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Infectious disease studies in zebrafish : the fish pathogen Edwardsiella tarda as a model system

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Citation
Soest, J. J. van. (2011, November 29). *Infectious disease studies in zebrafish : the fish pathogen Edwardsiella tarda as a model system*. Retrieved from https://hdl.handle.net/1887/18149

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Expression profiling of the response to *Edwardsiella tarda* infection in a zebrafish *myd88* knockout mutant

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Abstract

The availability of knockout mutants has greatly assisted functional studies in many model organisms. However, zebrafish knockout mutants in genes of the innate immune system have thus far been lacking. Here we describe the functional analysis of a knockout mutant in the gene encoding Myd88, a central adaptor in Toll like and IL1 receptor signalling. We focus on the responsiveness of *myd88* mutant embryos to infection by the natural fish pathogen *Edwardsiella tarda*. The results of microarray expression profiling show that, as expected, *myd88* plays a crucial role in the response to infection. We have annotated the genes whose expression is highly dependent on *myd88*. Besides impaired induction of immune response genes in *myd88* mutants, we also observed a positive influence of *myd88* deficiency on the induction of several gene groups after *E. tarda* infection. The *myd88*-dependent gene set includes many genes that in previously published data were shown to be responsive to Salmonella infection in zebrafish embryos. In the Salmonella infection studies, we identified *il1b*, *mmp9* and *irak3* as *myd88*-dependent targets. Here we show the *myd88*-dependence of these genes in *E. tarda* infection and add many other immune response genes as *myd88*-dependent targets, for example genes encoding transcription factors, chemokines, and the flagellin receptor Tlr5. The *cxcl-c1c* chemokine gene is an example of a *myd88*-dependent gene that could not be identified in previous morpholino knockdown studies due to off target effects of these treatments. In addition, our analysis of the knockout mutant showed that *E. tarda* induction of *tnfa* was independent of the Myd88 pathway, whereas *tnfb* induction did require *myd88* function. The significantly impaired innate immune response to *E. tarda* infection demonstrates that the *myd88* knockout mutant represents an immunodeficient model that will be a valuable asset to immunological and cancer studies in zebrafish.

Introduction

The innate immune system is the first line of defence against invading microbes. Pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs), of which the Toll-like receptors (TLRs)

have been studied most extensively. There are to date 10 functional TLRs identified in humans, each of which recognizes distinct groups of PAMPs [1]. Following ligand binding, TLRs recruit one or more cytosolic adaptor molecules, thereby initiating a signalling cascade that ultimately leads to the activation of the innate immune response. Myeloid differentiation factor 88 (MYD88) is an important adaptor protein in the TLR signalling pathway, and is used by all TLRs except TLR3. It contains two domains, a C-terminal TIR-domain (Toll/IL-1 receptor-domain) separated with a short linker sequence from the N-terminal death domain. The TIR-domain enables interaction with the TLRs, while the death domain enables interaction with IRAKs (IL-1 receptor associated kinase), leading to an initiation of signalling to MAP-kinases (mitogen activated protein kinases) and NF-κB (nuclear factor κB) [2-4]. MYD88 is the only adaptor used by TLR5, -7, -8 and -9. TLR2, forming heterodimers with TLR1 and -6, requires an additional adaptor, Mal to bridge the interaction with MYD88. TLR4 signals through a MYD88-dependent and a MYD88-independent (TRIF/TRAM-dependent) signalling pathway, leading to the production of pro-inflammatory cytokines and type I interferons, respectively. In addition to its pivotal role in the TLR pathway, MYD88 is an adaptor molecule downstream of the interleukin-1 receptor (IL-1R) and it has been associated with IFN-γ receptor signalling, leading to p38 activation [5, 6].

