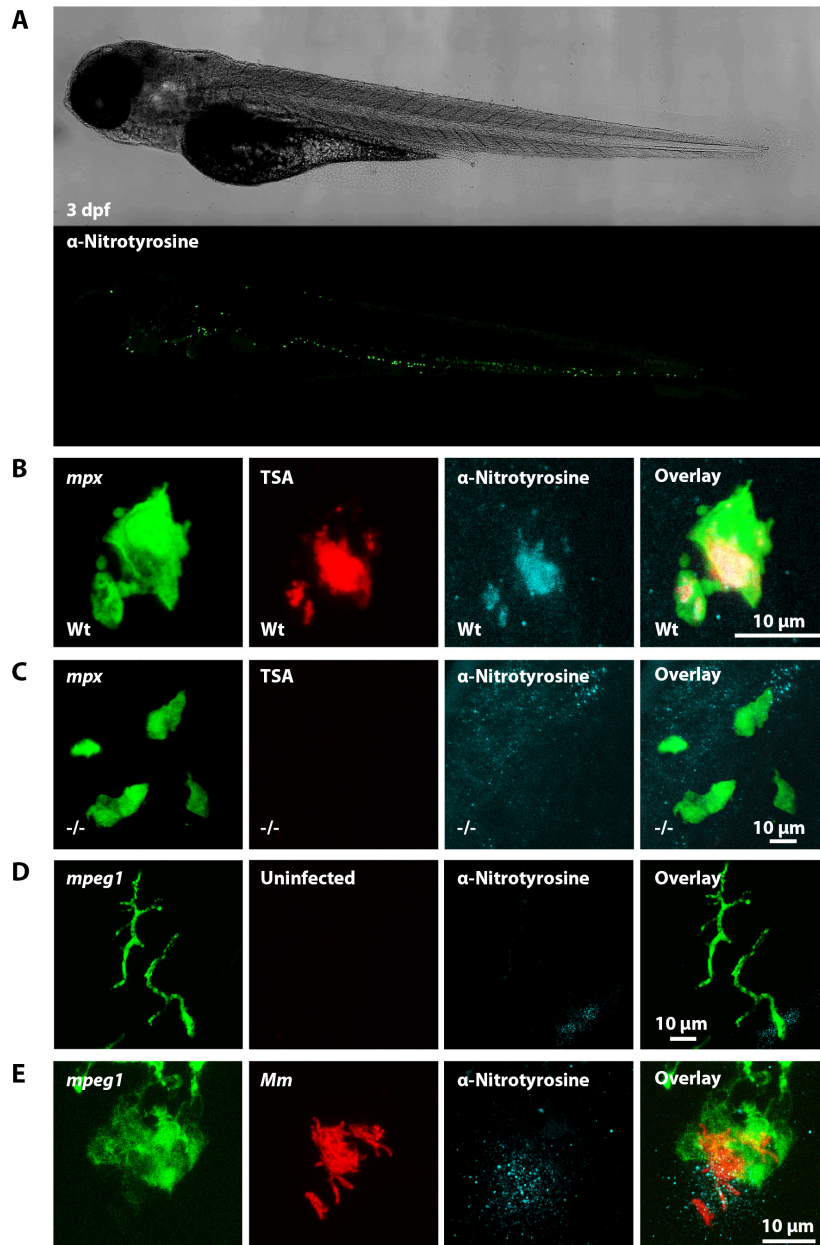


Figure 1: Tyrosine nitration is detectable in leukocytes (A) Fluorescence micrographs of confocal images stitched together to show the location of anti-nitrotyrosine staining in a whole-mount wild type zebrafish embryo of 3 dpf. (B and C) Fluorescence micrographs of confocal images showing the co-localization of *mpx:GFP*, TSA staining, and anti-nitrotyrosine staining for an embryo with normal Mpx activity (B) or an inactive mutant version of Mpx (C). (D) Fluorescent micrographs of confocal images showing no co-localization between *mpeg1:GFP* and anti-nitrotyrosine staining in the absence of infection, (C) overlap between *mpeg1:GFP* and anti-nitrotyrosine staining was observed when embryos were infected with *Mm*.

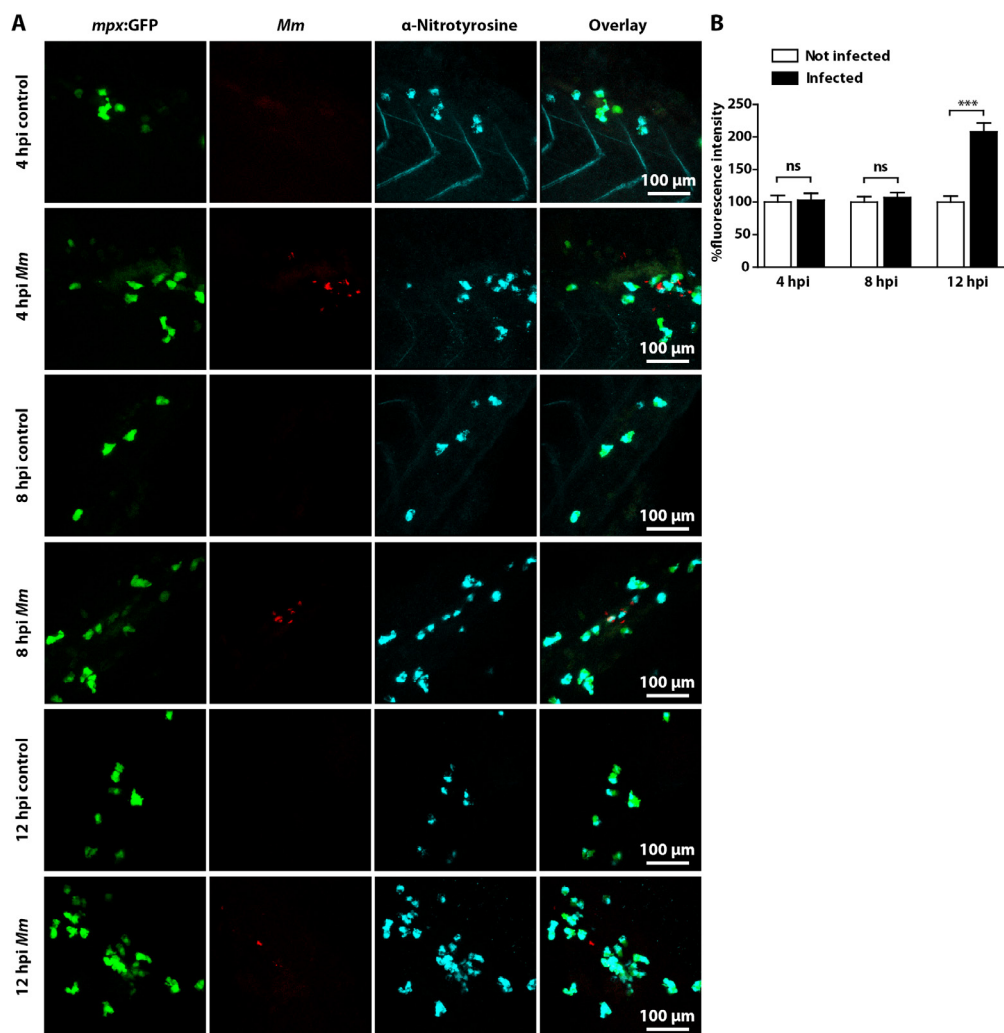


Figure 2: *Mm* infection significantly increased tyrosine nitration levels at 12 hpi (A) Example fluorescence confocal z-stacks of the caudal vein region of embryos stained with anti-nitrotyrosine antibody, imaged at 4, 8 or 12 hpi in the presence or absence of *Mm* infection. (B) Corrected fluorescence intensity levels of anti-nitrotyrosine antibody confocal z-stacks at 4, 8 or 12 hours after *Mm* infection relative to the control group per time point. Data shown are mean $\pm$ SEM, $n = 50$ -85 cells from 5 embryos representative of 3 independent experiments.

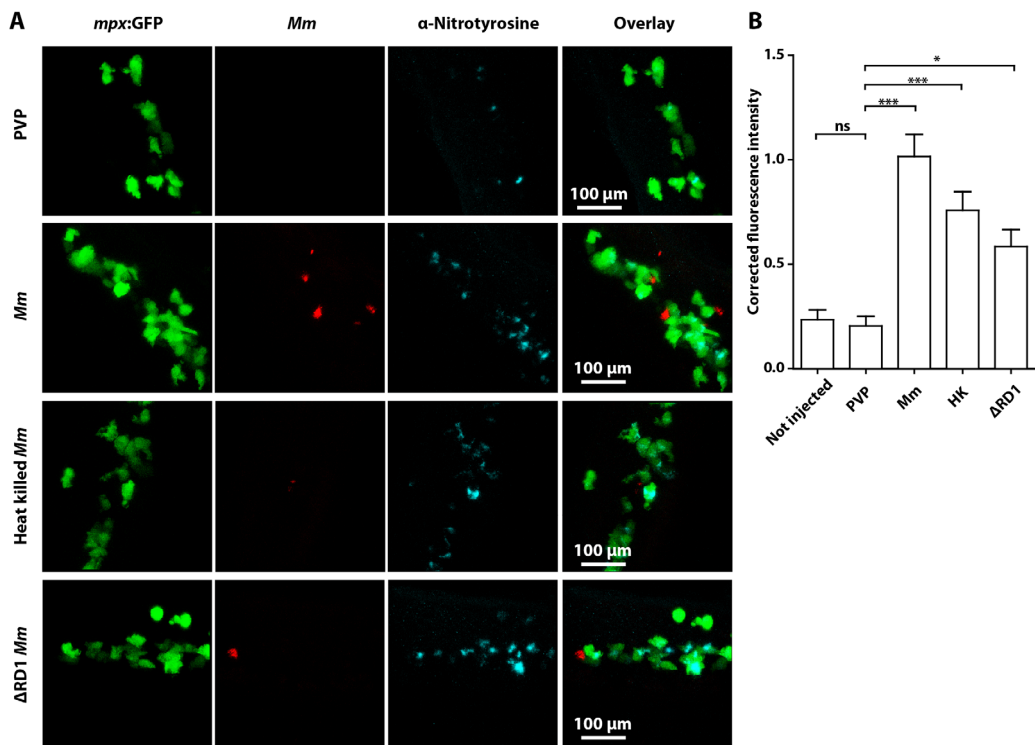


Figure 3: Injection with live, heat killed or Δ RD1 *Mm* significantly increased tyrosine nitration levels (A) Example fluorescence confocal z-stacks of the caudal vein region of embryos stained with anti-nitrotyrosine antibody, imaged 1 day after injection with live, heat killed or Δ RD1 *Mm*. (B) Corrected fluorescence intensity levels of anti-nitrotyrosine antibody confocal z-stacks 1 day after injection with live, heat killed or Δ RD1 *Mm*. Data shown are mean $\pm$ SEM, $n = 58-99$ cells from 5 embryos representative of 3 independent experiments.

and detectable increased tyrosine nitration is consistent with the fact that infection-induced RNS production first requires *de novo* synthesis of iNOS²⁷. Increased nitration levels in leukocytes were observed after injection with live, heat-killed, or *Mm* with a deleted RD1 (Δ RD1) locus (figure 3A and B, supplementary figure 1). The RD1 virulence locus of pathogenic mycobacteria contains genes for the ESX-1 secretion system and secreted factors required for survival and replication inside macrophages^{46, 47}. These results demonstrate that the presence of *Mm* is sufficient to induce a leukocyte RNS response shortly after encountering bacteria, irrespective of their infectivity.

TLR/IL1R-MyD88 signaling is required for *Mm* induced RNS production

Pattern recognition receptors, like Toll-like receptors (TLRs), can recognize pathogen associated molecular patterns (PAMPs) present on the exterior of mycobacteria and initiate an appropriate immune response^{43, 48}. Stimulation of mouse macrophages with

TLR ligands increased the expression of *iNOS in vitro*⁴⁹, and we have previously shown that a zebrafish mutant for the important TLR/IL1R signaling adaptor MyD88 (*myd88*^{-/-}) was more susceptible to mycobacterial infection⁵⁰. The basal level of anti-nitrotyrosine staining in neutrophils was unchanged in uninfected *myd88*^{-/-} embryos compared to *myd88*^{+/+} embryos (figure 4A). Anti-nitrotyrosine staining was significantly increased in infected *myd88*^{+/+} embryos, whereas infected *myd88*^{-/-} embryos did not show an increase compared to uninfected controls (figure 4B and C). Therefore, we conclude that MyD88-dependent signaling is required for mycobacterial infection induced RNS production, most likely following TLR recognition of the pathogen.

Nitrotyrosine levels are attenuated during *Mm* pathogenesis in a live-bacteria dependent mechanism

We evaluated RNS production at later stages of mycobacterial pathogenesis by performing anti-nitrotyrosine staining on zebrafish embryos with clustered bacteria. At 3 days post infection (dpi) anti-nitrotyrosine staining co-localized with, or was in proximity of, bacterial clusters, demonstrating that nitrosative defense mechanisms remain active at a later time point of pathogenesis (figure 5A). Interestingly, we observed that the anti-nitrotyrosine staining that co-localizes with heavily infected areas was less intense than the staining at the periphery of these areas, or in their direct proximity (figure 5B). The same phenomenon was observed for larger and more organized mycobacteria-containing developing granulomas at 5 dpi (supplementary figure 2). Thus, while *Mm* infection initially increased nitrotyrosine levels of infected and bystander leukocytes, the host RNS response was attenuated in developing granulomas.

Signaling via the hypoxia inducible factor α (HIF- α) was shown to be involved in leukocyte activation and host defense⁵¹. Our previous results showed that activated HIF-1 α signaling due to injection of dominant active *hif-1 α* RNA was able to upregulate anti-nitrotyrosine staining in zebrafish embryos prior to infection⁴². However, when HIF-1 α signaling was activated, *Mm* infection led to a decrease in tyrosine nitration levels, in contrast to the increase that occurs following *Mm* infection of wild type embryos (figure 6A and B)⁴². By injecting live, heat killed, or Δ RD1 *Mm* into embryos with activated HIF-1 α signaling we found that the decrease in anti-nitrotyrosine levels is dependent on live bacteria (figure 6A and B). Exposure to heat killed *Mm* further increased tyrosine nitration levels of leukocytes, while infection with living wild type or Δ RD1 *Mm* significantly decreased tyrosine nitration levels compared to mock-injected controls (figure 6B). These data confirmed that recognition of mycobacteria increased host NO output, even when RNS production of leukocytes was genetically increased prior to infection. However, live *Mm* lowered the efficacy of this nitrosative defense via a mechanism independent of the RD1 virulence locus.

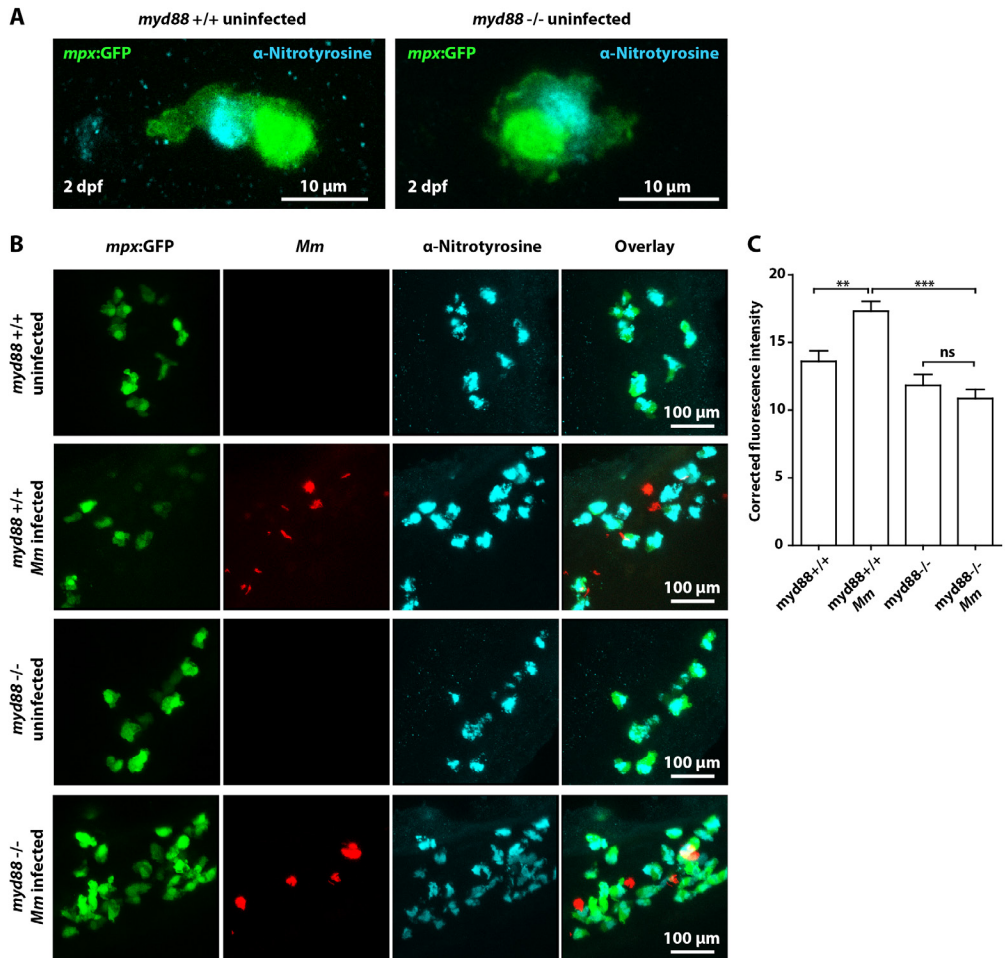


Figure 4: Increased tyrosine nitration following *Mm* infection is dependent on MyD88 (A) Fluorescent micrographs of confocal images showing the overlap between *mpx:GFP* and anti-nitrotyrosine staining in *myd88*^{+/+} or *myd88*^{-/-} embryos. (B) Example fluorescence confocal z-stacks of the caudal vein region of *myd88*^{+/+} or *myd88*^{-/-} embryos stained with anti-nitrotyrosine antibody, imaged at 1 dpi in the presence or absence of *Mm* infection. (C) Corrected fluorescence intensity levels of anti-nitrotyrosine antibody confocal z-stacks 1 day after injection with *Mm* in *myd88*^{+/+} or *myd88*^{-/-} embryos. Data shown are mean ± SEM, n = 45-52 cells from 5 embryos representative of 3 independent experiments.

