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

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SUMMARY
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 signaling. The zebrafish is increasingly used as a complementary model system for disease research and drug screening. Here, we describe a zebrafish 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 analysis revealed that expression of genes for transcription factors central to innate immunity (including NF-kB and AP-1) and the pro-inflammatory cytokine Il1b, is dependent on MyD88 signaling 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 for further study of the role of IL1R and TLR signaling in the 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 response (Medzhitov and Janeway, 2000). 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 (Medzhitov and Janeway, 2000; Matzinger, 2002). Myeloid differentiation factor 88 (MYD88) is an important adaptor protein in the TLR signaling pathway because it is used by all TLRs except for TLR3 (Takeda and Akira, 2004). Its C-terminal TIR domain enables interaction with TLRs, and 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’ signaling complex, activating NF-kB (nuclear factor kB) and MAPK (mitogen-activated protein kinase) signaling (Muzio et al., 1997; Wesche et al., 1997; Burns et al., 1998; Lin et al., 2010; Gay et al., 2011). 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-Y receptor signaling, adding to its central role in inflammation and host defense (Adachi et al., 1998; Sun and Ding, 2006; Lin et al., 2010).

Patients with a deficiency in MYD88 suffer from a primary immunodeficiency syndrome (von Bernuth et al., 2008; Picard et al., 2010). This syndrome is characterized by an increased susceptibility to pyogenic bacteria like Streptococcus pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa and Salmonella species (Netea et al., 2012). Mice deficient in MyD88 were shown to be hyporesponsive to lipopolysaccharide (LPS) and IL1 stimulation and resistant to endotoxic shock (Adachi et al., 1998; Kawai et al., 1999; Akira and Takeda, 2004). As a consequence of their reduced ability to recognize PAMPs, MyD88-deficient mice are more susceptible to infection by Toxoplasma gondii parasites (Scanga et al., 2002) and several bacterial pathogens, including S. aureus (Takeuchi et al., 2000), Listeria monocytogenes (Edelson and Unanue, 2002), Chlamydia pneumoniae (Naiki et al., 2005) and Mycobacterium tuberculosis (Ryffel et al., 2005).

The zebrafish (Danio rerio) is an excellent model for studying the innate immune system that can complement research in mouse models and human cell lines (Meijer and Spaink, 2011). 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 1 day post fertilization (dpf), zebrafish embryos are capable of mounting an effective innate immune response against microbial infections (Herbomel et al., 1999). This immune response has a transcriptional signature similar to responses observed in mammalian and cell culture systems (Stockhammer et al., 2009).
**RESOURCE IMPACT**

**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 diseases associated with immune dysfunction is growing rapidly, owing to the many advantages of this model. These include the potential for intravital imaging, genetic screening and high-throughput drug discovery. Therefore, zebrafish mutants for key components of the immune system are eagerly awaited.

**Results**

In this study, the authors characterized a zebrafish mutant with a premature stop-codon in the myd88 gene. This is the first zebrafish mutant for a TLR-IL1R signaling component. The mutant line was 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 analysis of gene expression revealed that expression of transcription factors central to innate immunity (such as NfκB and AP-1) and pro-inflammatory genes (such as 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 the growth of an attenuated *S. typhimurium* strain.

**Implications and future directions**

The zebrafish *myd88* mutant is a new and valuable addition to mammalian knockout models, especially when combined with transgenic lines 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 provide the possibility to study functions of the innate immune system with minimal interference of adaptive immunity (at embryo and larval stages). In this study, this potential was exploited using a zebrafish model for tuberculosis. The authors demonstrated that MyD88 has a protective role during early mycobacterial pathogenesis in zebrafish larvae, i.e. when only innate immunity is functional. The zebrafish *myd88* mutant model will be a valuable tool for studying innate immune responses relevant to other human infectious diseases, as well as complex disorders involving immune dysregulation.