Mice deficient in Myd88 were shown to be hyporesponsive to LPS, which in wild type mice induces endotoxic shock [7]. Furthermore they were shown to be unresponsive to PAMPs recognized by Tlr2, Tlr4, Tlr5, Tlr7 and Tlr9, as well as to Il-1 [5, 8]. Myd88-deficient mice were also found to be more susceptible to infection by *Toxoplasma gondii* parasites [9] and by a number of bacterial pathogens, including *Staphylococcus aureus* [10] , *Listeria monocytogenes* [11], *Chlamydia pneumoniae* [12], and *Mycobacterium tuberculosis* [13].

Elucidating pathways of the innate immune system in animal models is complicated by the interaction of the innate immune system with the adaptive immune system. To circumvent these interactions, *in vitro* studies using peripheral blood monocytes, polymorphonuclear cells or peritoneal macrophages as well as cultured cell lines have been used to elucidate innate immunity signalling. The zebrafish embryo model provides a useful *in vivo* alternative to study vertebrate innate immunity, since it has a temporal segregation of the development of the innate and adaptive immune system. At

one day post fertilization (dpf) the zebrafish embryo has a functional innate immune system, capable of mounting an effective immune response against different pathogens [14-16]. In contrast, the adaptive immune system does not reach maturity until 2 - 4 weeks post fertilization [17-19]. In addition, zebrafish embryos are transparent, facilitating the visual study of the immune response with the use of fluorescent protein expressing immune cells and bacteria [14, 16, 20-24].

In recent years several infection models have been developed which enabled validation of the zebrafish embryo as a model for vertebrate innate immunity [25-28]. The highly conserved TLR family was identified in zebrafish and its members were found to be highly expressed in one day old zebrafish embryos [29-31], at which stage functional macrophages are first present. As in mammals, Tlr3 was linked to the response against viral infections and Tlr5 to recognition of flagellin [32, 33]. However, zebrafish Tlr4 is not activated by LPS, indicating that there are also differences in TLR ligand recognition between fish and mammals [34, 35].

The TLR adaptor molecules, Myd88, Mal and Trif are conserved in zebrafish, while Tram is not found in the zebrafish genome [30, 36, 37]. Similar to the TLR receptors, the adaptor molecules are broadly expressed during zebrafish embryogenesis [31]. Using a transgenic reporter line, *myd88* expression was detected in adult immune cell lineages and its expression was also confirmed in embryonic and larval leukocytes that contribute to wound healing and phagocytosis of injected bacteria [38]. Morpholino knockdown of Myd88 in zebrafish embryos affected their ability to clear an infection with the *Salmonella enterica* serovar *Typhimurium* Ra mutant [31]. In addition, morpholino knockdown studies showed that, like in mammals, Myd88-dependent and Myd88-independent signalling pathways are involved in the response to Salmonella infection: the Myd88-dependent pathway leading to expression of pro-inflammatory genes such as *il1b* and *mmp9*, and the Myd88-independent pathway to expression of *ifn1* [33]. Induction of *il1b* by TLR ligands, such as peptidoglycan and lipoteichoic acid, was also impaired under Myd88 knockdown conditions in zebrafish embryos [37]. Furthermore, knockdown analysis demonstrated the role of Myd88 in LPS- or TNBS-induced intestinal inflammation and microbial-dependent intestinal epithelial cell proliferation in zebrafish larvae [39-41].

A limitation of morpholino knockdown is that it can only achieve a temporal and incomplete block of gene activity. For further analysis of *myd88* in the zebrafish model, it is therefore desirable to have a knockout mutant available. In this report we describe a *myd88* mutant allele that was identified by resequencing of an ENU (N-ethyl-N-nitrosourea)-mutagenized population and contains an early stop codon in the N-terminal death domain. This leads to a truncated protein, unable to signal, since it lacks most of its death domain, necessary for downstream interaction with IRAK, and the complete TIR-domain, necessary for the interaction with the TLRs. We used a natural fish pathogen, *Edwardsiella tarda*, to compare the immune response of individual *myd88*-knockout embryos to that of their wild-type siblings. Microarray analysis revealed that a large set of known immune response genes as well as many genes not previously associated with the immune response are dependent on *myd88* function during *E. tarda* infection.