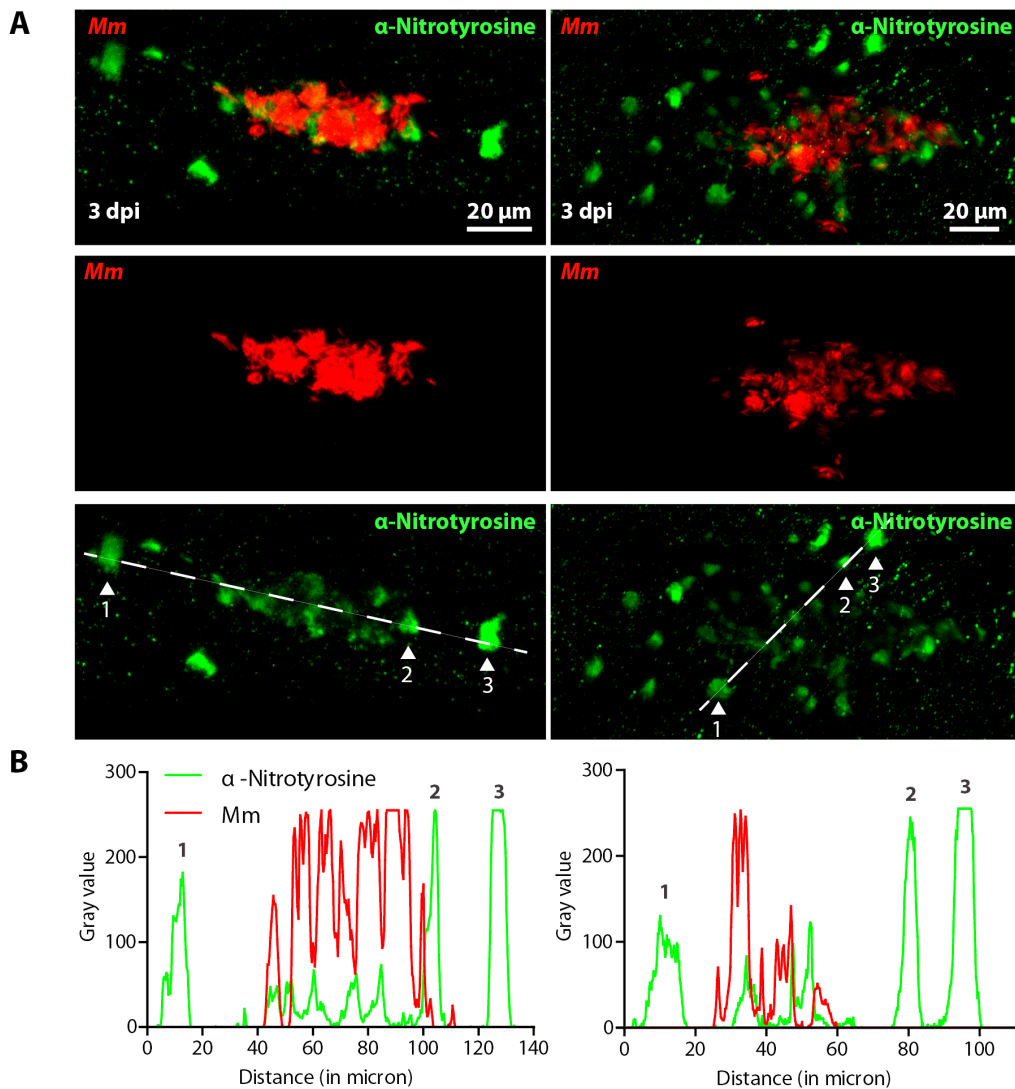


Figure 5: Anti-nitrotyrosine staining levels are lower when co-localizing with bacteria (A) Example fluorescence confocal micrographs of anti-nitrotyrosine staining performed on 3 dpi granuloma structures in wild type embryos infected with *Mm*. (B) Gray values of the bacterial and anti-nitrotyrosine fluorescence signals measured along a straight line through the center of the granuloma (see white lines in (A)), intensity of the fluorescent signal was measured using ImageJ. Numbered peaks in the graphs correspond to numbered patches of tyrosine nitration in (A).

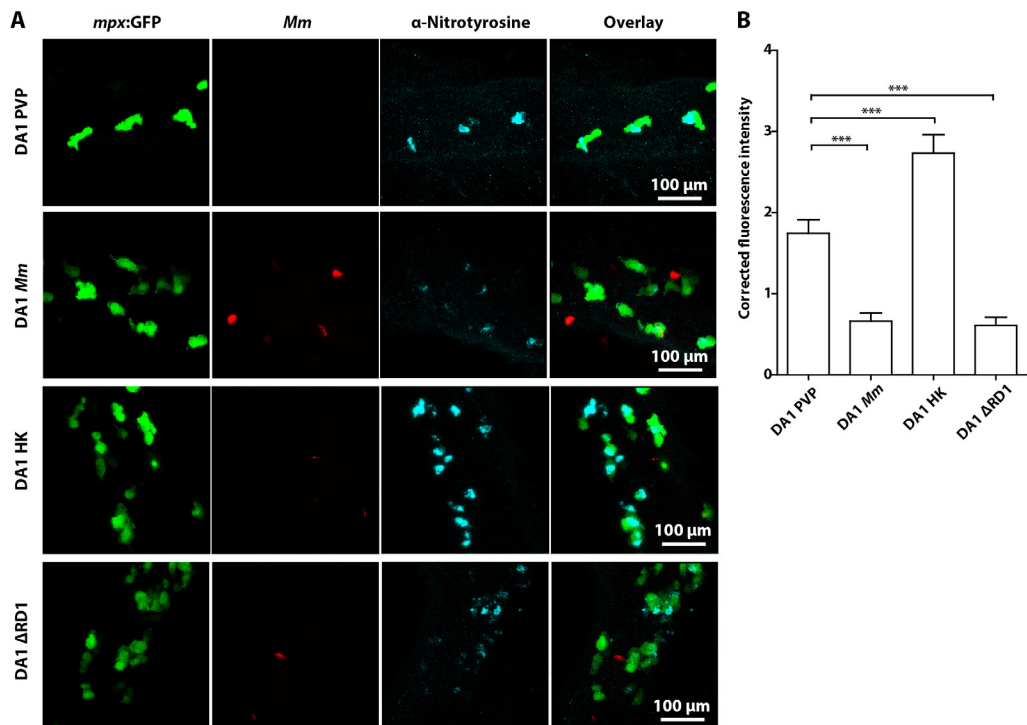


Figure 6: Genetically increased tyrosine nitration in leukocytes due to activated HIF-1 α signaling is attenuated by live wild type or Δ RD1 *Mm*, but not heat killed *Mm* (A) Example fluorescence confocal z-stacks of the caudal vein region of dominant active *hif-1 α* (DA1) mRNA injected embryos stained with anti-nitrotyrosine antibody, imaged 1 day after injection with live, heat killed or Δ RD1 *Mm*. (B) Corrected fluorescence intensity levels of anti-nitrotyrosine antibody confocal z-stacks on dominant active *hif-1 α* (DA1) mRNA injected embryos, 1 day after injection with live, heat killed or Δ RD1 *Mm*. Data shown are mean $\pm$ SEM, n= 74-108 cells from 5 embryos representative of 3 independent experiments.

Discussion

In this study we observed that TLR/IL1R-MyD88 signaling was required for the induction of a nitrosative defense mechanism when zebrafish embryos were infected with *Mm*. We found earlier that this mechanism is dependent on iNOS, which is expressed at higher levels in both infected and bystander leukocytes, predominantly neutrophils^{42, 52}. However, neither a morpholino targeting iNOS, encoded by the *nos2a* gene in zebrafish⁵², nor the use of pan-NOS or iNOS specific inhibitors resulted in significantly increased *Mm* bacterial burdens⁴². Similarly, treatment with ROS scavengers did not increase *Mm* bacterial burdens of wild type zebrafish embryos⁵², despite that we find components of the NADPH oxidase complex (including *nox1*, *noxo1a*, *cyba*) to be induced during *Mm* infection in a TLR/IL1R-MyD88-dependent manner (RNASeq dataset GSE49188). Apparently, the activation of these potent anti-microbial defense mechanisms has

little or no effect on the survival of *Mm* inside the zebrafish host. Indeed, in this study we found that live *Mm* are capable of attenuating the nitrosative stress generated by leukocytes during the innate immune response.

Pathogenic mycobacteria have evolved as metabolically flexible bacteria that can sense and adapt to the continuously changing host environment during infection⁵³. *Mtb* is continuously exposed to endogenous ROS produced by normal aerobic respiration, but also exogenous ROS and RNS generated by the host immune system in the often hypoxic human granuloma^{53, 54}. The *Mtb* WhiB4 protein can sense O_2 and NO levels and activate the oxidative stress response, which maintains the redox balance and modulates virulence⁵⁵. Exposure of *Mtb* to H_2O_2 or NO initiated a robust transcriptional response, including the expression of cellular repair mechanisms and upregulation of genes that perform ROS and RNS scavenging functions^{35, 36}. The presence of macrophages and neutrophils expressing NOS proteins was demonstrated for *Mtb* granulomas in human and macaque tissue samples⁵⁶. However, the presence of NOS proteins does not guarantee a cytostatic or cytotoxic effect of NO production. We found that attenuation of the host RNS response occurred in developing granulomas of *Mm*-infected zebrafish embryos. Similarly, the host RNS response to *Mm* infection was attenuated in embryos with activated HIF-1 α signaling, an experimental strategy utilized to genetically increase RNS production of leukocytes prior to infection. This attenuation was independent of the RD1 virulence locus required for survival and replication inside macrophages, but could not be induced by heat-killed bacteria. Together, the data suggest that *Mm* infection initially induces increased NO production in bystander neutrophils and that this response is attenuated during the course of infection by a bacterial virulence mechanism (figure

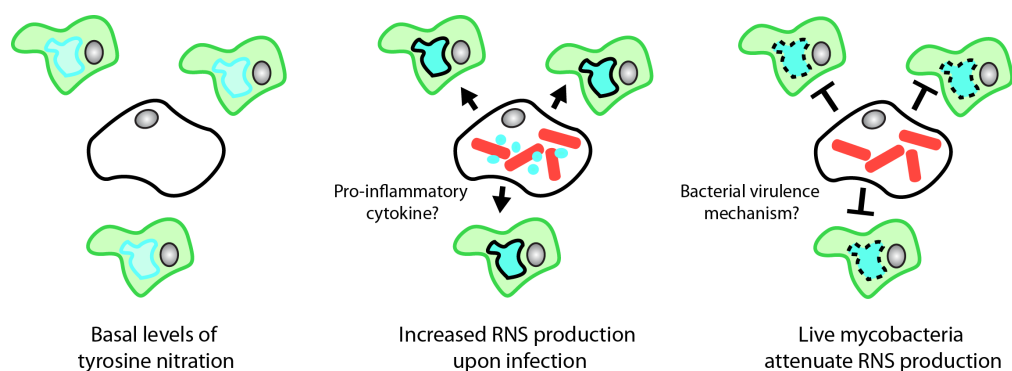


Figure 7: Model of RNS production and attenuation during mycobacterial infection Leukocytes have basal levels of tyrosine nitration, most commonly caused by the neutrophil specific enzyme Mpx. Tyrosine nitration levels resulting from RNS production are increased in infected and bystander leukocytes following mycobacterial infection, possibly via pro-inflammatory cytokine secretion. The effectivity and/or production of RNS is then attenuated by live mycobacteria, via a presently unknown virulence mechanism. Priming leukocytes for defense against mycobacteria by inducing RNS production via the hypoxia pathway reduced bacterial burdens.

7). A bacterial protein potentially involved in regulating this oxidative stress response is OxyR⁵⁷. Additionally, mycobacteria can directly protect themselves against oxidative stress via AhpC and MsrA and B, by catalyzing the breakdown of peroxynitrite or protecting *Mtb* against its detrimental effects⁵⁸⁻⁶⁰. These virulence mechanisms of *Mtb* and *Mm* have evolved to specifically counteract the oxidative defenses of their natural human and zebrafish hosts, which may partly explain why *Mtb* is more susceptible to RNS production in the non-natural mouse model of TB disease^{61, 62}.

The presence of heat killed, live or Δ RD1 *Mm* was sufficient to increase the host nitric oxide output 12 hours after encountering the microbes. This demonstrated that the recognition of mycobacterial PAMPs, rather than virulence or viability of the bacteria was responsible for this effect. Since the increase in tyrosine nitration was absent in *myd88*^{-/-} embryos infected with *Mm*, we concluded that TLR recognition of mycobacteria was required to initiate this defense mechanism. The mycobacterial cell wall contains lipoproteins and lipoglycans that serve as TLR ligands⁶³. Recognition of the cell wall component lipoarabinomannan (LAM) by macrophages resulted in iNOS-NO production directly⁶², or via induction of the *iNOS* expression regulating cytokines TNF- α and IL-1 β ^{64, 65}. It is possible that production of IL-1 β played a role in the increased RNS production in leukocytes following *Mm* infection, since the interleukin 1 receptor (IL1R) also relies on MyD88 for its downstream signaling^{50, 66}. Furthermore, we found that most leukocytes near bacteria, particularly neutrophils, displayed increased tyrosine nitration, not just those leukocytes directly interacting with *Mm*, suggesting a potential role for a locally secreted pro-inflammatory cytokine like IL-1 β in increased RNS production. Likewise, the dampening of RNS production at later stages of infection may be caused by secretion of anti-inflammatory cytokines. NO produced by iNOS is also required for the infection-responsive expansion of hematopoietic stem and progenitor cells and controls immunopathology of TB by preventing overproduction of IL-1 β by limiting inflammasome activity^{52, 67}.

The search for new host- or pathogen-based anti-TB therapies has intensified with the increasing occurrence of *Mtb* strains that are resistant to multiple drug treatments^{1, 2}. The attenuation of RNS activity described here is directly linked to this problem, since resistance of clinical *Mtb* strains to first-line antibiotics was shown to be associated with decreased susceptibility to NO⁶⁸. We have previously shown that dominant active expression of the hypoxia inducible transcription factor Hif-1 α primed leukocytes for defense against mycobacterial disease by increasing iNOS-dependent RNS production prior to infection, resulting in a decrease in bacterial burden⁴². This suggested that mycobacteria were more susceptible to host nitrosative defenses before they had a chance to sense and adapt to the high levels of RNS present in primed phagocytes. Our current data indicate that targeting the bacterial response to nitrosative stress represents a potential therapeutic option for the treatment of multiple drug resistant TB strains.

Materials and methods

Fish husbandry

Zebrafish strains (wildtype strain used was AB/TL) were maintained according to standard protocols. Transgenic and mutant strains used were *Tg(mpx:GFP)ⁱ¹¹⁴*⁶⁹, *Tg(mpeg1:EGFP)^{gl22}*⁷⁰, *myd88^{hu3568}/Tg(mpx:GFP)ⁱ¹¹⁴*⁵⁰, and *mpx^{NL144}/Tg(mpx:GFP)ⁱ¹¹⁴*. The *mpx^{NL144}* mutant line contains a C to T point mutation at nucleotide position 1126 of the *mpx* RefSeq mRNA sequence (NM_212779) resulting in a premature stop codon (M.J. Redd, E. Murayama, W. Horsley, P. Herbomel, and N. Trede, unpublished). Adult fish were maintained on a 14 hour light and 10 hour dark cycle at 28°C in accordance with local animal welfare regulations.

Bacterial culture preparation

Infection experiments were performed using *M. marinum* strain M (ATCC #BAA-535)⁷¹, containing the pSMT3-mCherry vector⁷². Liquid cultures were prepared from bacterial plates as previously described⁷² with 50 µg/ml hygromycin. Injection inoculum was prepared from overnight liquid cultures (at OD₆₀₀<1) by washing three times in sterile 0.05% Tween80/PBS solution (BD Difco), assessing optical density at 600 nm and resuspending in a 2% polyvinylpyrrolidone40 (PVP40) solution (CalBiochem) in PBS (Benard et al., 2012).

Intravenous injection of bacteria into one-day old embryos

Injection of bacteria was performed into the blood-forming region above the caudal vein at 28hpf as previously described⁷³. An inoculum volume of 1nl and 100 colony-forming units (CFU) of bacteria were injected. CFU was checked by plating out the injection volume.

RNA injections of dominant active Hif-1α

Embryos were injected with dominant active Hif-1α (ZFIN: *hif1ab*) RNA at the one to two cell stage as previously described⁷⁴. Phenol red (Sigma Aldrich) was used as an injection control.

Stereoinaging and bacterial pixel quantification

Embryos were imaged at 4dpi on a Leica MZ16FA Fluorescence Stereo Microscope. Brightfield and fluorescence images were generated with a Leica DC500 (DFC420C) camera. Bacterial loads were analysed using dedicated pixel counting software as previously described⁷⁵.

Anti-nitrotyrosine antibody staining and TSA staining

Larvae were fixed in 4% paraformaldehyde in PBS overnight at 4°C and nitrotyrosine levels were immune-labelled using a rabbit polyclonal anti-nitrotyrosine antibody (Merck Millipore 06-284, MA, USA) at a 1:200 dilution of primary antibody, and were detected using an Alexa Fluor (Invitrogen Life Technologies, NY, USA) secondary antibody. Mpx activity was labelled with TSA (TSAplus kit, Fluorescence Systems, Perkin

Elmer Inc., Waltham, MA) as previously described ⁴⁴.

Confocal microscopy and quantification of corrected cell fluorescence of anti-nitrotyrosine levels

Embryos were imaged at 1dpi, in the presence or absence of infection, embedded in 1% low melting point agarose (Sigma Aldrich) and transferred to a Leica DMIRBE inverted microscope with a Leica SP1 confocal scanhead for imaging with 40 or 63 times lenses. For quantification purposes acquisition settings and area of imaging (in the caudal vein region) were kept the same across the groups. Corrected total cell fluorescence was calculated for each immune-stained cell using Image J measurements as previously described ⁴². The GFP of the Tg(*mpx:GFP*)ⁱ¹¹⁴ ⁷⁶ was used to assess the leukocyte cell boundaries.

Statistical analysis

All data were analysed (Prism 5.0, GraphPad Software, San Diego, CA) using unpaired, two-tailed t-tests for comparisons between two groups and one-way ANOVA (with Bonferroni post-test adjustment) for multiple group comparisons.