Furthermore, the major PRRs, their downstream signaling pathways and innate effector mechanisms are conserved between humans and zebrafish (van der Vaart et al., 2012). Expression of *myd88* in zebrafish leukocytes was confirmed using a transgenic reporter line (Hall et al., 2009). Maturation of the adaptive immune system is not complete until approximately three weeks post fertilization (Lam et al., 2004), 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 rapidly (Mione et al., 2009). Therefore, zebrafish mutants for important components of the immune system are eagerly awaited. As an alternative to mutant lines, morpholino antisense oligonucleotides can be used for blocking of protein production, but knockdown by morpholinos is only temporary and might also cause off-target effects (Bedell et al., 2011). Knockdown of MyD88 by morpholino in zebrafish embryos affected their ability to combat infection with the attenuated *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) Ra strain (van der Sar et al., 2006). 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 (Stockhammer et al., 2009; Liu et al., 2010). 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 larvae (Bates et al., 2007; Cheesman et al., 2011; Oehlerls et al., 2011).

Here, we characterize the first *myd88* mutant zebrafish line (*myd88<sup>−/−</sup>*) 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 stop codon, resulting in a truncated protein that lacks part of its death domain and the complete TIR domain, which are required for interaction with IRAK4 and TLRs, respectively. As expected, the homozygous mutant showed an immune-compromised phenotype. We show that *myd88<sup>−/−</sup>* 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<sup>−/−</sup>* embryos with that of wild-type embryos to distinguish between *MyD88*-dependent and *MyD88*-independent gene expression upon infection with different pathogens. This analysis revealed that gene expression of transcription factors central to innate immunity (including NF-κB and AP-1) and of many innate immunity signaling and effector genes is dependent on MyD88 signaling 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<sup>gua3568</sup>*, which carries a threonine to alanine point mutation creating a premature stop codon (Fig. 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 material Fig. S1). Zebrafish embryos homozygous for the mutation were found at Mendelian frequencies after crossing of heterozygous parents. They showed no developmental differences compared with their wild-type siblings and could only be distinguished by genotyping. Two groups of *myd88<sup>−/−</sup>* and *myd88<sup>−/+</sup>* 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 (Fig. 1B). After this period, *myd88<sup>−/−</sup>* larvae showed a significantly decreased survival rate compared with wild-type larvae (Fig. 1B). The steady decline of *myd88<sup>−/−</sup>* larvae ceased at around 20 dpf. Thus, under our rearing conditions, 8-20 dpf was a crucial period for rearing.
Fig. 1. Characterization of myd88 mutant zebrafish and their survival following infection with E. tarda and S. typhimurium. (A) Mutant sequence and protein structure. Point mutation (threonine to alanine) in the death domain sequence of zebrafish myd88 introduces a premature stop codon. 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, gray 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 with 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 iil1b and mmp9 at 2 hpi 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. (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 was determined by a logrank test. (I) E. tarda infection. At 28 hpf, myd88−/− and wild-type embryos were infected with ~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 are pooled from two individual experiments and statistical significance was tested with a contingency test. (J) S. typhimurium infection. Embryos were injected into the blood island at 28 hpf with ~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 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 pHrodo, a pH-dependent fluorogenic dye. 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 was observed between wild-type and mutant embryos, but both groups were significantly different from Pu.1 morpholino-injected controls by one-way ANOVA with Tukey’s Multple Comparison method as a post-hoc test. ***P<0.001; ns, not significant.
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 with wild-type individuals and heterozygotes, often showing pathological features linked with infection (data not shown). To conclude, we observed a reduced survival in embryonic and adult myd88−/− zebrafish, most probably caused by a primary immune deficiency due to the lack of functional MyD88.