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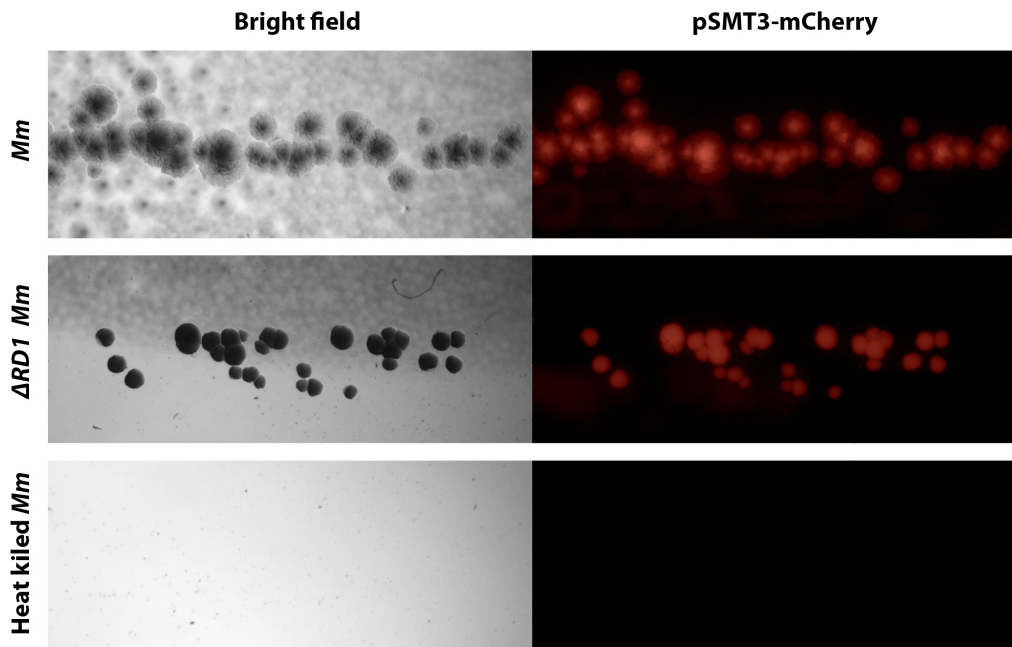
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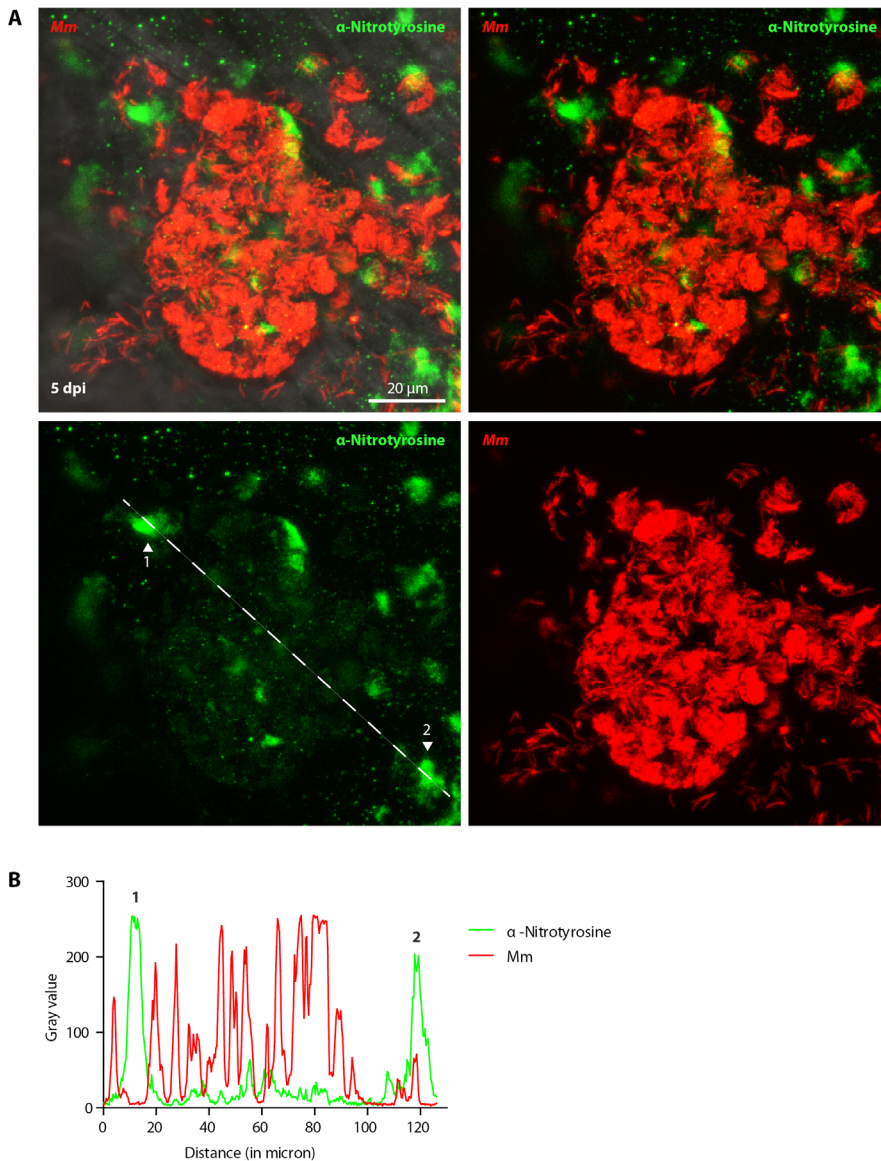
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Supplementary figure 1: Heat killed *M. marinum* The injection dose of live, heat killed or $\Delta RD1$ *Mm* were plated for CFU counts. As expected, heat killed bacteria did not grow.



Supplementary figure 2: Anti-nitrotyrosine staining levels in progression granulomas are lower when co-localized with bacteria (A) Example fluorescence confocal micrographs of anti-nitrotyrosine staining performed on 5 dpi granuloma structures in wild type embryos infected with *Mm*. (B) Gray values of the bacterial and anti-nitrotyrosine fluorescence signals measured along a straight line through the center of the granuloma (see white lines in (A)), intensity of the fluorescent signal was measured using ImageJ. Numbered peaks in the graphs correspond to numbered patches of tyrosine nitration in (A).

Chapter 5

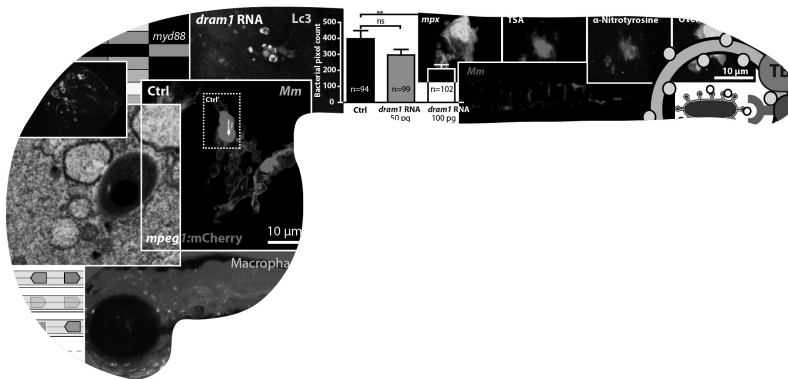
DRAM1 links pathogen recognition via TLR-MYD88 to autophagic defense against tuberculosis

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Abstract

Autophagy is an important immunological defense mechanism against the intracellular pathogens *Mycobacterium tuberculosis* and *Mycobacterium marinum*, the causative agents of tuberculosis in human and animal models. Here we place autophagy modulator DRAM1 in the TLR/IL1R-MYD88-NFκB signaling pathway central to innate immunity, in contrast to its known role as p53-target in autophagy-mediated cell death and cancer. Unraveling this innate defense pathway provides the missing link between pathogen recognition and autophagic host defense. Mycobacterial infection induced expression of *DRAM1* in both the zebrafish model and human macrophages. Knockdown of DRAM1 increased mycobacterial infection, whereas overexpression lowered infection by hyper-activating autophagic defense via the selective autophagy receptor p62/SQSTM1. We propose that this novel function of DRAM1 protects against a broader range of intracellular pathogens, since *DRAM1* expression was also induced by the common bacterial endotoxin LPS. Therefore, stimulating this selective autophagy pathway represents a potential therapeutic strategy for tuberculosis and other infectious diseases.

Introduction

5 A growing body of evidence firmly establishes autophagy as a defense mechanism against intracellular pathogens¹. Autophagy is an evolutionary conserved process in eukaryotes essential for cellular homeostasis in response to environmental and cellular stressors. During autophagy (or macroautophagy), cytoplasmic components are enveloped in double membrane vesicles that fuse with lysosomes for degradation of their content. The selective autophagy system specifically degrades unwanted protein aggregates or cellular contents via ubiquitin-mediated targeting. Selective autophagy receptors such as p62/SQSTM1 and NDP52 function to link ubiquitinated cargo to the autophagosome-associated protein LC3². Subsequently, other components of the general autophagy machinery, including the ATG12-ATG5-ATG16L1-complex, are required for maturation of the autophagosome³. Autophagic processes perform diverse roles in defense by contributing to cytokine secretion, targeting cytosolic or vacuolar microbes for lysosomal degradation, and acting as regulator of innate and adaptive immune responses^{3,4}.

Studies on *Mycobacterium tuberculosis* (*Mtb*) have been a leading example of how autophagy can counteract the ability of intracellular pathogens to avoid host defenses⁵⁻⁹. *Mtb* is the causative agent of pulmonary tuberculosis (TB), a common and often lethal infectious disease. Mycobacteria evade leukocyte bacterial killing mechanisms by preventing phagosome-lysosome fusion, which creates a niche that allows them to survive and proliferate¹⁰. Infected leukocytes then recruit other immune cells, including macrophages, neutrophils, and T-cells, to form highly organised structures known as granulomas¹¹. *Mtb* can persist for many years within granulomas, before eventually

escaping to disseminate and cause active TB disease¹². The importance of autophagy in host defense against *Mtb* is underscored by observations that first-line antibiotics against TB require autophagy activation for their effective anti-mycobacterial action¹³, and that autophagy enhances the efficacy of Bacille Calmette Guerin (BCG) vaccination¹⁴.

In this study we exploited the zebrafish model to investigate the impact of autophagy modulation during early stages of mycobacterial infection. *Mycobacterium marinum* (*Mm*) is a natural fish pathogen and a close relative of *Mtb*. It causes a phenotype in zebrafish that highly resembles human TB disease, including the formation of caseating granulomas and development of latency¹⁵. *Mm* infection of zebrafish embryos is a well-established *in vivo* model to study early stages of TB disease and has been successfully used to understand host cell signaling and mycobacterial virulence determinants¹⁶. An advantage of this model is that the host-pathogen interactions in the first days of mycobacterial pathogenesis can be visualized in the absence of an adaptive immune contribution, which has helped understanding the functions of macrophages and neutrophils in tissue dissemination and control of infection^{17, 18}.

Recent work on autophagy-mediated control of mycobacterial infection demonstrated that permeabilization of the *Mtb*-containing phagosome, dependent on the ESX-1 secretion system encoded by the bacterial region of difference 1 (RD1), allows cytosolic components of the selective autophagy pathway access to the bacteria inside these vacuoles¹⁹. Recognition of extracellular bacterial DNA by the STING-dependent DNA-sensing pathway leads to degradation of *Mtb* in mature autophagolysosomes. This process requires ubiquitination of bacteria, the selective autophagy receptors p62 and NDP52, and the DNA-responsive kinase TBK1¹⁹. TBK1 was shown to promote autophagosome maturation during defense against mycobacteria²⁰. This control of autophagic flux by TBK1 involves phosphorylation of p62, the autophagic receptor that targets ubiquitinated bacteria and delivers ribosomal and cytosolic proteins to autophagolysosomes where they are proteolytically converted into products capable of killing *Mtb*²¹.

As of yet, the molecular signaling pathway responsible for autophagic control of mycobacterial disease remains unclear, although there are strong links between Toll-like receptor (TLR) signaling and autophagy induction²⁰⁻²⁴. We took advantage of our recently characterized myeloid differentiation primary response 88 (*myd88*) mutant zebrafish line to study regulation of autophagy downstream of this central TLR/IL1R-signaling adaptor²⁵. We found that zebrafish DNA-damage regulated autophagy modulator 1 (*dram1*) is expressed in a MyD88-dependent fashion during mycobacterial infection. Human *DRAM1* is a known target gene of the tumor suppressor p53 and is required for p53-dependent cell death by inducing autophagy²⁶. There is considerable interest in *DRAM1* due to its relation with tumor development and cancer therapy²⁶⁻²⁸. The relation between *DRAM1* and autophagic control of *Mtb* has not been previously addressed.

In the present study we show that expression of zebrafish *dram1* during *Mm* infection did not require p53, but was dependent on functional MyD88 and NFκB. Knockdown of either *Dram1* or p62 resulted in increased mycobacterial burdens. This was associated with an apparent inability of macrophages to contain the pathogen in intracellular compartments, resulting in excessive intracellular replication during early pathogenesis and increased extracellular growth of the bacteria during later stages of infection. Imaging of autophagy in GFP-Lc3 transgenic zebrafish showed that mycobacterial-associated autophagosome formation was strongly reduced under knockdown conditions of *myd88* or *dram1* and significantly enhanced by *dram1* overexpression. Strikingly, *in vivo* activation of the general autophagy pathway proved to be detrimental to host defense during early stages of mycobacterial infection, whereas induction of the selective autophagy pathway by overexpressing *dram1* was protective. We propose that DRAM1 functions as an evolutionary conserved modulator of anti-mycobacterial autophagy via the ubiquitin receptor p62, since *DRAM1* and *p62/SQSTM1* expression was also increased in primary human macrophages infected with *Mtb*. Regulation of human *DRAM1* following *Mtb* infection was dependent on NFκB, confirming our findings in the zebrafish model. Taken together, we show for the first time that DRAM1 is involved in defense against intracellular bacterial pathogens, placing this important regulator of autophagy in the TLR/IL1R-MYD88-NFκB signaling pathway central to innate immunity.

Results

Blocking or overactivating general autophagy increases mycobacterial infection *in vivo*

The majority of studies on autophagy as anti-mycobacterial defense mechanism were performed using *in vitro* infection assays. We aimed to utilize the advantages of zebrafish embryo infection as a complementary *in vivo* model to study autophagy in immune defense. First, treatment conditions for widely used autophagy modulators were tested in transgenic zebrafish embryos expressing GFP-Lc3 driven by a constitutive cytomegalovirus (CMV) promoter²⁹ (Figure 1a). We confirmed that 3-methyladenine (3-MA) inhibits autophagosome formation in GFP-LC3 zebrafish, while chloroquine (Cq) and Ar-12 increased the number of Lc3-fluorescent punctae (Figure 1b) similar to the previously reported effect of rapamycin²⁹. The increase in Lc3 punctae by Cq and Ar-12 is consistent with the function of Cq in blocking autophagic flux by preventing autophagosome-lysosome fusion, whereas Ar-12 increases autophagosome formation by inhibiting PDK1, causing cellular stress due to accumulation of reactive oxygen species (ROS)³⁰. To confirm that autophagy functions as a defense mechanism against mycobacterial disease in this *in vivo* model, we infected GFP-Lc3 transgenic embryos with *Mm* and treated them with 3-MA, Cq or Ar-12 for the first 24 hours post infection (hpi). Co-localization of autophagosomes and bacteria was observed in ~30% of control infected embryos, consistent with a previous study in cultured macrophages showing

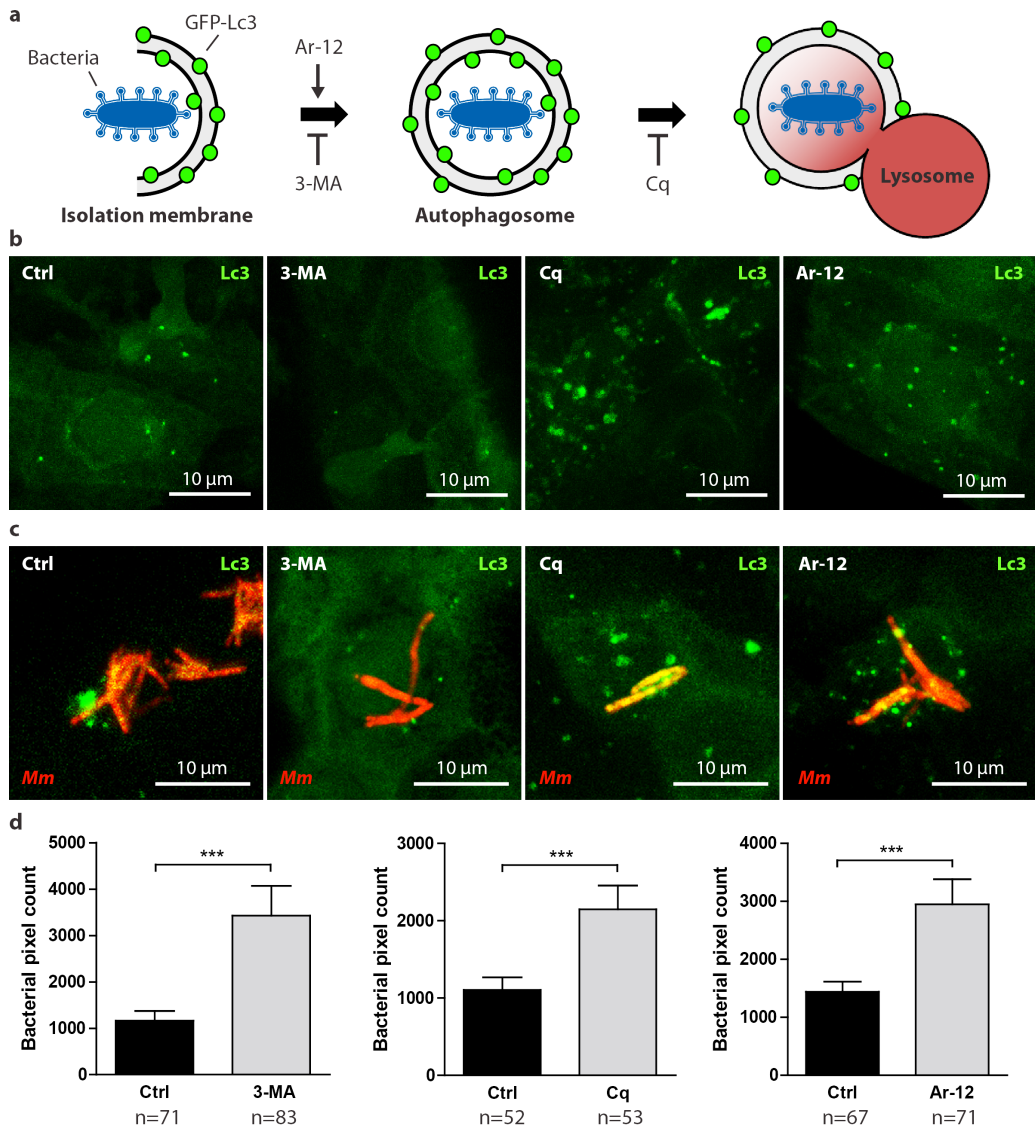


Figure 1. Modulating general autophagy increases mycobacterial infection.

(A) Schematic representation of the effects of 3-MA, Cq, and Ar-12 on autophagosome formation and autophagic flux. (B) GFP-Lc3 embryos 2 days post fertilization (dpf) were treated for 24 hours with DMSO (control), 3-MA (10 mM), Cq (100 μ M), or Ar-12 (1 μ M). Representative confocal micrographs of endothelial cells at 3dpf are shown. (C) The same treatments were applied to GFP-Lc3 embryos injected with *Mm*. Representative confocal micrographs of infected cells at 3dpf are shown. (D) The same treatments were applied to non-transgenic embryos infected with *Mm*. Bacterial pixel counts were determined at 3 dpi. Data (mean $\pm$ SEM) is pooled from at least two individual experiments ($n \geq 50$ embryos per group).

that *Mm* resides temporarily in GFP-LC3 labeled compartments devoid of lysosomal enzymes³¹. As expected, treatment with 3-MA decreased autophagy induction in infected cells and prevention of autophagic flux by Cq treatment led to an accumulation of autophagosomes co-localized with bacteria (Figure 1c). Infected embryos treated with Ar-12 displayed a large number of small Lc3-punctae throughout their cells, which mostly not associated with bacteria. Subsequently we applied the same treatment conditions to infected non-transgenic embryos and determined the effect on bacterial burden by quantifying bacterial fluorescence at 3 days post infection (3 dpi). We observed increased bacterial burdens after treatment with all three compounds (Figure 1d). The observation that blocking autophagosome formation or autophagic flux led to increased infection is consistent with results obtained from mammalian cell cultures infected with mycobacteria⁷ and supports that autophagy functions as a defense mechanism against mycobacterial infection in zebrafish. Furthermore, stimulating the ROS-inducible general autophagy pathway was ineffective to control mycobacterial infection in this *in vivo* model.

Induction of autophagy modulator *dram1* by mycobacterial infection is MyD88-dependent

An important illustration of the immunological role of autophagy is the control of this process by TLR recognition of pathogen-associated molecular patterns^{22, 23}. However, it remains largely unknown how TLR signaling and autophagy are connected. We have previously demonstrated that *myd88* mutant (*myd88*^{-/-}) zebrafish embryos, lacking a signaling adaptor vital to TLR recognition, show impaired induction of genes central to innate immunity and are more susceptible to infection by *Mm* strains²⁵. Microarray profiling of *myd88*^{+/+} and *myd88*^{-/-} embryos infected with *Mm* confirmed that expression of important pro-inflammatory genes, such as *il1b*, *tnfa*, and *mmp9*, is significantly lower in the absence of MyD88-signaling (Figure 2a). Intriguingly, we found that expression of the gene *DNA-damage regulated autophagy modulator 1* (*dram1*) was significantly reduced in the absence of functional MyD88 (Figure 2a). This gene encodes a transmembrane protein that is highly conserved among vertebrates in terms of protein homology and gene synteny²⁶ (Figure S1). We evaluated the expression levels of *dram1* over a time course of mycobacterial infection (Figure 2b). Starting at 2 dpi, *dram1* expression progressively increased over the following days in infected *myd88*^{+/+}, but not in *myd88*^{-/-}. The MyD88-dependency of *dram1* during mycobacterial infection was confirmed by RNA deep sequencing and qPCR in independent experiments (Figure 2a and c). Increased expression of the genes encoding p53, Atg5, Lc3, or p62 was not detected on the whole embryo level.

In silico analysis of the promoter regions of human and zebrafish *DRAM1/dram1* revealed the presence of conserved binding sites for the hematopoietic transcription factor Pu.1 (Figure S2). Expression of zebrafish *dram1* in leukocytes was confirmed by qPCR on FACS-sorted immune cell populations, showing its enrichment in the Mpx+, Mpeg1+, and Lck+ fractions of dissociated transgenic embryos specific for neutrophils,

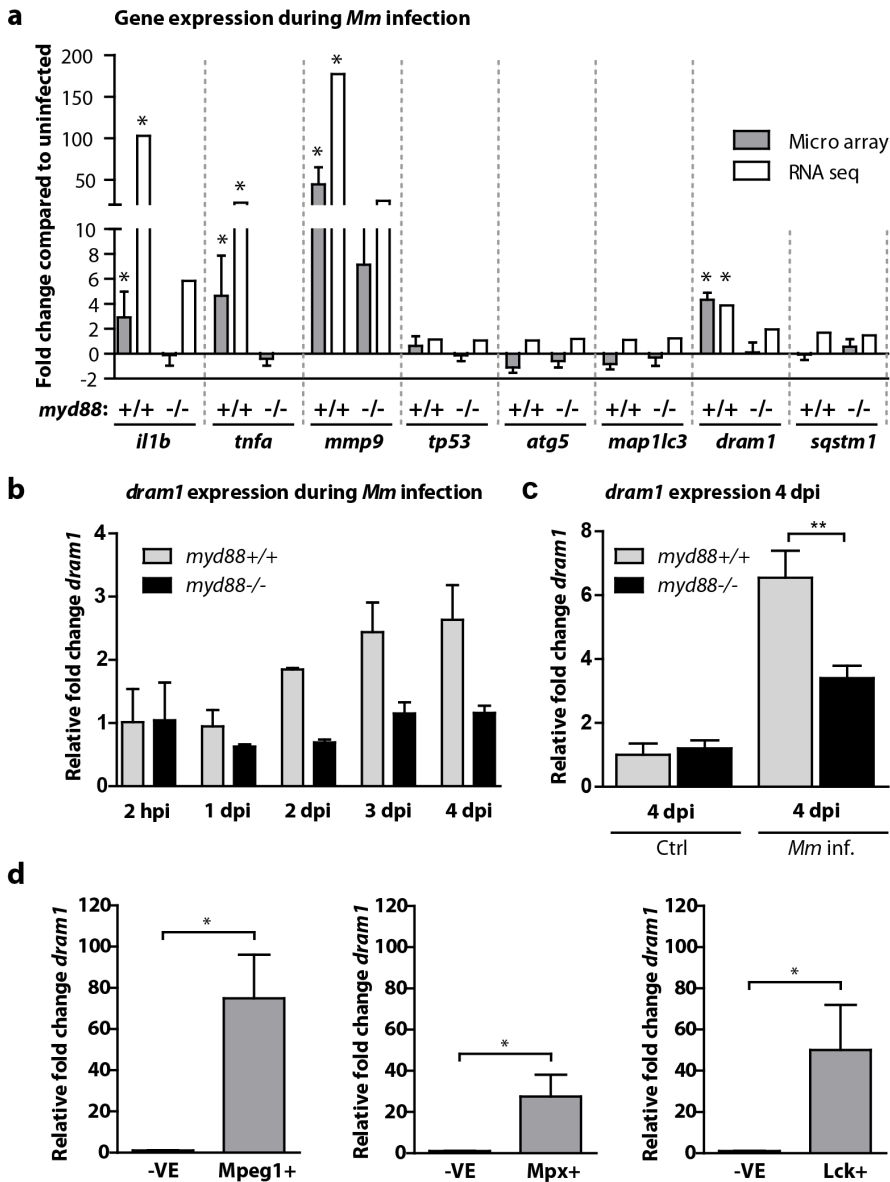


Figure 2. *dram1* is induced by mycobacterial infection in a Myd88-dependent fashion.

(A) *myd88*^{+/+} and *myd88*^{-/-} embryos infected with *Mm* were snap frozen individually at 4 dpi and triplicate samples were compared with PBS-injected controls using a common reference microarray design. Significant differences between infected wild types versus infected mutants were calculated using Rosetta Resolver re-ratio analysis (* = fold change > 2, $p < 10^{-5}$). Observed differences were confirmed by RNA sequencing of pools (n=20 embryos per pool) of uninfected and *Mm* infected *myd88*^{+/+} and *myd88*^{-/-} embryos. Significant differences between infected wild types versus infected mutants were calculated by DESeq analysis of transcript count data (* = fold change > 2, $p < 0.05$). (B) Expression of *dram1* at multiple time points after infection was analysed by qPCR on pools of *Mm* infected *myd88*^{+/+} and *myd88*^{-/-} embryos, relative to

Figure 2 continued: PBS-injected controls (mean $\pm$ SEM of $n=2$ biological replicates with 20 embryos per pool). (C) Expression levels of *dram1* at 4 dpi in individual *myd88*^{+/+} and *myd88*^{-/-} embryos with or without infection were determined by qPCR (mean $\pm$ SEM of $n=3$ biological replicates with 10 embryos per pool). (D) Macrophages, neutrophils, and leukocytes from 5-6 dpf larvae were isolated by FACS. Enrichment of *dram1* in immune cell populations was analyzed by qPCR. Expression levels of *dram1* in the positive fractions (e.g. Mpeg1+) relative to the negative fractions (-VE) are shown (mean $\pm$ SEM of $n=4$ biological replicates).

macrophages, and lymphocytes, respectively (Figure 2d). We conclude that zebrafish *dram1* is expressed in myeloid and lymphoid immune cell lineages, and is induced in a MyD88-dependent fashion following infection by mycobacteria.

Bacterial induced expression of *dram1* is independent of p53, dependent on NFkB

Since the known functions of *dram1* are dependent on p53-signaling, we analyzed expression levels of *dram1* in embryos with a mutation in the DNA-binding domain of p53 (*p53*^{-/-})³². In contrast to *myd88*^{-/-} mutants, *Mm*-infected *p53*^{-/-} mutants showed upregulation of *dram1* to wild type levels, indicating that infection-induced *dram1* expression is p53-independent (Figure 3a). As a control, we treated embryos with the p53-stabilizing agent roscovitine³². Unlike wild type, *p53*^{-/-} embryos were insensitive to roscovitine-induced malformations and did not express *dram1* upon treatment (Figure S3, Figure 3b). These results show that the canonical p53-dependent route to *dram1* induction is functional in zebrafish, but is not employed during *Mm* infection. Autophagy is known to be regulated by NFkB³³, and the promoter regions of human and zebrafish *dram1* contain predicted NFkB consensus binding sites (Figure S2). We hypothesized that NFkB, rather than p53, is an essential transcription factor downstream of MyD88 in regulating *dram1* expression during mycobacterial infection. Unfortunately, the application of a previously described NFkB activation inhibitor (NAI) for the duration of time needed to observe early *Mm* pathogenesis (2 to 4 dpi) was harmful to embryonic development³⁴. We therefore developed an alternative assay to address the possible involvement of MyD88-NFkB signaling in immune related expression of *dram1* based on two previous findings: (1) *dram1* expression was upregulated in zebrafish embryos infected by *Salmonella enterica* serovar Typhimurium (*St*)³⁵, and (2) TLR recognition of *St* derived lipopolysaccharide (LPS) in zebrafish depended on MyD88²⁵. Since LPS-induced gene expression occurs rapidly after exposure, this allowed us to avoid harmful effects of NAI treatment by incubating zebrafish embryos with NAI for only 4 hours. We show that LPS injection significantly increased *dram1* expression in control treated embryos and that this effect was completely abrogated in the presence of NAI (Figure 3c). As expected, *myd88*^{-/-} embryos did not increase *dram1* expression in response to LPS exposure, while *p53*^{-/-} embryos behaved identical to wild type controls (Figure 3c). Expression levels of the known NFkB target gene serum amyloid A (*saa*) displayed a similar pattern following LPS injection (Figure 3d). Together, these data demonstrate that bacterial induced expression of *dram1* was dependent on MyD88-NFkB, but not on p53.

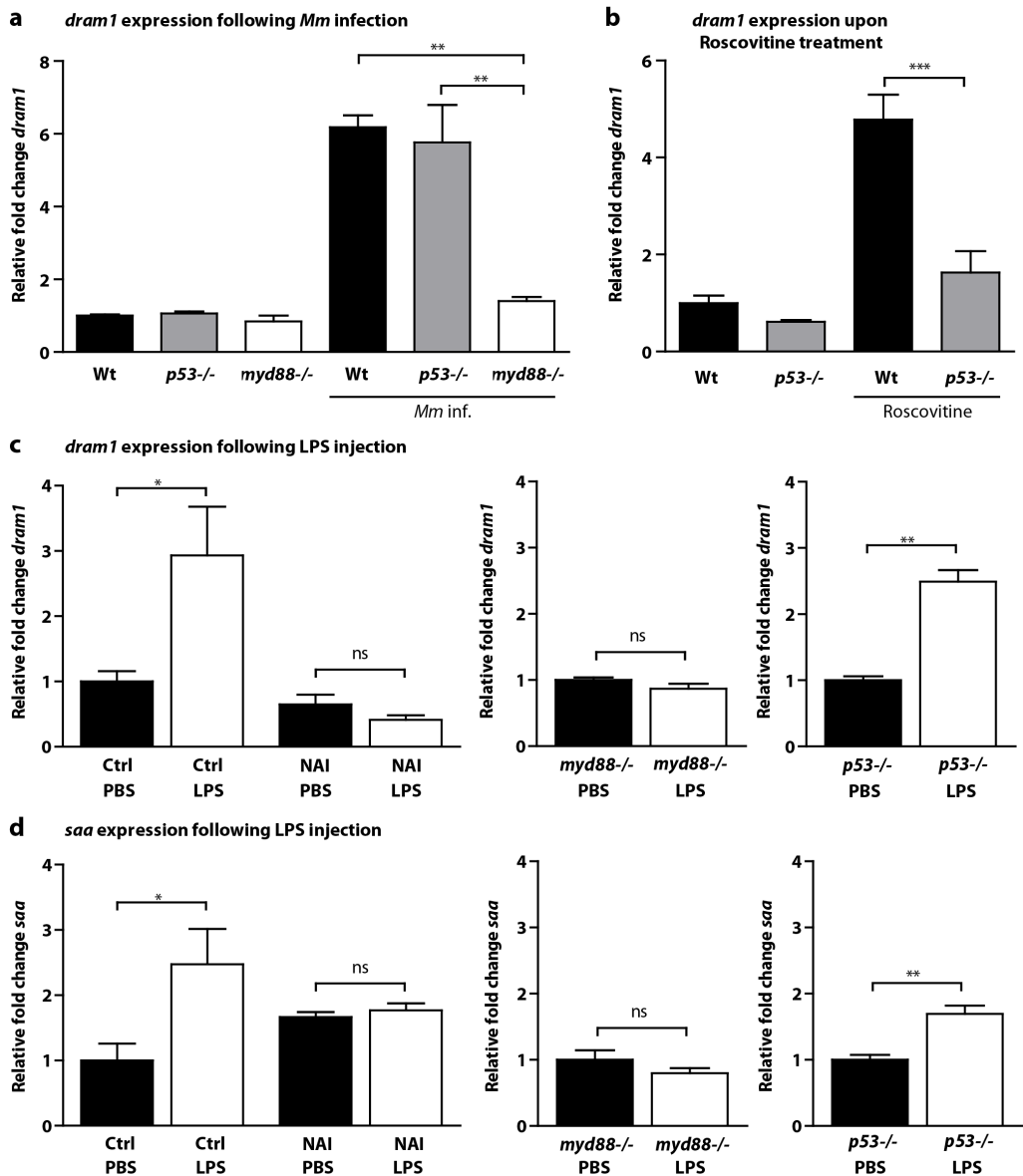


Figure 3. *dram1* expression during mycobacterial infection is independent of p53, but dependent of NFκB. Expression levels of *dram1* were determined by qPCR for (A) wild type, *p53*^{-/-}, and *myd88*^{-/-} embryos 4 days after infection with *Mm*, relative to mock injected controls; (B) wild type and *p53*^{-/-} embryos at 5 dpf after 24 hours of treatment with roscovitine, relative to untreated controls (see also Figure S3); (C) one day old embryos at 2 hours after injection with LPS, relative to their respective PBS injected controls; left panel: wild type embryos with (NAI) or without (Ctrl) NFκB inhibitor treatment (50 nM, 4 hours of total treatment, including 2 hours pre-treatment); middle panel: *myd88*^{-/-} embryos; right panel: *p53*^{-/-} embryos. (D) Expression levels of *saa* were determined by qPCR under the same conditions as described for (C). All graphs show data (mean ± SEM) from three biological replicates with n=20 embryos pooled per replicate.

***Mtb* infection of human primary macrophages leads to increased expression of *DRAM1* dependent on NFκB**

In view of the strong evolutionary conservation of *DRAM1*, we hypothesized that the signaling pathway controlling *dram1* expression during mycobacterial infection is conserved between human and zebrafish. We obtained human primary macrophages type 1 (M1) and 2 (M2) from peripheral blood mononuclear cells (PBMCs) and infected them with *Mtb*. At 4 hpi we detected elevated expression levels of *DRAM1* in infected M1 and M2 (Figure 4a). Whereas we could not detect upregulation of *sqstm1* (p62) by *Mm* infection on the whole zebrafish embryo level, we did find *SQSTM1* to be significantly induced in both types of *Mtb*-infected macrophages (Figure 4b). *IL1β* expression was also induced in *Mtb*-infected M1 and M2 macrophages, confirming the inflammatory response to infection (Figure 4c). Next, we tested whether NAI could block the increased expression of *DRAM1* in *Mtb*-infected macrophages. The results show that inhibition of NFκB activation prevented the expression of *DRAM1* in infected M1 and M2 macrophages (Figure 4a). Infection induced expression of *SQSTM1* and *IL1β* was partially dependent on NFκB activity in M1, and independent of NFκB in M2 macrophages (Figure 4b and c). These results show that the NFκB-dependent induction of *DRAM1* expression during mycobacterial infection is a conserved mechanism between zebrafish and human. The co-expression of *SQSTM1* suggests a collaboration between the autophagy-related proteins *DRAM1* and p62 during mycobacterial infection.

5

***Dram1* is required to contain mycobacterial growth inside macrophages**

Due to the infection-inducible and immune cell specific expression of *dram1*, we hypothesized that it functions in autophagic defense against mycobacterial infection. We used an antisense morpholino oligonucleotide approach to block intron-exon splicing events for zebrafish *dram1*, knocking down normal *dram1* mRNA production (Figure S4). *Dram1* deficiency significantly increased *Mm* bacterial burdens and we could phenocopy this effect by using a second splice-blocking morpholino targeting *dram1* (Figure 5a). Both human *DRAM1* and *SQSTM1* were shown to be expressed in tumor-initiating glioblastoma stem cells, in which knockdown of *DRAM1* decreased p62/*Sqstm1* localization to autophagosomes, suggesting a role for *DRAM1* in p62-dependent selective autophagy²⁸. Furthermore, p62 itself is required for targeting autophagy to *Mtb* bacteria inside phagosomes¹⁹. Similar to *dram1* deficiency, knockdown of zebrafish *sqstm1* also significantly increased mycobacterial burdens (Figure 5a; Figure S4). Interestingly, highly infected embryos with abrogated *dram1* or *sqstm1* expression displayed a similar phenotype with accumulation of bacteria inside intersegmental blood vessels, indicative of extracellular growth of bacteria (Figure 5b). In contrast, highly infected control embryos only displayed large, dense granuloma-like aggregates of *Mm*-infected cells. The increased infection and extracellular localization of bacteria was not caused by a reduced number of neutrophils or macrophages in *dram1* knockdown embryos (Figure 5c). Neither did *dram1* knockdown lead to decreased expression of the pro-inflammatory genes *il1b* and *mmp9*, ruling out the possibility that *Dram1* is

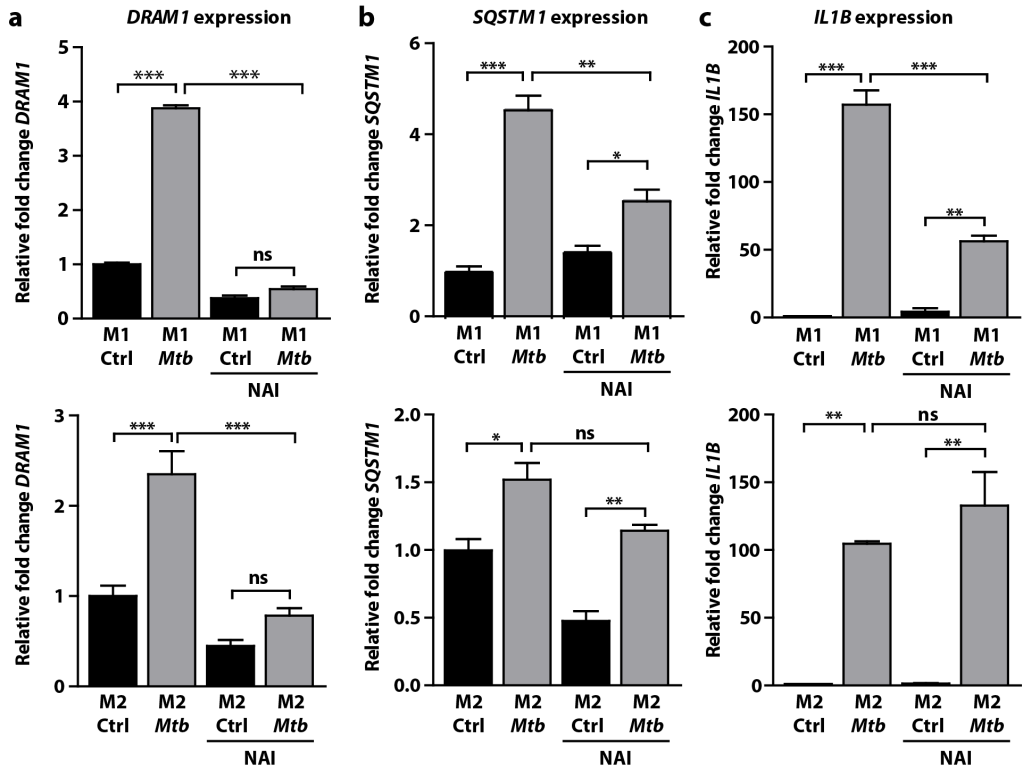


Figure 4. Human macrophages infected with *Mtb* increase *DRAM1* expression. The effect of NAI treatment (30 nM) on expression of (D) *DRAM1*, (E) *SQSTM1*, and (F) *IL1B* in primary human macrophages type 1 (M1) and type 2 (M2) in the presence or absence of *Mtb* was determined by qPCR, relative to uninfected controls. All graphs show data (mean $\pm$ SEM) from three biological replicates.

required for the initiation of inflammation (Figure 5d). In contrast, *dram1* knockdown resulted in significantly higher expression of *il1b* during infection with *Mm*.