**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 mpox:GFP transgene that specifically marks neutrophils (Renshaw et al., 2006). 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 (mpox::GFP/L-plastin double fluorescent cells) and macrophages (L-plastin-positive and mpox::GFP-negative) (Fig. 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 at 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 (Sepulcre et al., 2009; Sullivan et al., 2009). Nevertheless, we show here that recognition of LPS in zebrafish embryos does require MyD88, because expression of both il1b and mmp9 after injection with LPS was significantly lower in mutants compared with wild types (Fig. 1D,E). We also injected groups of embryos with flagellin, a known ligand for the Tlr5-MyD88 pathway in zebrafish (Stockhammer et al., 2009). The observed expression levels of il1b following injection confirmed the MyD88-dependency for flagellin recognition (Fig. 1F). Interestingly, flagellin-induced mmp9 expression occurred independently of MyD88 (Fig. 1G). 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 ~24 hours of incubation (Fig. 1H), which was similar for both myd88−/− and wild-type embryos, showing that poly I:C recognition and toxicity occurs independently of MyD88, 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 (van der Sar et al., 2003; van Soest et al., 2011). 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 (Fig. 1I). Lethality of S. typhimurium infection also occurred significantly faster in mutant than in wild-type embryos (Fig. 1I). 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 labeled with the pH-dependent fluorogenic dye, pHrodo. This provides a quantitative measure for phagocytosis because pHrodo only becomes fluorescent in acidic environments, like those encountered in the phagolysosomal pathway (Hall et al., 2009). The results showed similar levels of phagocytosis in myd88−/− and wild-type embryos at 2 hpi (Fig. 1K). A control group, injected with a Pu.1 morpholino to severely deplete leukocyte populations (Hsu et al., 2004), 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.

**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 colony-forming units (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). At 8 hours after infection with E. tarda or S. typhimurium, a large number of probes (619 and 1214, respectively) were upregulated in wild-type embryos (Fig. 2A). In comparison, the numbers of upregulated probes were about threefold lower in myd88−/− embryos (190 for E. tarda and 339 for S. typhimurium) (Fig. 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) (Fig. 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.
(Fig. 2C). However, whereas all GO terms belonging to the ‘Immune system process’ subclass were enriched for infected wild-type embryos, only one or three out of five 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 downregulated by the different infections in myd88−/− mutants did not reveal a specific MyD88-independent signaling pathway that was 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 levels of genes in these categories was lower in myd88−/− embryos. The number of upregulated genes in these categories was lower in myd88−/− embryos, whereas myd88−/− embryos showed a much weaker transcriptional response (Fig. 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 the expression level of genes that did respond was lower compared with wild types. The residual induction of immune-related transcription factors in myd88−/− embryos was reflected in the number of cytokine genes that were regulated upon infection, because 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: NF-κB (irf9 and ire1), 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 E. tarda infection, whereas irf9 and irf11 appeared to be 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 with wild-type embryos. The other major pro-inflammatory cytokine gene, infa, 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, whereas 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 that 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 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 MyD88-independent genes are highly variable during infection

Our transcriptome analysis of myd88−/− and wild-type embryos infected with E. tarda FL6-60 or S. typhimurium SL1027 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 with controls during S. typhimurium infection; four- to eightfold change difference in S. typhimurium infection) (Fig. 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 fourfold 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−/−), whereas 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, whereas 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 (Fig. 4). We found that expression of mmp9 and cxcl-c1c was dependent on MyD88 during infection with either pathogen, similarly to il1b. Expression of il8 was significantly lower in myd88−/− embryos infected with E. tarda compared with 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 was the induction 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.

**MyD88-deficient embryos can limit growth of the attenuated S. typhimurium Ra strain**

On the basis of 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 embryos (van der Sar et al., 2003). Four hours after injecting the bacteria into the bloodstream, we observed clustered fluorescent bacteria, indicative of phagocytosis, in both myd88−/− and wild-type embryos (Fig. 5A,C). By contrast,

![Image](https://via.placeholder.com/150)

Fig. 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, ifnphi1 was analyzed by qPCR in wild-type (Wt) and mutant (Mu) embryos infected with E. tarda (A) or S. typhimurium (B). 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 were calculated by one-way ANOVA with Tukey's multiple comparison method as a post-hoc test; *P<0.05; **P<0.01; ***P<0.001; ns, not significant.
clustering was greatly decreased in a Pu.1 morpholino-injected control group deficient in leukocytes (Fig. 5C). At 1 dpi, we noted increased infection in myd88−/− embryos compared with wild types, which was quantifiable by bacterial pixel counting (Fig. 5D,E,G). At the same time point, embryos in the Pu.1 morpholino-injected control group were completely overgrown with infection, with the bacteria spreading throughout the entire vasculature (Fig. 5F). The bacterial pixel count in both myd88−/− and wild-type embryos gradually declined over the next 4 days. At the 5 dpi time point, 58% of the wild types had already cleared the infection, whereas wild types with remaining infection showed greatly decreased numbers of 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 attenuated S. typhimurium Ra bacteria in zebrafish embryos can be limited in a MyD88-independent manner.