Upon infection of zebrafish embryos, mycobacteria are rapidly phagocytosed by macrophages¹⁷. We used a macrophage-specific transgenic reporter line with mCherry localized to all cellular membranes (*mpeg1*:mCherry; Dr. Georges Lutfalla, unpublished data) to study the intracellular localization of *Mm* bacteria upon knockdown of *dram1*. In both the control and *dram1* knockdown group, all *Mm* were phagocytosed at 1dpi and bacteria inside macrophages were enclosed by membranes (Figure 5e). At 2dpi, bacteria remained intracellular and enclosed by membranes in control infected embryos (Figure 5e). In contrast, we frequently found overgrown macrophages unable to contain *Mm* inside vesicles in the *dram1* knockdown group (Figure 5e). This observation coincided with the appearance of extracellular bacteria, demonstrating that *Dram1* was required to restrict bacterial growth inside macrophages during early mycobacterial pathogenesis.

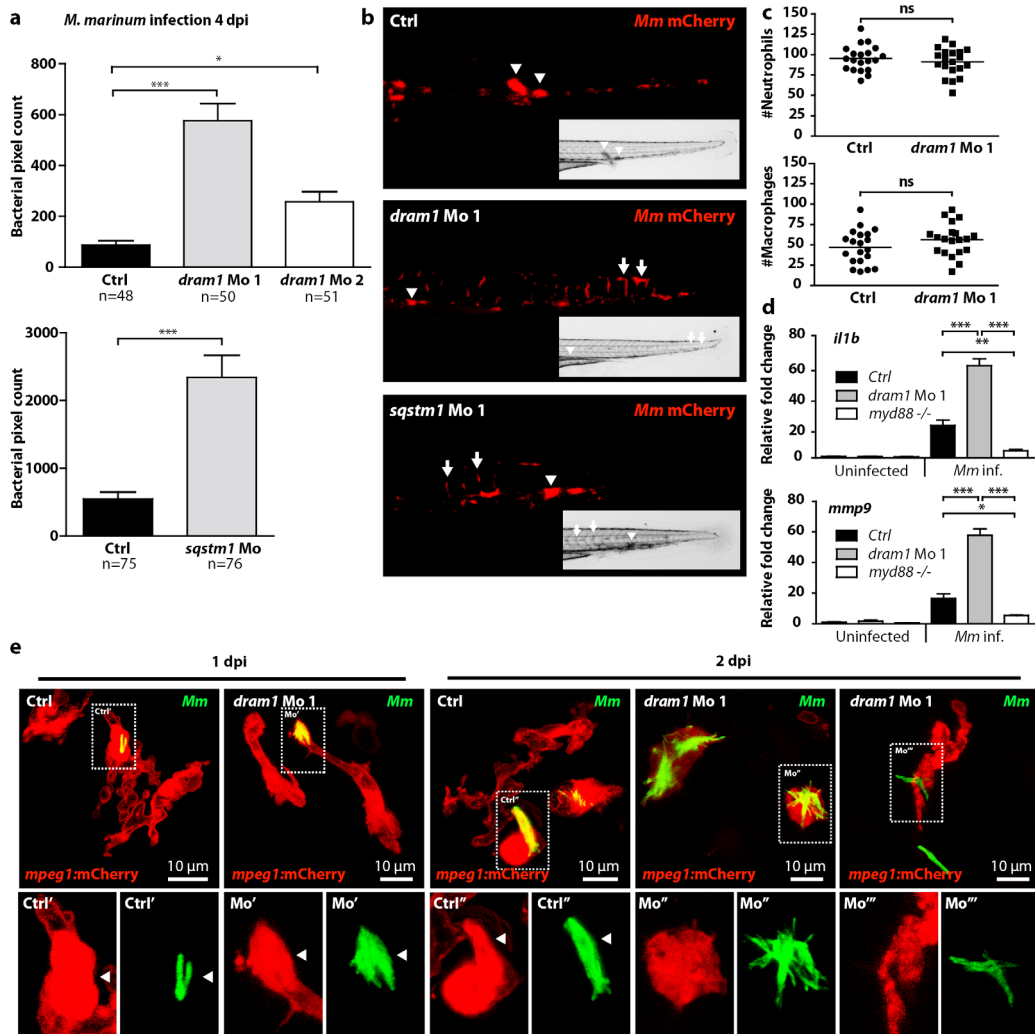


Figure 5. *Dram1* is required to contain *Mycobacterium marinum* growth inside macrophages. Zebrafish embryos injected with standard control morpholino, morpholino against *dram1* (*dram1* Mo 1 and Mo 2) or *sqstm1* were injected with *Mm* (see also Figure S4). Bacterial pixel counts were determined at 4 dpi. Data (mean $\pm$ SEM) is pooled from at least two individual experiments ($n = 48$ -76 embryos per group). (B) Stereo micrographs of the tail region of highly infected standard control, *dram1* Mo 1, or *sqstm1* Mo injected embryos. Arrowheads indicate granuloma formation and arrows indicate accumulation of bacteria in intersegmental veins. (C) Total numbers of neutrophils and macrophages in 2 dpf standard control or *dram1* morpholino injected embryos were counted under a fluorescence stereo microscope ($n=20$ per condition, blinded). Total numbers of macrophages were determined by performing whole mount L-plastin immunohistochemistry and deducting the number of cells positive for neutrophil specific myeloperoxidase activity from the number of L-plastin-positive total leukocytes per embryo. Each data point represents an individual embryo and lines indicate the mean. No significant difference (ns) in numbers of neutrophils or macrophages was observed with a t-test. (D) Expression levels of *il1b* and *mmp9* at 4 dpi in standard control morpholino injected, *dram1*

Mo 1 injected, and *myd88*^{-/-} embryos with or without *Mm* infection were determined by qPCR (mean $\pm$ SEM from n=3 biological replicates with 20 embryos per pool). (E) Representative confocal micrographs (1 or 2 dpi) of *mpeg1*:mCherry transgenic embryos injected with *Mm*. Boxed areas are detailed below with the green and red channel shown separately. Arrowheads indicate bacteria enclosed by membranes.

Selective autophagy induced by *dram1* overexpression restricts mycobacterial infection

To further test the hypothesis that Dram1 is involved in autophagic defense against mycobacterial infection, we cloned zebrafish *dram1* and injected mRNA into GFP-Lc3 transgenic embryos at the 1-cell stage, leading to ubiquitous overexpression of *dram1* in the developing embryo. Embryos overexpressing *dram1* developed normally and showed no apparent phenotypes (data not shown). At 3 days post fertilization (dpf), we assessed autophagy induction in GFP-Lc3 embryos with *dram1* overexpression and found that they had significantly increased numbers of autophagosomes compared to controls (Figure 6a and b). This demonstrated that the function of DRAM1 as an inducer of autophagy is conserved between human and zebrafish^{26, 36}. We then tested the effect of *dram1* overexpression during mycobacterial infection and observed that it reduced bacterial burden in a dose-dependent manner (Figure 6c and d). The number and the size of mycobacterial clusters was decreased in embryos injected with the highest dose of *dram1* RNA (Figure S5), showing that Dram1 is part of a defense mechanism against mycobacterial infection. To demonstrate that this defense mechanism involves the targeting of mycobacteria by selective autophagy, we manipulated the expression of *myd88* and *dram1* in GFP-Lc3 embryos and infected them with *Mm*. We quantified the number of Lc3 punctae per infected cell and found that morpholino knockdown of either *myd88* or *dram1* expression significantly reduced autophagosome formation (Figure 7a and b). Strikingly, overexpression of *dram1* RNA increased the number of autophagosomes in infected cells, showing a clear co-localization between Lc3-puncta and bacteria (Figure 7a and b). The induction of autophagy in *Mm* infected cells was dependent on functional bacterial RD1 virulence (Figure 7c). The increase in bacterial burden upon *dram1* knockdown was also RD1-dependent (Figure 7d), indicating bacterial escape or permeabilization of the phagosome as a trigger for Dram1-dependent autophagy. Morpholino knockdown of p62/*sqstm1* significantly lowered the number of autophagosomes per infected cell in zebrafish embryos (Figure 7e and f), consistent with previous observations in human cell cultures^{19, 21}. If Dram1 defends against mycobacterial infection via a p62-dependent mechanism, Dram1-dependent autophagy should be blocked by p62 deficiency. Indeed, co-injection of *sqstm1* morpholino with *dram1* RNA counteracted the increased formation of *Mm*-associated autophagosomes caused by *dram1* overexpression (Figure 7e and f). Together, these data demonstrate that Dram1 stimulates the targeting of autophagosomes to bacteria or bacteria-containing compartments, which required the selective autophagy receptor p62. We conclude that this Dram1/p62-mediated mechanism downstream of MyD88 has a protective function during mycobacterial infection *in vivo*.

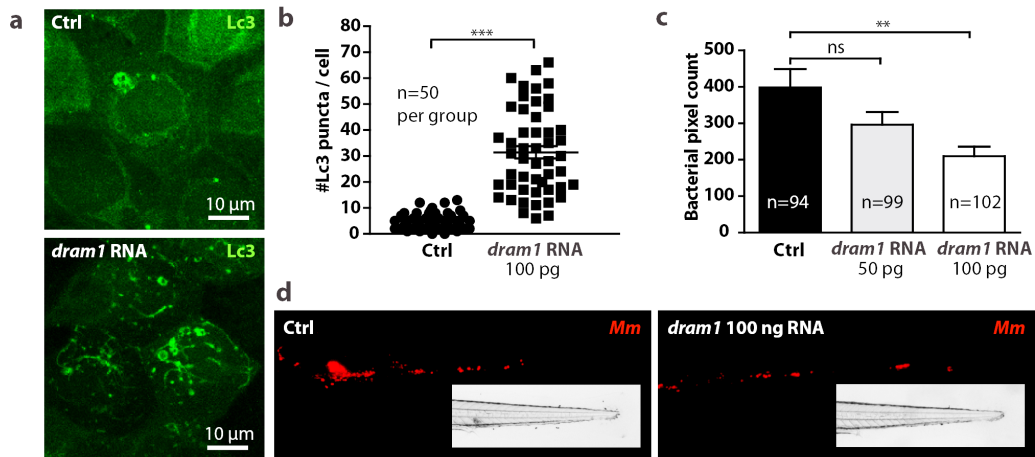
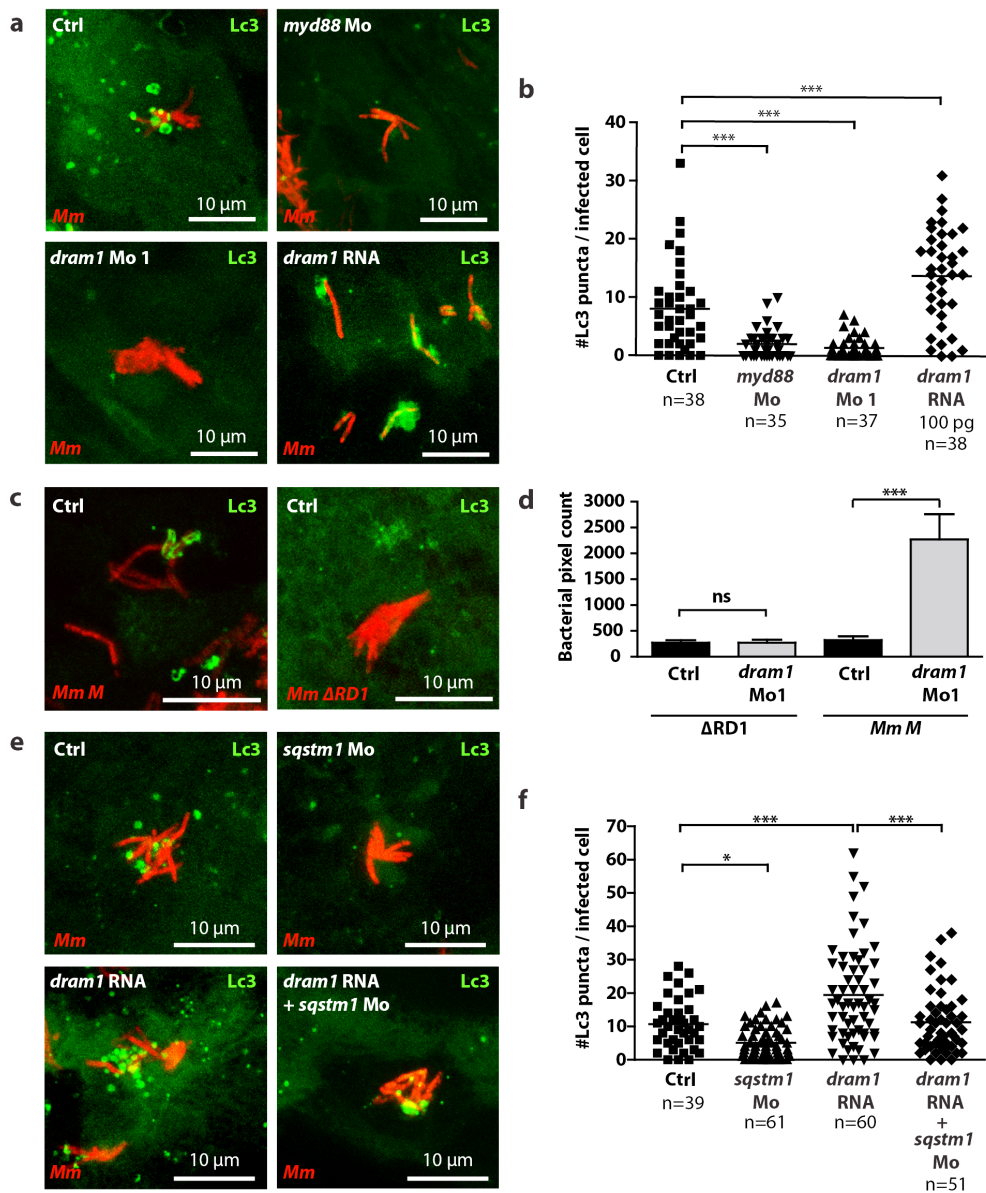


Figure 6. *Dram1* overexpression reduces mycobacterial burden. (A) GFP-Lc3 embryos were injected with 100 pg *dram1* RNA. Representative confocal micrographs of epithelial cells at 3 dpf are shown. (B) The number of GFP-Lc3 punctae was determined for $n=50$ cells per group, quantified based on confocal micrographs of control and *dram1* RNA injected embryos ($n \geq 5$, blinded). (C) Bacterial pixel counts were determined at 3 dpi for infected control or *dram1* RNA injected embryos. Data (mean $\pm$ SEM) is pooled from two individual experiments ($n \geq 94$ embryos per group). (D) Representative stereo micrographs of the tail of infected control or *dram1* RNA injected embryos at 3 dpi.

Figure 7. *Dram1* modulates an autophagic defense mechanism that requires bacterial RD1 virulence and host p62/Sqstm1. (A and B) GFP-Lc3 embryos injected with standard control morpholino, *myd88* morpholino, *dram1* morpholino 1, or *dram1* RNA. All groups were injected with *Mm*. Confocal micrographs ($n \geq 5$ embryos per group) were used to quantify GFP-Lc3 punctae per infected cell ($n \geq 5$ embryos per group, blinded). (C) Representative confocal micrographs of GFP-Lc3 embryos infected with *Mm* Δ RD1 (400 CFU) and wild type *Mm* *M* bacteria (200 CFU). (D) Bacterial pixel counts following these infections were determined with or without *dram1* knockdown. Data (mean $\pm$ SEM) is pooled from two individual experiments ($n > 62$ embryos per group). (E and F) Using the same approach as described for figure 6E and F, Lc3-punctae formation in infected zebrafish embryos was quantified ($n \geq 6$ embryos per group, blinded) following injection with *sqstm1* morpholino, *dram1* RNA (100 pg), or a combination of the two ($n \geq 6$ embryos per group, blinded). »



Discussion

Autophagy is a highly conserved process that acts as a quality control system for eukaryotic cells. It functions in programmed cell survival and cell death, normally resulting in cell survival by providing energy and nutrients and ridding the cytoplasm of unwanted components, such as defective organelles³. Besides this fundamental cellular homeostatic function, selective autophagy has emerged as an important effector mechanism of immune defense against intracellular pathogens². Up till now, the autophagy modulator DRAM1 was exclusively known as a p53-target gene that induces autophagy and cell death in response to cellular stresses related to cancer²⁶ and HIV infection of CD4⁺ T cells³⁷. In this study, we show how this important modulator of autophagy also functions downstream of the TLR/IL1R-MYD88-NFκB pathway in controlling infection with intracellular mycobacteria independently of p53.

We demonstrate that the strong conservation between human and zebrafish *DRAM1* on the level of gene synteny and protein homology extends to a conserved function in antimicrobial defense. We show that, like its human orthologs^{26, 36}, zebrafish *Dram1* is capable of modulating autophagy and that *DRAM1* expression is increased in response to infection with mycobacterial pathogens in human macrophages as well as zebrafish embryos. The zebrafish embryo model proved highly suitable for *in vivo* imaging of GFP-LC3 localization during mycobacterial infection and gain/loss-of-function studies in this model demonstrated the requirement of *Dram1* for autophagosome formation in infected cells and co-localization between Lc3-punctae and mycobacteria. To find support for a function of *DRAM1* in TB disease progression, we examined published microarray data sets of human patient material for *DRAM1* expression levels, which revealed that *DRAM1* was upregulated in the whole-blood transcript signature of active TB patients³⁸ and in macrophages obtained from *Mtb* infected patients³⁹.

Macrophages in *dram1*-deficient zebrafish embryos had difficulty maintaining mycobacteria inside vesicles and were frequently overgrown by bacteria. This resulted in a large accumulation of extracellular bacteria at later stages of infection. Both *DRAM1* and *SQSTM1* (p62) are expressed in human macrophages upon infection with *Mtb* and knockdown of zebrafish *sqstm1* had the same effect on mycobacterial pathogenesis as observed for *dram1* knockdown. We found that the defense mechanism controlled by MYD88 and DRAM1 required p62 for the formation of autophagosomes in response to mycobacterial infection. These results reinforce the importance of p62 in autophagic resistance to *Mtb* infection. Sensing of *Mtb* DNA by the STING-pathway was shown to induce autophagosome formation via the ubiquitin receptors p62 and NDP52¹⁹. Furthermore, p62 has been shown to enhance the bactericidal properties of autophagosomes by delivering cytosolic substrates that are proteolytically cleaved into neo-antimicrobial peptides²¹. Autophagy induction in macrophages via the STING pathway followed permeabilization of the phagosomal membrane by mycobacteria,

which depended on the RD1 virulence locus¹⁹. In contrast, RD1 virulence was linked with inhibition of autophagy in *Mtb* infected dendritic cells⁴⁰. While mycobacteria are likely to have evolved mechanisms to evade autophagic defense, our results in the zebrafish *in vivo* model corroborate the essential requirement of RD1 for LC3 recruitment and the function of p62-mediated selective autophagy in anti-mycobacterial host defense^{19,31}. Based on our present results, we cannot exclude the possible involvement of other selective autophagy receptors in anti-microbial autophagy regulated by DRAM1².

The IKK-related kinase TBK1, which phosphorylates p62, can be recruited to autophagosomes and drive their maturation into bactericidal organelles²⁰. TBK1 can be activated by TLR signaling via both the MYD88- and TRIF-dependent pathways, which can lead to inhibition of the canonical IKK complex (α , β , and γ)⁴¹. The canonical IKKs are involved in the initiation of autophagy³³, consistent with our finding that NF κ B regulated the expression of *DRAM1/dram1* in human macrophages and zebrafish embryos in response to infection. It is becoming clear that autophagy is regulated by both the canonical and non-canonical NF κ B activation pathway, as well as the stress-induced p53 pathway³³. Our discovery of DRAM1 as an autophagy modulator that functions downstream of these pathways will help understanding the complex signaling networks involved in autophagy initiation and flux in response to different stress factors.

The diverse roles of autophagy as effector and regulator of immune processes are receiving much attention⁴. Here, we showed that the TLR/IL1R-MYD88-NF κ B dependent expression of *dram1* is required for targeting of mycobacteria to autophagosomes. Zebrafish *dram1* expression was previously shown to be upregulated by *Salmonella* infection³⁵, and in the current study we showed it to be responsive to the common bacterial endotoxin LPS. Furthermore, autophagic defense against bacteria that invade the intestines was recently shown to be dependent on MYD88, but how MYD88 is linked with autophagosome induction remained unknown⁴². In light of these observations, we expect this novel TLR-MYD88-DRAM1 defense pathway to protect against a broader range of intracellular pathogens than mycobacterial species alone. The role of DRAM1 in immunity might even be broader than that. First, autophagy also functions as a regulator of inflammation by targeting inflammasomes for degradation, which limits the production of IL1 β ⁴³. Knockdown of zebrafish *dram1* increased *il1b* expression levels following mycobacterial infection, possibly linking DRAM1 to this regulatory immune function of autophagy as well. Second, particles that stimulate TLRs during phagocytosis by macrophages trigger the rapid recruitment of LC3 to the phagosome in an ATG5 and ATG7 dependent manner²³. Based on our findings that TLR-MYD88 signaling leads to *DRAM1* expression and LC3 aggregation, DRAM1 might also be involved in this concerted effort of the autophagy and phagocytosis pathways on control of infection.

The increasing occurrence of *Mtb* strains with resistance to multiple drug treatments makes TB a key priority for infectious disease research. Understanding of the host-pathogen interactions during *Mtb* pathogenesis is necessary to develop novel host-

directed therapeutic strategies that may complement antibiotic interventions^{44, 45}. The role of DRAM1 as an inducer of anti-mycobacterial autophagy makes this pathway a highly interesting target for a therapeutic strategy against TB disease, since we have shown *in vivo* that hyper-activation of the DRAM1-dependent autophagy pathway significantly lowered mycobacterial burden.

Experimental procedures

Zebrafish culture and lines

Zebrafish were handled in compliance with the local animal welfare regulations. Embryos were grown at 28.5°C and kept under anaesthesia during bacterial injections or imaging as described (van der Vaart et al., 2013). We used AB/TL (wild type strain), *myd88*^{hu3568}²⁵, *tp53*^{m214k}³², *Tg(CMV:GFP-Lc3)*²⁹, *Tg(mpx::egfp)*ⁱ¹¹⁴⁴⁶, *Tg(mpeg1:Gal4-VP16)*^{gl24}; *Tg(UAS-E1b:Kaede)*^{s1999t}⁴⁷, *Tg(lck::egfp)*^{cz2}⁴⁸, and *Tg(mpeg1::mCherry-F)*^{UMSF001} (Georges Lutfalla, kind gift) fish lines.

Injection conditions

Mycobacterium marinum *Mma20*, *Mma M*, or *Mma M* Δ RD1 labeled with mCherry or GFP¹⁵ were prepared for the infection of zebrafish embryos as described²⁵. Embryos were manually dechorionated at 24 hpf. 200 colony forming units (CFUs) were injected into the caudal vein after the onset of blood flow (28 hpf), or PBS was injected as a control. The same conditions were used for injection of 1 nl of LPS from *S. enterica* serovar *typhimurium* dissolved in PBS (10 μ g/ml, Sigma, #L6511). Splice morpholinos (supplementary table 1) and RNA was injected into the yolk sac at the one-cell stage as described²⁵. Morpholino knockdown of mRNA was tested using the SuperScript® One-Step RT-PCR System (Invitrogen, #10928-034; supplementary table 2 for primer sequences).

Gene expression analysis

RNA was isolated using TRI reagent (Life Technologies Europe BV, Bleijswijk, the Netherlands) and purified using the Rneasy MinElute Cleanup kit (QIAGEN Benelux B.V., Venlo, Netherlands). cDNA synthesis reactions and qPCR (supplementary table 2 for primer sequences) were performed as previously described²⁵, gene expression was normalized against the expression of housekeeping genes. Microarray and RNA deep sequencing analysis of the transcriptome was performed as previously described^{25, 49}. Gene Expression Omnibus (GEO) database accession for micro array and RNASeq: GSE49188. Macrophages, neutrophils, and lymphocytes were isolated by FACS from 5-6 dpf *Tg(mpeg1:Gal4-VP16)*^{gl24}; *Tg(UAS-E1b:Kaede)*^{s1999t}⁴⁷, *Tg(mpx::egfp)*ⁱ¹¹⁴⁴⁶, and *Tg(lck::egfp)*^{cz2}⁴⁸ zebrafish larvae, as described⁴⁹.

Drug treatments

The following drugs were used: 3-methyladenine (3-MA, 10mM, Sigma, #M9281); chloroquine (Cq, 100μM, Sigma, #C6628); Ar-12 (OSU-03012, 1 μM, Selleck Chemicals, #S1106); NFκB activation inhibitor (NAI, 50 nM in embryos, 30 nM in macrophages, Calbiochem, #481406); Roscovitin (50 μM, Sigma, #R7772). Drugs were administered via the water or culture medium for zebrafish embryos and human macrophages, respectively.

Microscopy and fluorescent pixel quantification

Zebrafish embryos were imaged using a Leica MZ16FA stereo fluorescence microscope with Leica DFC420C camera or a Leica TCS SPE confocal microscope. Maximal intensity projections of confocal micrograph Z-stacks are shown. Total fluorescent pixels per infected fish were determined on whole embryo stereo fluorescent micrographs using dedicated software⁴⁹.

Immunohistochemistry

Identification of neutrophils and macrophages was done by immunolabeling with a leukocyte specific L-plastin antibody and Alexa568-conjugated secondary antibody, combined with neutrophil specific staining for myeloperoxidase activity⁴⁹.

Infection of human macrophages

Type 1 and type 2 human macrophages were generated and maintained as previously reported⁵⁰. Mycobacterium tuberculosis was cultured in Difco Middlebrook 7H9 broth (Becton Dickinson) supplemented with 10% ADC (Becton Dickinson), 0.5% Tween-80 (Sigma-Aldrich). Primary human macrophages were infected at a MOI of 10. Wells containing the cell cultures were inoculated with 100 μl of the bacterial suspension, centrifuged for 3 minutes at 800 rpm and incubated at 37°C/5% CO₂ for 60 minutes. Plates were then washed with medium containing 30 mg/ml gentamicin sulfate (Lonza BioWhittaker, Basel, Switzerland) and incubated for 48 hours at 37°C/5% CO₂ in medium containing 5 mg/ml gentamicin and inhibitors, if appropriate.

Statistical analysis

All data (mean ± SEM) were analysed (Prism 5.0, GraphPad Software, San Diego, CA) using unpaired, two-tailed t-tests for comparisons between two groups and one-way ANOVA with Tukey's Multiple Comparison method as a post-hoc test for other data. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). For micro array, significant differences were calculated using Rosetta Resolver re-ratio analysis (* = fold change > 2, $p < 10^{-5}$). For RNA deep sequencing, significant differences were calculated by DESeq analysis of transcript count data (* = fold change > 2, $p < 0.05$).

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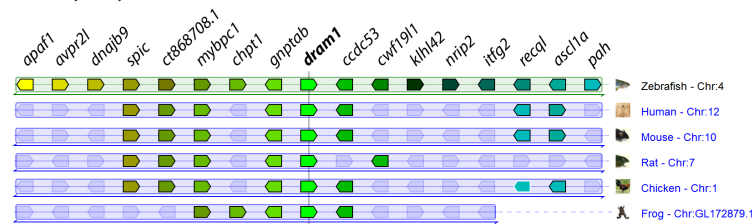
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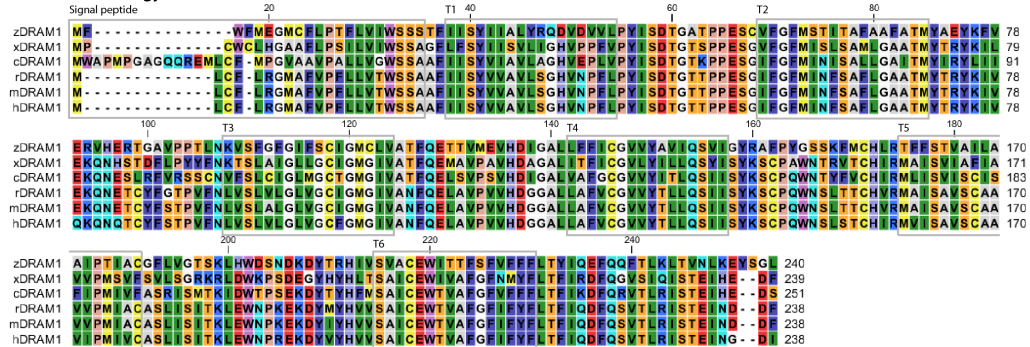
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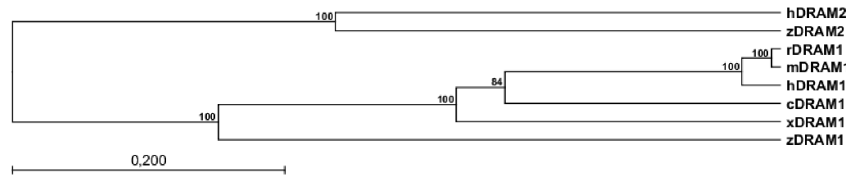
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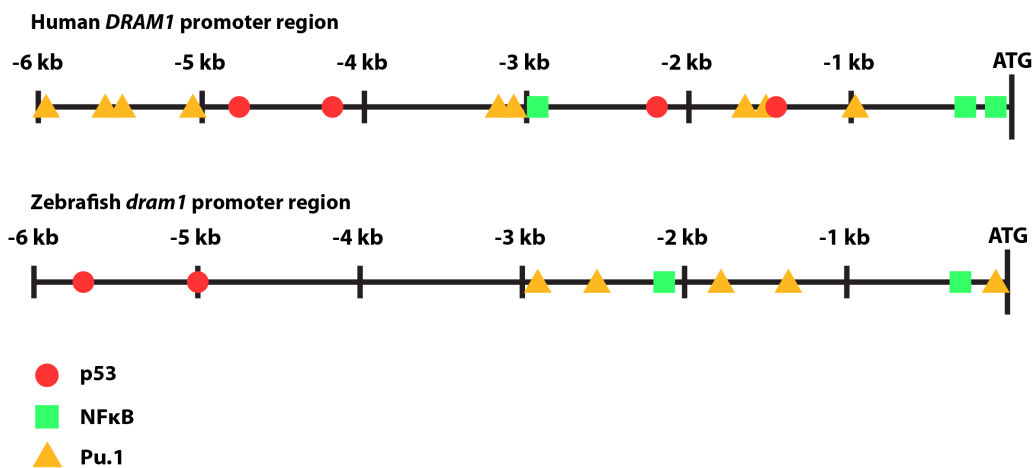
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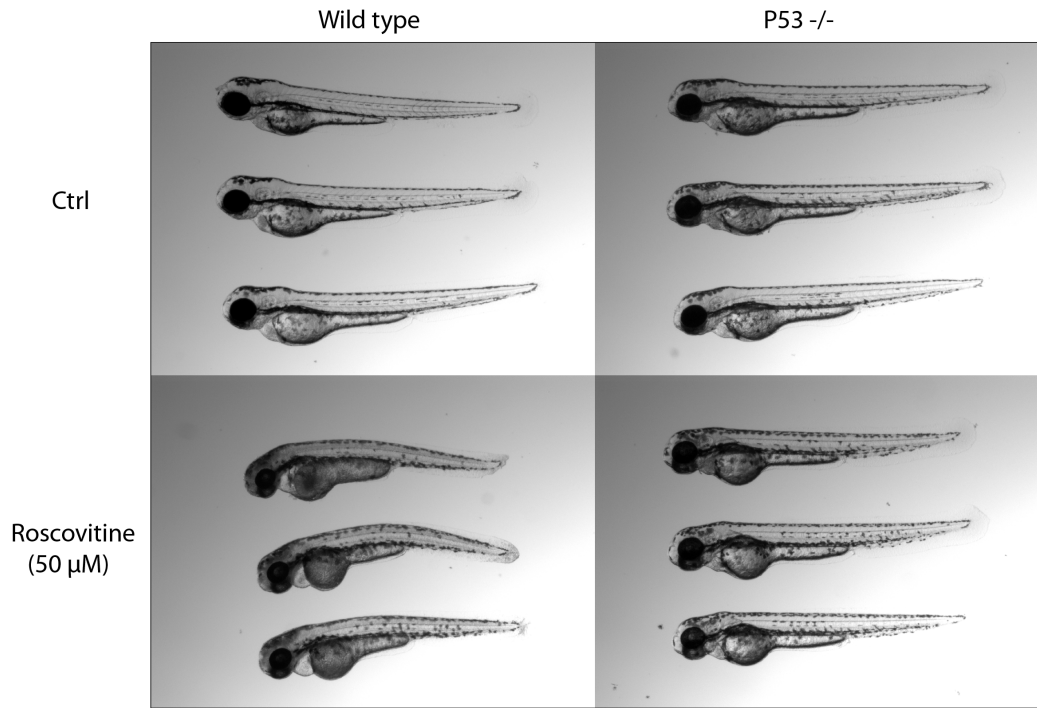
c Alignment tree



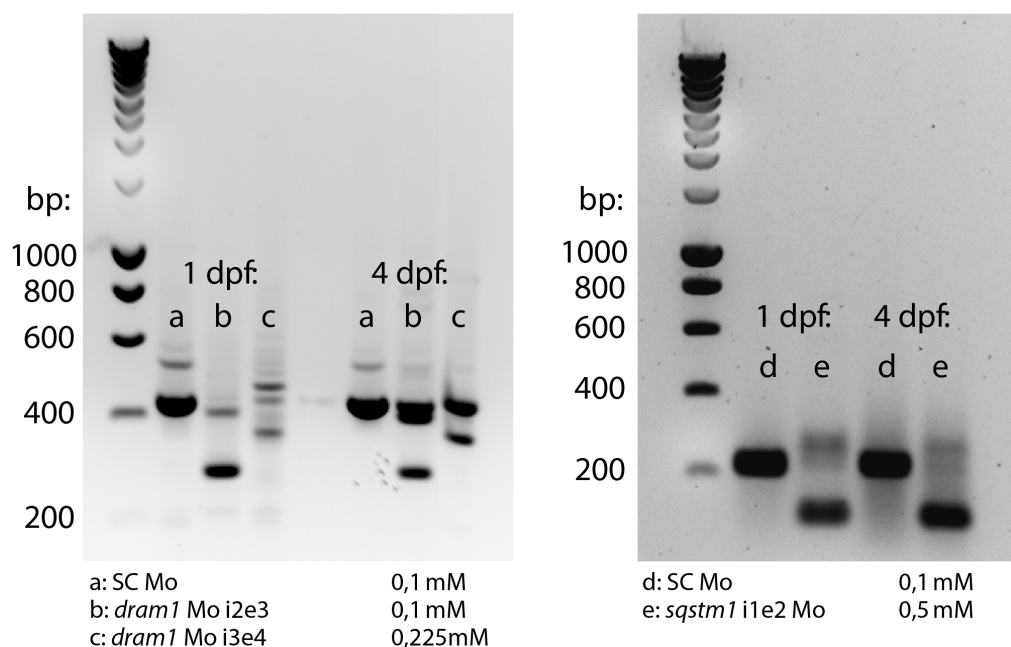
Supplementary figure 1. Conservation of vertebrate DRAM1. Genomic structure and sequence analysis of the zebrafish *dram1* gene. (A) Physical synteny of the region containing the *dram1* gene in zebrafish chromosome 4 (Chr:4) with human chromosome 12 (Chr:12), mouse chromosome 10 (Chr:10), rat chromosome 7 (Chr:7), chicken chromosome 1 (Chr:1) and frog scaffold GL172879.1. Surrounding *dram1* the genes SPIC, CT8868708.1, MYBPC1, GNPTAB, CCDC53, RECQL, ASCL1A are arranged in the same order on zebrafish Chr:4 as on human Chr:12, and appeared in the same position in the majority of the vertebrate species analyzed. Coding direction of the genes is indicated by the pointed end. Empty pale blue symbols indicate non-syntenic genes. (B) Multiple alignment of the vertebrate DRAM1 proteins. Identical residues identified are indicated with the same color. Grey boxes indicate the signal peptide and the transmembrane domains based on human DRAM1 (Crichton et al., 2006). (C) Unrooted phylogenetic tree of DRAM1 amino acid sequences. The tree was created by UPGMA method using ClustalW multiple alignment and bootstrapped 5000 times. The scale for the given branch length indicates 0.200 amino acid substitutions per site. Sequences were retrieved from the following accession numbers in public databases: zebrafish (zDRAM1): ENSDARG00000045561; xenopus (xDRAM1): ENSXETG00000027990; chicken (cDRAM1): ENSGALG00000012761; rat (rDRAM1): ENSRNOG00000038916; mouse (mDRAM1): ENSMUSG00000020057; human (hDRAM1): ENSG00000136048.



Supplementary figure 2. Transcription factor binding sites in the promoter region of *DRAM1*. We used Patch 1.0 (<http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi>) to predict the presence of p53, NFκB, and Pu.1 transcription factor binding sites in a 6 kb region upstream of the transcription start site of human and zebrafish *DRAM1*. Patch is a pattern-based program for predicting transcription factor binding sites in DNA sequences. It uses the set of binding sites from TRANSFAC® Public 6.0. Since the consensus binding sites for zebrafish transcription factors are unknown, we depict predicted vertebrate binding sites.