Fig. 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 ~150 cfu of DsRed-labeled S. 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 at 1 dpi (D-F) are shown. (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 are accumulated from two individual experiments. Significant differences 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 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 whereas the difference for mmp9 was significant.**P<0.01, ***P<0.001; ns, not significant.
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 cells (Davis et al., 2002). 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. For comparison, wild-type and myd88-morpholino-injected embryos were infected with the same dose of M. marinum Mma20 (E,F). (G-I) Quantification of bacterial burden. Red symbols indicate which infected individuals are shown as representative images in A-F. Bacterial pixel counts were determined based on stereo fluorescence images. Significant differences were determined by one-way ANOVA with Tukey’s Multiple Comparison method as a post-hoc test; ***P<0.001.

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 (Tobin et al., 2010; Tobin et al., 2012). We performed qPCR analysis on RNA from individual myd88−/− and wild-type embryos infected with M. marinum Mma20 at a time point (4 dpi) with clear granuloma formation. Both il1b and tnfα were expressed at significantly lower levels in infected myd88−/− embryos (Fig. 7). 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 MyD88 (Volkman transcript in both wild-type and mutant embryos (van der Sar et al., 2006). The AUG-morpholino increased infection in wild-type individuals compared with mismatch morpholino-injected wild types, but did not increase infection in myd88−/− compared with mismatch-control injected mutants (supplementary material Fig. S2). These results support the idea that myd88hu3568 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.
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 crucial residue (lysine 95) for signaling (Loiarro et al., 2009). 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 might be explained by a lower stability of myd88hu3568 mRNA and/or by a possible feedback 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 material Fig. S1). 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 facts 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 (Fig. 6; supplementary material Fig. S2).

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. The mortality rate of developing myd88−/− larvae decreased after this period, which might correlate with the onset of active adaptive immunity. Nevertheless, adult myd88−/− zebrafish still display increased mortality compared with wild types. For instance, fin clipping of adult myd88−/− for genotyping purposes is followed by a higher incidence of death over the next few 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 cells (Jima et al., 2009). Interestingly, humans with defective MYD88 suffer from a primary immunodeficiency, with life-threatening infections occurring during early infancy (Ku et al., 2007). Children with this deficiency have a cumulative mortality of 30-40% (Ku et al., 2007), whereas adult patients with this immune deficiency had no major infections (Bousfiha et al., 2010). This difference was ascribed to the development of proficient adaptive immune responses later in life that compensate for the defects in the inflammatory reaction (Neta et al., 2012), 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 is an ideal tool for further investigation of this phenomenon.

**myd88−/− mutant as a research model for immunity, cancer and development**

The myd88 mutant presented here is a valuable model for determining the role of TLR and IL1R signaling and innate immunity in numerous important processes. Not only could it be used to study host-pathogen interactions in zebrafish (Meijer and Spaink, 2011), but also to study the role of TLR signaling in the tumor microenvironment (Sato et al., 2009), as well as the role of MyD88 in the establishment of normal intestinal microbiota and maturation of the immune system during development (Frantz et al., 2012). 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 knockdown 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 bacteria (Deng et al., 2012) and phagocytosis of bacteria are not affected by the absence of MyD88 (Fig. 1F and Fig. 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 NF-kB and AP-1 and encoding cytokine Il1b (Fig. 3). These differences could not have been caused by variations in the injection dose because we visually controlled for this by using fluorescently labeled bacteria. Because 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 mutant parents, also demonstrating that the induction levels of innate immune-response genes were in significantly lower ranges in mutant embryos (Figs 5, 7).

An important finding is that we identified genes, including the chemotactic cytokine Il8, for 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 from wild type was only significant during E. tarda infection. The natural fish pathogen E. tarda might have evolved more specialized methods of manipulating or evading the immune system of their aquatic hosts, compared with 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 twofold higher in S. typhimurium infection compared with E. tarda infection (Fig. 2A,B). A similar situation occurs when the transcriptome of zebrafish embryos exposed to E. tarda is compared with embryos exposed to Pseudomonas aeruginosa, a broad host range pathogen capable of infecting plants, invertebrates and vertebrates (van Soest et al., 2011).

The myd88 dependency of il1b and mmp9 expression induced by E. tarda and S. typhimurium 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-dependent (Stockhammer et al., 2009). However, in the previous studies it was not possible to discriminate between complete or partial myd88 dependency because the morpholino knockdown effect itself could be incomplete. With the use of the mutant line we were able to establish that the E. tarda- or S. typhimurium-induced il1b expression at 8 hpi is lower but not completely absent in myd88-/- embryos. We also showed that mmp9 induction at this time point is completely inhibited by MyD88 deficiency during E. tarda infection, whereas partial induction occurs during S. typhimurium infection. Furthermore, a partial induction of ifna was observed in myd88 mutants during M. marinum infection, whereas ifna 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 (Adachi et al., 1998; Kawai et al., 1999; Takeuchi et al., 2000; Hirotani et al., 2005; Weighardt et al., 2006; Kissner et al., 2010). The gene for Lfnp1, which belongs to the type I interferon group (Hamming et al., 2011), was dependent on MyD88 for its induction during S. typhimurium infection, as was described for mouse IFN1 when macrophages lacking MYD88 were stimulated with LPS (Hirotani et al., 2005).

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 (Fig. 5I). Approximately half of the infected myd88-/- larvae showed il1b expression levels comparable with uninfected controls, whereas the other half displayed il1b expression at a similar level to 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 (Fig. 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-antigen (Tsolis et al., 1999). Transcriptome analysis of infected myd88-/- embryos revealed that expression of genes involved in complement is for a large part independent of MyD88 (Fig. 3). It has also been shown that complement component C5a is a potent endogenous pro-inflammatory peptide, capable of triggering production of Il1b upon infection (Montz et al., 1991). TLRs and complement can independently induce pro-inflammatory responses, but their synergistic interaction results in amplified responses (Holst et al., 2012). This might explain why il1b induction in myd88-/- embryos infected with pathogenic bacteria was significantly lower, but not completely absent (Fig. 4). Finally, some TLRs, like TLR4, can signal via both MyD88-dependent and MyD88-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, whereas mmp9 induction was similar to the 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 infections (Lesley and Ramakrishnan,
2008), the role of MYD88 in mycobacterial disease remains controversial. Mice deficient in MYD88 are more susceptible to infection by *M. tuberculosis* (Ryffel et al., 2005), 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 *M. tuberculosis* (Sánchez et al., 2012). 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. That MyD88 is important for early control of these infections was demonstrated by the finding that homozygous myd88 mutant and MyD88-morpholino-injected larvae showed accelerated formation of granulomas and at least fivefold higher bacterial burdens compared with 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 (Clay et al., 2008; Volkman et al., 2010). In agreement, the impaired control of *M. marinum* in the absence of functional MyD88 is most probably caused by a marked reduced induction of mmp9 and pro-inflammatory cytokines, like il1b and tnfα (Fig. 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 that have been developed in recent years for human infectious diseases, inflammatory disorders and cancer.

**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 anesthesia in egg water containing 0.02% buffered 3-aminobenzoic acid ethyl ester (Tricaine).