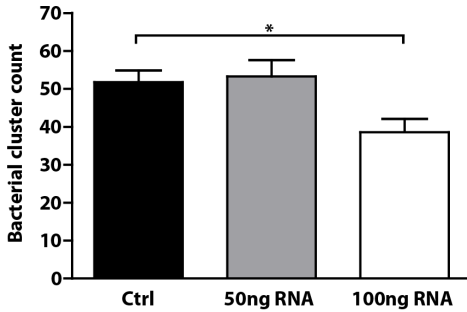
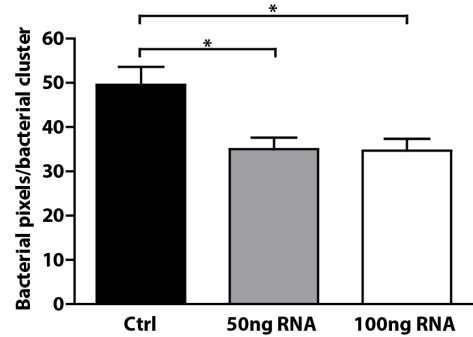
Supplementary figure 3. *p53*^{-/-} embryos are resistant to roscovitin-induced cell death. Representative images are shown for AB/TL (wild type) and *p53*^{-/-} embryos at 5 days post fertilization after 24 hours of treatment with 50 μ M roscovitin.



Supplementary figure 4. RT-PCR confirmation of splice-blocking morpholinos. Reverse transcription polymerase chain reaction (RT-PCR) was used to confirm antisense morpholino blocking of intron-exon splicing events in zebrafish *dram1* or *sqstm1* at 1 and 4 days post fertilization. (A) and (D) 0,1 mM Standard control (SC) morpholino; (B) 0,1 mM *dram1* Mo 1 (i2e3, targeting the splicing event between the second intron and the third exon); (C) 0,225 mM *dram1* Mo 2 (i3e4, targeting the splicing event between the third intron and the fourth exon); (E) 0,5 mM *sqstm1* Mo (i1e2, targeting the splicing event between the second intron and the third exon). For both *dram1* and *sqstm1*, the RT-PCR product (approximately 400 and 200 bp in size, respectively) is disrupted upon morpholino injection. Morpholino sequences and primers used for the RT-PCR are listed in supplementary table 1.

Supplementary table 1: Morpholino sequences

Gene	Name	Location	Sequence
<i>dram1</i>	Dram1 Mo1	Intron 2 > exon 3	AAGGCTGGAAAACAAACGTACAGTA
<i>dram1</i>	Dram1 Mo2	Intron 3 > exon 4	GTCGTCTCTGTAAACAAAACATGCA
<i>sqstm1</i>	Sqstm Mo1	Intron 1 > exon 2	CTTCATCTAGAGACAAAGTTCAGGA
<i>myd88</i>	MyD88 Mo	Exon 2 > intron 2	GTAAACACTGACCCTGTGGATCAT

a Number of bacterial clusters per embryo**b** Average bacterial cluster size

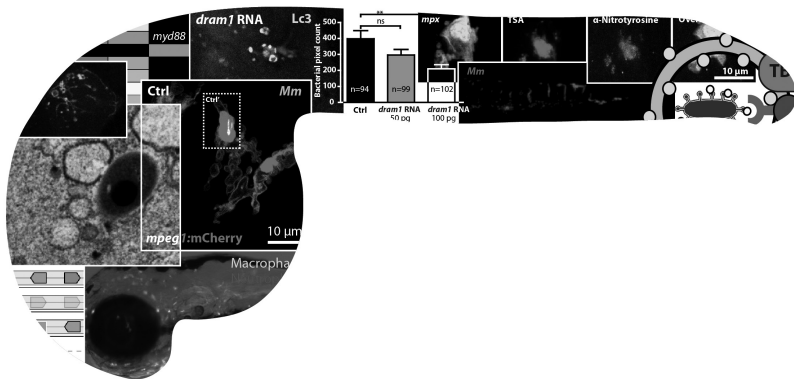
Supplementary figure 6. *dram1* overexpression effect on the formation of granuloma-like aggregates. Wild type embryos were mock-injected or injected with 50 or 100 pg *dram1* RNA immediately after fertilization. Embryos were infected with 200 CFU *Mm* at 1 dpf. Whole embryo stereo fluorescent micrographs were taken at 3 dpi and (A) the total number of bacterial clusters and (B) the average bacterial cluster size were determined using dedicated software (Stoop et al., 2010). Data is pooled from two individual experiments (n>94 embryos per group). Significant differences were calculated by one-way ANOVA with Tukey's Multiple Comparison method as a post-hoc test (*= $p < 0.05$). Significant reduction of both the total number of clusters and cluster size by *dram1* overexpression is in agreement with the reduction of total bacterial pixel counts shown in figure 6D.

Supplementary table 2: PCR primers

Gene	Type	Species	Accession #	Forward qPCR primer	Reverse qPCR primer
<i>dram1</i>	qPCR	ZF	NM_001006049.1	ATGGTGATGAGCCGATTAGC	TGCTCGATGACCAGATAACC
<i>ppial</i>	qPCR	ZF	AY391451	ACACTGAAACACGGAGGCAAAG	CATCCACAACCTTCCCGAACAC
<i>il1b</i>	qPCR	ZF	NM_212844	GAACAGAATGAAGCACATCAAACC	ACGGCACTGAATCCACCAC
<i>mmp9</i>	qPCR	ZF	NM_213123	CATTAAAGATGCCCTGATGTATCCC	AGTGGTGGTCCGTGGTTGAG
<i>saa</i>	qPCR	ZF	NM_001005599.1	CGCAGAGGCAATTCAGAT	CAGGCCTTTAAGTCTGTATTGTTG
<i>dram1</i>	RT	ZF	NM_001006049.1	GGATTATGTCGACAATTACGGC	CCGACAAGAAACCCACATGC
<i>sqstm1</i>	RT	ZF	NM_213173.1	ATTGTCAGCGAAAAGTGCTC	AGTGAACGGAACCCAGGAA
<i>dram1</i>	Cloning	ZF	NM_001006049.1	TCCGCCAGTCTCTTCACTTT	AACTGCACCAAAACAGTGG
<i>DRAM1</i>	qPCR	Human	NM_018370.2	TGCTGTGCTTCACTAATTTCCA	TCACAGATCGCACTCACTACG
<i>SQSTM1</i>	qPCR	Human	NM_003900.4	TCCGCCGGCACTCTTTTTT	TCCAGTGACGAGGAATTGACAATGG
<i>IL1B</i>	qPCR	Human	M15840.1	CCACGCCACATTTGGTT	AGGGAAGCGGTTGCTCATC
<i>GAPDH</i>	qPCR	Human	NM_002046.4	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA

Chapter 6

Summary and discussion



Humans and other animals are constantly exposed to attacks by various potential pathogenic microorganisms. The discovery of Toll-like receptors (TLRs) as gatekeepers of the immune response triggered an explosion of research in the field of innate immunity and was later awarded with a Nobel Prize in medicine^{1,2}. TLRs are an important class of pattern recognition receptors (PRRs) that detect the presence of pathogenic microorganisms via evolutionary conserved pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Pathogen recognition by the innate immune system activates anti-microbial defense mechanisms, such as production of defense proteins, and reactive oxygen and nitrogen species (ROS and RNS), but is also indispensable for activation of adaptive immunity³. The discovery of TLRs paved the way for identification of other families of PRRs: NOD-like receptors for cytoplasmatic detection of PAMPs and DAMPs; RIG-I-like receptors for detection of viruses; C-type lectin receptors that recognize and take up carbohydrates and other macromolecules; and sequestosome-like receptors (SLRs) that recognize ubiquitinated microbes and microbial proteins (**chapter 2**). The subsequent discovery of the inflammasome demonstrated how cytokines are processed into excreted pro-inflammatory signals upon pathogen recognition by PRRs⁴. The most recent paradigm-shift in immunity came from the discovery that autophagy functions as an anti-microbial defense mechanism, alongside its function in cellular homeostasis⁵. With its extensive opportunities for genetic manipulation and the unparalleled *in vivo* imaging possibilities during embryonic and larval stages, the zebrafish has become an important model organism for the study of innate immunity and infectious diseases⁶.

Zebrafish embryos with defective TLR/IL1R-MyD88 signaling are more susceptible to bacterial infection

6 Myeloid differentiation factor 88 (MYD88) is an essential signaling adaptor used for downstream signaling by all TLRs, except for TLR3⁷. MYD88 also functions downstream of the interleukin 1 receptor (IL1R) family, and has been associated with IFN- γ receptor signal transduction, further establishing it as a signaling adaptor with a central role in inflammation and innate immunity⁸⁻¹⁰. In humans, MYD88-deficiency causes a primary immunodeficiency syndrome characterized by an increased susceptibility to pyogenic bacteria, including *Salmonella* species¹¹. Mice deficient in MyD88 were unresponsive to the common bacterial endotoxin LPS and interleukin 1 (IL1) stimulation, and were more susceptible to infection by several bacterial pathogens, including *Mycobacterium tuberculosis* (*Mtb*)^{7,12}.

The major classes of innate immune receptors, their downstream signaling pathways, and innate effector mechanisms are conserved between human and zebrafish (**chapter 2**). We used well-established zebrafish embryo infection models to further examine the role of MyD88 in innate immune defense against bacterial pathogens⁶. We found that zebrafish embryos mutated in *myd88* (*myd88*^{-/-}) were more susceptible to infection by the acute bacterial pathogens *Salmonella enterica* serovar Typhimurium and *Edwardsiella tarda*, as well as the chronic bacterial pathogen *Mycobacterium marinum*

(*Mm*) (**chapter 3**), similar to the effect of MYD88 deficiency in humans and in the mice model. The increased infection susceptibility of *myd88*^{-/-} embryos cannot be explained by a defect in a single innate immune effector mechanism, since microarray analysis revealed that infection induced gene expression downstream of MyD88 is involved in a plethora of defense mechanisms (**chapter 3**). This included the expression of genes encoding transcription factors central to innate immunity, such as NFκB and AP-1; genes encoding pro-inflammatory cytokines such as Il1b and Tnfa; genes for proteins involved in the respiratory burst; and genes for matrix metalloproteinases, such as Mmp9, which play a role in various aspects of immunity and inflammation. Based on the work presented in this thesis, we conclude that MyD88-deficiency during bacterial infection affects several innate defense mechanisms, including production of cytokines, ROS and RNS, and initiation of autophagic defense (figure 1).

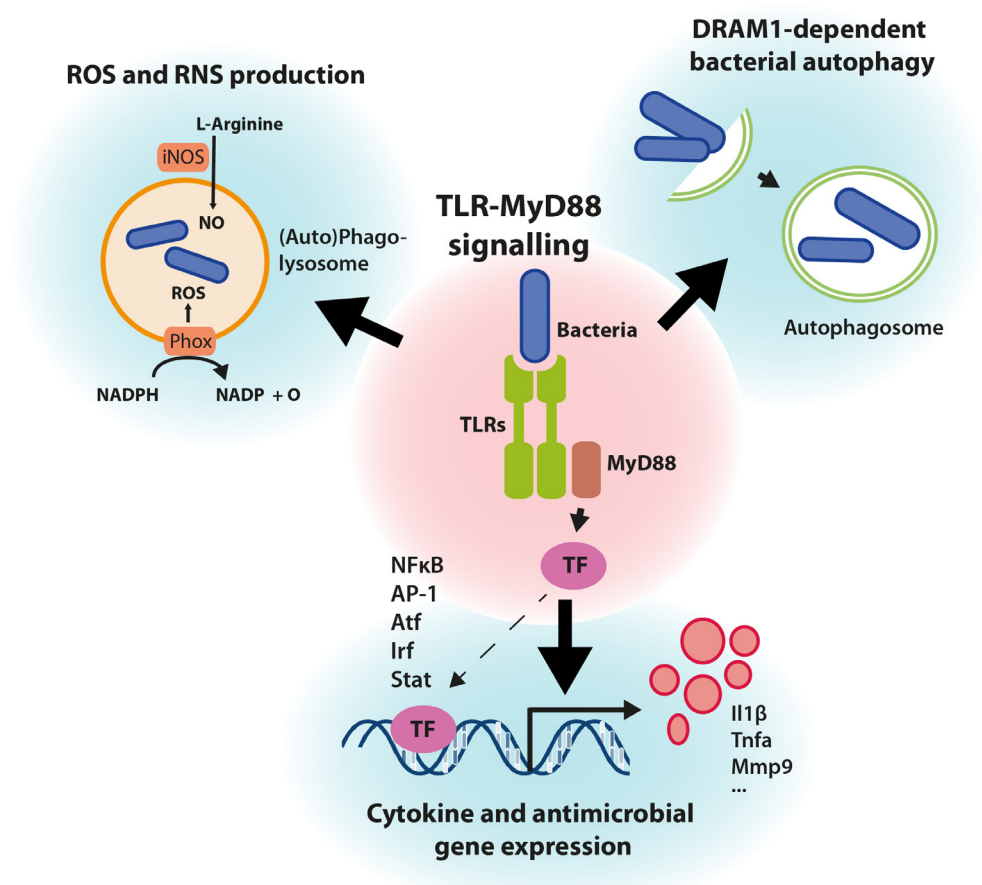


Figure 1: TLR-MyD88 signaling is involved in several innate immune defense mechanisms. These mechanisms include the activation of pro-inflammatory transcription factors and cytokines (chapter 3); the production of ROS and RNS to directly kill microorganisms or to modulate the immune response (chapter 4); and the DRAM1 regulated initiation of autophagic defense (chapter 5).

Mycobacteria counteract a nitrosative host defense mechanism initiated upon TLR/IL1R-MyD88 signaling

The production of ROS by the NADPH-oxidase complex (or Phox, for phagocytic oxidase) during the respiratory burst plays an important role in innate immune processes¹³. Nitric oxide (NO) is produced by inducible nitric oxide synthase (iNOS) in response to infection, and RNS resulting from the reaction between O₂ and NO radicals are important in the inflammatory response of innate immune cells¹⁴. Although ROS and RNS production are potent defense mechanisms against intracellular pathogens^{13, 14}, their role in defense against mycobacterial infection remains controversial. Mutations in components of the NADPH-complex increased susceptibility to *Mtb*¹⁵⁻¹⁷, but ROS production itself was relatively ineffective in killing *Mtb*¹⁸. A recent study using the zebrafish model for mycobacterial infection investigated the effects of TNF-induced ROS production under conditions where TNF was produced in excess due to overexpression of an enzyme involved in the production of proinflammatory leukotrienes (LTA4H). ROS production due to excess TNF increased macrophage microbicidal activity initially, but, subsequently caused necrosis releasing mycobacteria into the extracellular environment¹⁹. This study also suggested that *Mm* can counter the effects of ROS production when TNF levels are normal. We did not study the effect of ROS production on mycobacterial infection directly, but found *nox1*, *noxo1a*, and *cyba*, components of the NADPH-oxidase complex, to be upregulated during *Mm* infection in a MyD88-dependent manner (**chapter 3**).

NO was shown to be essential for the killing of *Mtb* by phagocytes in the mouse model and *Mtb* susceptibility was increased in iNOS deficient mice^{16, 20-23}. However, the role of NO during *Mtb* infection in humans is debated, since mycobacteriostatic activity of human macrophages functioned independent of NO^{24, 25}. Nevertheless, evidence for a protective role for NO during mycobacterial infection in humans is accumulating and polymorphisms in the gene encoding iNOS have been associated with susceptibility to tuberculosis^{23 26-31}.

6

As described in **chapter 4**, we found that mycobacterial infection of zebrafish embryos led to an increase of nitrosative stress in leukocytes which was dependent on functional IL1R/TLR-Myd88 signaling. This host nitrosative defense mechanism was attenuated during the course of infection by live mycobacteria. The bacterial virulence mechanism used for adaptation to NO produced in host leukocytes functioned independent of the well-characterized mycobacterial RD1 virulence locus³². Interestingly, increasing RNS production in leukocytes via genetic upregulation of the hypoxia pathway prior to infection decreased mycobacterial burdens³³. Together, this suggested that mycobacteria were more susceptible to host nitrosative defenses before they could sense and adapt to the high levels of RNS present in primed phagocytes. We therefore hypothesize that mycobacteria use oxidative stress sensors, such as OxyR, to upregulate bacterial proteins that can scavenge or protect against the detrimental effects of RNS^{34, 35}. Elevated oxidative stress caused by ROS production in phagocytes might induce a bacterial response analogous to the attenuation of nitrosative defense mechanisms, since increasing ROS production via genetically upregulated levels of TNF prior to infection was required for increased anti-mycobacterial activity of macrophages¹⁹.

Autophagy regulator DRAM1 functions downstream of MYD88 in defense against tuberculosis

Autophagy, the process well-known for its role during starvation and cellular stress, has emerged as a defense mechanism against intracellular pathogens³⁶. Studies on the autophagic control of *Mtb* infections have demonstrated how the host deals with pathogens that otherwise circumvent host defenses³⁷. In fact, selective autophagy receptors such as p62/sequestosome 1 (SQSTM1) and NDP52 are collectively referred to as a novel group of PRRs, called sequestosome like receptors (SLRs)⁵. SLRs recognize ubiquitinated targets for autophagy, in this case mycobacteria, and connect them to a forming autophagic isolation membrane interacting directly with the autophagosome marker LC3³⁸. The resulting autophagosome eventually fuses with a lysosome to degrade its pathogenic contents. Furthermore, the same SLRs can target ubiquitinated proteins to the autophagosome, which endows it with unique microbicidal properties³⁹. Several intracellular pathogens have evolved mechanism to evade autophagy, notably *Listeria* and *Shigella* species that escape into the cytosol to spread from cell to cell by actin-based motility^{40, 41}. Some bacteria, for example *Staphylococcus aureus*, exploit the autophagosome to their advantage to facilitate intracellular survival⁴². Most likely, mycobacteria can also evade or exploit autophagy to a certain extent, but several studies show that autophagy is an effective defense mechanism against these pathogens^{39, 43-49}.