**MyD88 mutant, genotyping and morpholino injection**

The myd88hu3568 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::egfpGFP parents, obtained by increasing the heterozygous offspring of myd88+/−/mpx::egfpGFP male and their wild-type siblings. For genotyping, genomic DNA was amplified using forward primer 5′-GAGGCGATATTCCAGTACACGC-3′ and reverse primer 5′-GAAGCAGCAAGAGAAAAGCAAAGGCTCAGGTC-3′; 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 ~300 base pairs (bp) into products of 200 and 100 bp. Determining the stability of the mutant transcript versus the wild-type transcript was done with RT-PCR using forward primer 5′-GAGGCGATTTCCAGTACACGC-3′ and reverse primer 5′-GAAGCAGCAAGAGAAAAGGCTCAGGTC-3′; the product of this reaction was digested with MseI. Knockdown of myd88 by splice morpholino was performed as previously described (Bates et al., 2007). Knockdown of myd88 by AUG-morpholino was previously described (van der Sar et al., 2006).

**Immunohistochemistry**

Immunolabeling with L-plastin antibody (Mathias et al., 2009) and Alexa-Fluor-568-conjugated secondary antibody was as described (Cui et al., 2011).

**Injection conditions**

*E. tarda* strain FL6-60 labeled with mCherry (Lagendijk et al., 2010); *S. typhimurium* wild-type strain SL1027 and its isogenic LPS derivative SF1592 (Ra), both containing the DsRed expression vector pGMD3 (Stockhammer et al., 2009); and *M. marinum* strains Mma20 and E11 labeled with mCherry (van der Sar et al., 2004) 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 cfu 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 control. After injection, embryos were kept at 28°C. Purified TLR ligands: LPS from *S. enterica* serovar *typhimurium* (#L6511, Sigma), pol I/C high molecular weight (tirl-pic, Invivogen), flagellin from *S. enterica* serovar *typhimurium* (fla-st ultrapure, Invivogen).

**Phagocytosis assay**

*E. coli* pHrodo particles (Life Technologies Europe BV, Bleijswijk, The Netherlands) were injected into the blood island at 28 hpf (1 nl 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) was performed as previously described (de Jong et al., 2010). 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 glycinogen as a co-precipitant. RNA from the aqueous phase was purified using the Rneasy MinElute Cleanup kit (Qiagen Benelux BV, Venlo, The Netherlands).

**Microarray analysis**

Microarray was performed using our custom-designed Agilent 44k platform GPL10042, as previously described (van Soest et al., 2011). The raw data were submitted to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under accession number GSE39274. To compare wild-type control, mutant control, infected wild-type and infected mutant samples, a re-ratio experiment was performed as described previously (van Soest et al., 2011). The raw data were submitted to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under accession number GSE39274. To compare wild-type control, mutant control, infected wild-type and infected mutant samples, a re-ratio experiment was performed as described previously.
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 software (Stoop et al., 2011).

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

All authors conceived and designed the experiments. M.V. and J.S. performed the experiments and analyzed the data under supervision of A.M. M.V. wrote the manuscript and the final version was read and approved by all authors.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.010843/-/DC1

REFERENCES


Fig. S1. Mutant transcript stability is lower than wild type transcript stability. To compare the stability of the wild type transcript with that of the mutant transcript, RT PCR was performed on three biological replicates of wild type and heterozygous myd88 mutants (n=20 embryos of 3 dpf per group). The PCR product was digested using MseI, which specifically cuts the mutant transcript (at bp 200 out of 400). The 400 bp wild type transcript and the two 200 bp halves of the mutant transcript were separated by gel electrophoresis, demonstrating that the mutant transcript was less abundant than the wild type transcript.

Fig. S2. AUG-morpholino targeting myd88 does not increase bacterial burdens in myd88 mutants. At 28 hpf, myd88−/− (Mu) and wild type (Wt) embryos injected with either an AUG-morpholino targeting the myd88 transcript or a 5′ mismatch control morpholino were infected with approximately 200 cfu of mCherry-labeled M. marinum strain Mma 20 by injection into the blood island and stereo fluorescence images of infected embryos were taken at 3 dpi. Bacterial pixel counts were determined based on stereo fluorescence images. Significant differences (**P<0.01; ***P<0.001; *P<0.05) were determined by one-way ANOVA with Tukey’s multiple comparison method as a post-hoc test.
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