The immunological function of autophagy is underscored by the fact that pathogen detection by TLRs can induce the formation of autophagosomes or Lc3-associated phagosomes⁵⁰. However, the signaling events that connect TLR recognition with autophagic defense against mycobacterial infection remained unclear. One link between TLRs and autophagy is that the TLR adaptors MYD88 and TRIF were shown to directly trigger autophagy via interaction with the autophagy modulator Atg6/Beclin⁵¹. In **chapter 5**, we have discovered a new connection between pattern recognition and autophagy. We have used zebrafish embryos and human primary macrophages to demonstrate that anti-mycobacterial autophagy is mediated by DNA-damage regulator of autophagy 1 (DRAM1) in an IL1R/MYD88-NFκB dependent fashion (figure 2). Previously, the only known function of DRAM1 was mediating autophagy-dependent cell death in response to cellular stress such as DNA-damage, cancer, or HIV infection⁵²⁻⁵⁴. As a regulator of cell death and survival, DRAM1 expression was induced by the tumor suppressor protein p53. By imaging of autophagy in GFP-Lc3 transgenic zebrafish, we found that autophagosomes targeting to mycobacteria requires the selective autophagy receptor p62 (**chapter 5**; figure 3A and B). This dependency on p62 is similar to a previous observation concerning autophagy induction by DRAM1 in glioblastoma stem cells⁵⁵. The requirement of p62 in autophagic defense against tuberculosis fits perfectly with recent literature on the initiation and maturation of *Mtb* containing phagosomes^{48, 49}. When mycobacteria permeabilize the bacteria containing phagosome, their extracellular DNA is sensed by the STING-pathway, which ultimately leads to ubiquitination of the bacteria and autophagosome formation via p62⁴⁸ (figure 3C). The non-canonical IKK family member TBK1 then phosphorylates p62 and regulates the maturation of *Mtb*

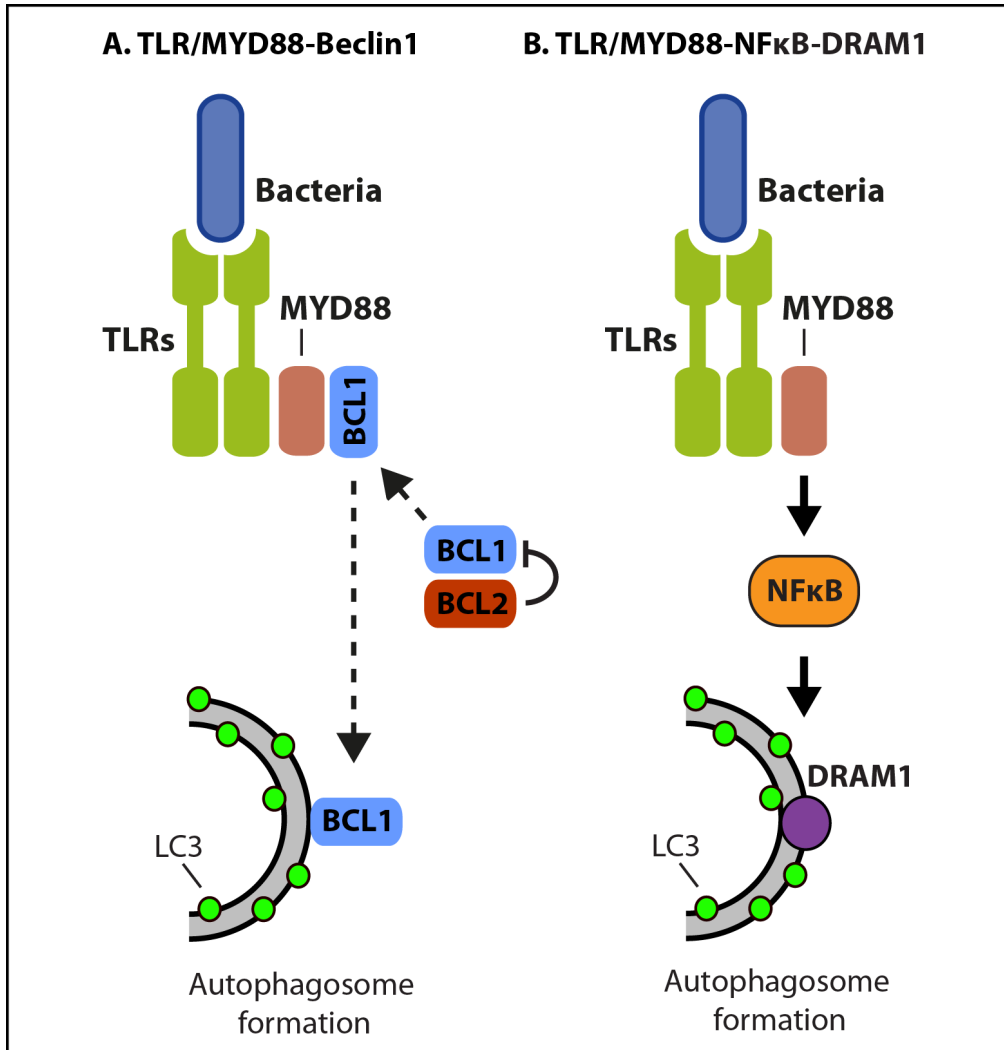


Figure 2: MyD88-dependent routes to autophagy induction. (A) MYD88 can interact directly with autophagy inducer Beclin1 (BCL1), removing it from an inhibitory complex also containing Beclin2 (BCL2). A similar function has been reported for another TLR adaptor protein, TRIF (Shi and Kehrl, 2008). (B) We now found that anti-mycobacterial autophagy is initiated when TLR-MYD88-NFκB signaling increases expression of the autophagy regulator DRAM1 (chapter 5).

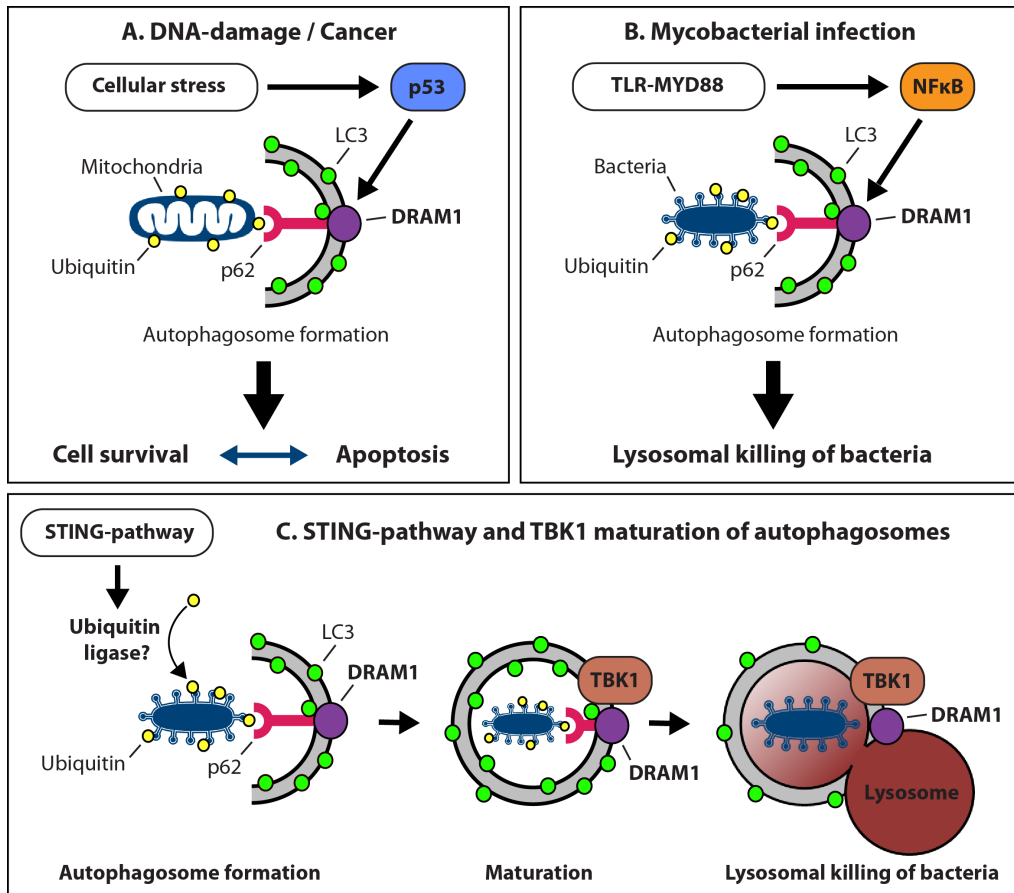


Figure 3: Autophagy initiation by DRAM1. (A) In the context of cellular stresses like DNA-damage or cancer, DRAM1 induces autophagy downstream of p53 activation (Crighton et al., 2006; Galavotti et al., 2013); (B) we now place p62-dependent induction of autophagy by DRAM1 downstream of NFκB signaling for defense against tuberculosis (chapter 5). (C) The STING DNA sensing pathway recognizes membrane permeabilization of the *Mtb* containing phagosome by detecting extracellular bacterial DNA (Watson et al., 2012). We propose that subsequent autophagosome formation via p62 requires DRAM1. TBK1 then phosphorylates p62, which leads to autophagosome maturation and lysosomal destruction of its contents (Pilli et al., 2012).

containing autophagosomes⁴⁹. We have shown that hyper-activation of this DRAM1-dependent autophagy pathway significantly lowered mycobacterial burden *in vivo*, while knockdown of this pathway increased infection levels and resulted in extracellular mycobacterial growth (**chapter 5**).

Conclusion

Tuberculosis remains a worldwide health problem and the increasing occurrence of multiple drug resistant *Mtb* strains calls for novel therapeutic strategies which can complement current antibiotic therapies⁵⁶⁻⁵⁸. A broad understanding of innate immunity in general, and especially those processes providing protection against tuberculosis disease, is required for the development of host-targeted approaches for the treatment of tuberculosis. The *myd88*^{-/-} mutant zebrafish line described in **chapter 3** proved highly useful for the study of innate immune mechanisms underlying tuberculosis (**chapters 4 and 5**). In view of the central role of MYD88 in the innate immune system, we expect that this *myd88*^{-/-} mutant zebrafish line will also facilitate the study of other infectious diseases, inflammatory disorders, and cancer. The work presented in this thesis has added to the understanding of the host-pathogen interactions that occur during mycobacterial infections and may also contribute to the development of novel therapeutic strategies. The role of DRAM1 as an inducer of anti-mycobacterial autophagy makes this pathway a highly interesting target for host-directed therapeutic strategies against human tuberculosis disease (**chapter 5**), and therapeutically deactivating the mycobacterial oxidative stress response might sensitize *Mtb* to phagocytic killing (**chapter 4**).

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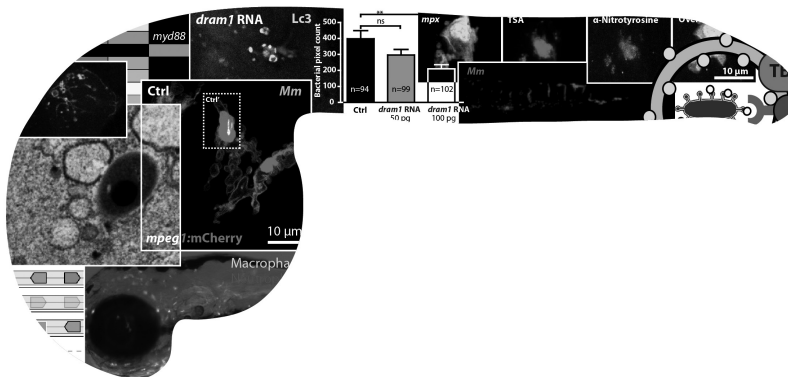
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Chapter 7

Dutch summary (Nederlandstalige samenvatting)



Verdedigingsmechanismen van het aangeboren immuunsysteem tegen intracellulaire pathogenen

De mens wordt continu aangevallen door pathogenen, micro-organismen die potentieel ziekten kunnen veroorzaken. Bacteriën, schimmels, parasieten en virussen kunnen het lichaam van de gastheer binnendringen en daar een infectie starten. Ter verdediging tegen deze infecties heeft de mens de beschikking over twee afweersystemen: (1) het aangeboren immuunsysteem en (2) het adaptieve immuunsysteem. Het aangeboren immuunsysteem bestaat uit cellen en eiwitten die samen de eerste verdedigingslinie tegen binnendringers vormen. Hun functie is om zo snel mogelijk micro-organismen te elimineren die een fysieke barrière, zoals de huid, hebben doorbroken. Het adaptieve (of selectieve) immuunsysteem wordt geactiveerd wanneer de reactie van het aangeboren immuunsysteem alleen niet afdoende is om de infectie de kop in te drukken. Het adaptieve immuunsysteem produceert antilichamen die aan ziekteverwekkers kunnen binden en ze neutraliseren. Daarnaast worden zogenaamde T-cellen geactiveerd die pathogenen direct herkennen en uitschakelen. Het aangeboren immuunsysteem is al vroeg in de evolutie ontstaan en is aanwezig in zowel ongewervelde als gewervelde dieren. Het adaptieve immuunsysteem is later geëvolueerd en is alleen in de mens en andere gewervelde dieren aanwezig.

Dit proefschrift beschrijft de studie naar de werking van het aangeboren immuunsysteem in reactie op bacteriële infectie. Hiervoor hebben we gebruik gemaakt van het zebravisembryo als modelorganisme. In **Hoofdstuk 2** worden de receptoren besproken die het aangeboren immuunsysteem gebruikt om pathogenen te herkennen. Daarnaast worden de verschillende verdedigingsmechanismen beschreven die na herkenning van een microbiële infectie worden aangezet. Tenslotte blijkt in **Hoofdstuk 2** dat het immuunsysteem van de mens en de zebravis op cruciale punten overeen komt.

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De best bestudeerde pathogeen-herkende receptoren zijn de Toll-achtige receptoren. De ontdekking dat deze receptoren verantwoordelijk zijn voor de activatie van het aangeboren en adaptieve immuunsysteem is recent bekroond met een Nobelprijs. In **Hoofdstuk 3** van dit proefschrift wordt een zebravismutant beschreven in een gen dat codeert voor een eiwit dat cruciaal is voor het doorgeven van signalen van Toll-achtige receptoren. Zonder dit eiwit, MyD88, komen de juiste genen niet tot expressie en blijft een groot gedeelte van de immunoreactie achterwege. Het was dan ook niet verwonderlijk dat zebravismutanten in het *myd88*-gen veel vatbaarder waren voor infecties veroorzaakt door de pathogene bacteriën *Salmonella typhimurium*, *Edwardsiella tarda* en *Mycobacterium marinum*. De mutant die in dit hoofdstuk beschreven wordt is een belangrijk hulpmiddel in het onderzoek naar de immunoreactie, zowel tijdens infecties als bij auto-immun- en ontstekingsziektes, maar ook bij het ontstaan van tumoren.

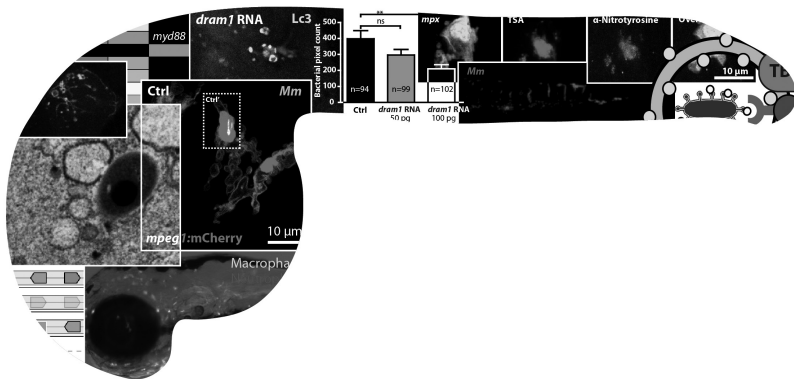
Mycobacterium marinum is een nauwe verwant van de menselijke pathogeen *Mycobacterium tuberculosis*, de veroorzaker van tuberculose. Tuberculose is een zeer gevaarlijke infectieziekte voor de mens, waar jaarlijks meer dan een miljoen mensen wereldwijd aan overlijden. Vooral de opkomst van bacteriestammen die resistent zijn tegen veelgebruikte antibiotica is zorgelijk. Meer onderzoek naar de interacties tussen gastheer en pathogeen is daarom noodzakelijk voor de ontwikkeling van nieuwe of complementaire geneesmiddelen.

Zowel *M. marinum* als *M. tuberculosis* zijn in staat om te overleven in immuuncellen van hun gastheren. Een potent verdedigingsmechanisme van immuuncellen tegen pathogene bacteriën is de productie van zuurstofradikalen en stikstofradikalen. In **Hoofdstuk 4** hebben we onderzoek gedaan naar het effect van productie van stikstofradikalen tijdens mycobacteriële infectie in de zebravis. Het blijkt dat MyD88 noodzakelijk is voor het activeren van dit afweersysteem. Helaas hebben *M. marinum*-bacteriën echter een methode ontwikkeld om deze productie vervolgens te remmen, waardoor ze de schadelijke effecten hiervan ontlopen. Opmerkelijk genoeg waren zebravisembryo's waarvan de stikstofradicalenproductie voorafgaand aan de infectie artificieel verhoogd was vervolgens beter beschermd tegen een mycobacteriële infectie.

In **hoofdstuk 5** hebben we de rol van een ander verdedigingsmechanisme tegen tuberculose onderzocht: autofagie, oftewel cellen die zichzelf opeten. Jarenlang werd dit proces gezien als hét huishoudelijke mechanisme van de cel, waarbij defecte organellen en andere overbodige dingen werden opgegeten en afgebroken. Inmiddels is bekend dat autofagie ook een belangrijke rol speelt bij het opruimen van pathogenen die binnen in een cel kunnen overleven. In **Hoofdstuk 5** hebben we aangetoond dat anti-mycobacteriële autofagie in zowel zebravisembryo's als in menselijke immuuncellen (macrofagen) afhankelijk is van Toll-achtige receptoren en MyD88. We hebben ontdekt dat een eiwit genaamd DRAM1 door MyD88 wordt geactiveerd en het autofagieproces controleert. Zodra we de hoeveelheid DRAM1-eiwit in zebravisembryo's verhoogden, waren ze significant beter in staat om zich te verdedigen tegen tuberculose. De resultaten gepresenteerd in **Hoofdstuk 4** en **Hoofdstuk 5** laten zien hoe een beter begrip van het ontstaan van ziektes hopelijk ooit kan leiden tot de ontwikkeling van nieuwe geneeskundige therapieën. In **Hoofdstuk 6** worden de bevinding uit dit proefschrift in het perspectief van bestaande kennis over het aangeboren immuunsysteem geplaatst.

Chapter 8

Curriculum Vitae

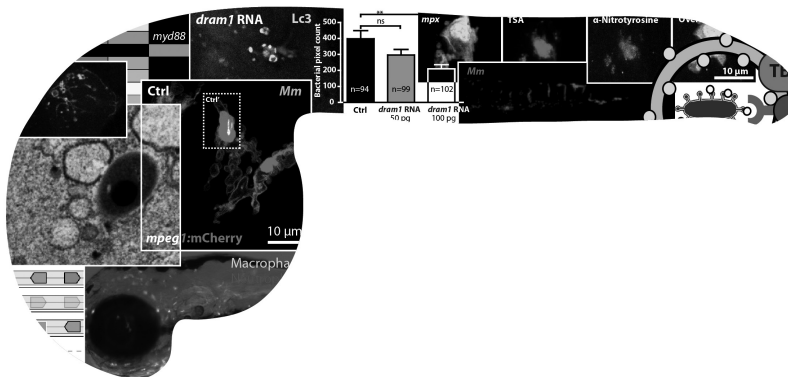


Curriculum Vitae

Michiël van der Vaart was born on April 23rd, 1985 in Maasland, The Netherlands. He also grew up there and finished his high school at the Christelijk Lyceum Delft. In 2003 he started studying for his BSc in Biology at Leiden University, during which he performed an internship at the Leiden University Medical Center under supervision of Dr. Louise Veltrop-Duits and Dr. Marco Schilham. Directly after obtaining his BSc degree in 2006, he started working on obtaining his MSc degree in Biology at the same university, with specialisations in Molecular and Cellular Biosciences and Science Based Business. During his MSc studies, he completed two internships, one at the Leiden University at the department of Molecular Cell Biology (under supervision of Dr. Oliver Stockhammer and Dr. Annemarie Meijer), and one at a company called Groen Agro Control (under supervision of Dr. Adriaan Vermunt and Dr. Bert van Tol). He obtained his MSc degree (cum laude) early in 2009, and shortly after he started as a PhD-student in the infectious disease group of Dr. Annemarie Meijer at the Molecular Cell Biology department headed by Prof. Dr. Herman Spaink. During his PhD-training he studied the innate immune response against intracellular bacterial pathogens, using the zebrafish embryo as a model organism, which resulted in the work before you. After publishing chapters 2 and 3 during his PhD-studies, he and his co-authors are currently working on publishing chapters 4, 5, and 6 of this thesis. In 2014 he will start his postdoctoral research in the laboratory of Prof. Dr. David Traver at the University of California, San Diego.

Chapter 9

Publication list



Publication list

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