Results

Identification of a zebrafish knockout mutant in myd88

Previous morpholino knockdown studies indicated a central role for *myd88* in the innate immune response of zebrafish embryos and larvae [31, 33, 37, 39-41]. A stable knockout mutant of this gene is therefore highly useful. Resequencing of an N-ethyl-N-nitrosourea (ENU)- mutagenized zebrafish library resulted in the identification of a *myd88* mutant allele, $myd88^{hu3568}$, which carries a T to A point mutation creating an early stopcodon (figure 1). The mutation is in the N-terminal death domain leading to a truncated protein lacking part of its death domain required for interaction with the downstream Irak1 kinase and lacking also the complete TIR-domain required for interaction with cytoplasmic domains of the upstream TLR receptors (figure 1).

Zebrafish embryos homozygous for the mutation ($myd88^{-/-}$) were found at Mendelian frequencies after crossing of heterozygous parents. $Myd88^{-/-}$ embryos and larvae showed no developmental differences with their wild type siblings and could only be distinguished by genotyping. Until 5 dpf we observed no differences in survival between mutants and wild type siblings. However,

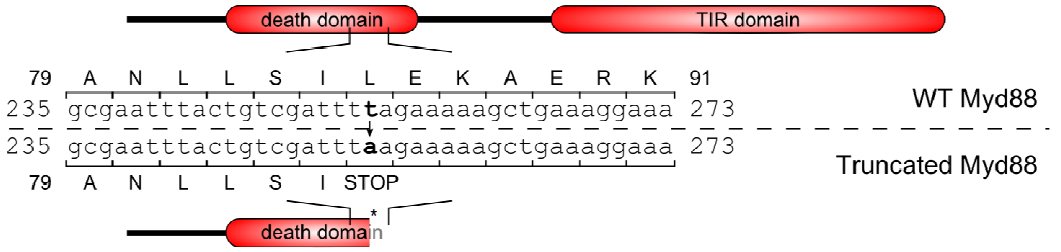


Figure 1. Wild type and mutant Myd88 sequence and protein structure. A random mutagenized zebrafish library was screened for *myd88* knockout mutants. One mutant had a T to A point mutation at nucleotide position 254 with respect to the ATG start codon, leading to a stopcodon that truncates the protein at amino acid position 85 (GenBank accession of wild type sequence: AY388401). The schematic drawing shows the resulting truncation of the death domain and lack of the TIR domain in the Myd88 mutant protein, with the DNA and amino acid sequences surrounding the mutation.

until now only a few zebrafish carrying the *myd88* mutation reached adulthood under our standard aquarium conditions. Similar as reported for *rag1*$^{-/-}$ zebrafish [42, 43], these few m*yd88*$^{-/-}$ fish died after finclips were taken for genotyping, suggesting a severely compromised immune system. Since we have not yet been able to obtain sufficient adults for further analysis, we concentrated this study on the characterization of *myd88*$^{-/-}$ embryos.

Gene expression profiles of myd88$^{-/-}$ embryos during development

To determine the effect of the *myd88*$^{-/-}$ mutation at the transcriptional level, we isolated RNA from wild type and *myd88*$^{-/-}$ sibling embryos at 1, 2, and 3 dpf for analysis on our previously described custom Agilent 44k microarray platform [33]. This platform is a combination of the commercial Agilent 22k and Sigma-Genosys 16k probe sets that we extended with probes for immune response genes. On this platform, a number of immune response genes are represented by multiple probes to increase reliability of the microarray data for this subset of genes. During the RNA isolation procedure, the chromosomal DNA fraction was retained for genotyping, based on which RNA samples from three individual wild type and three individual *myd88*$^{-/-}$ embryos were selected for microarray analysis.

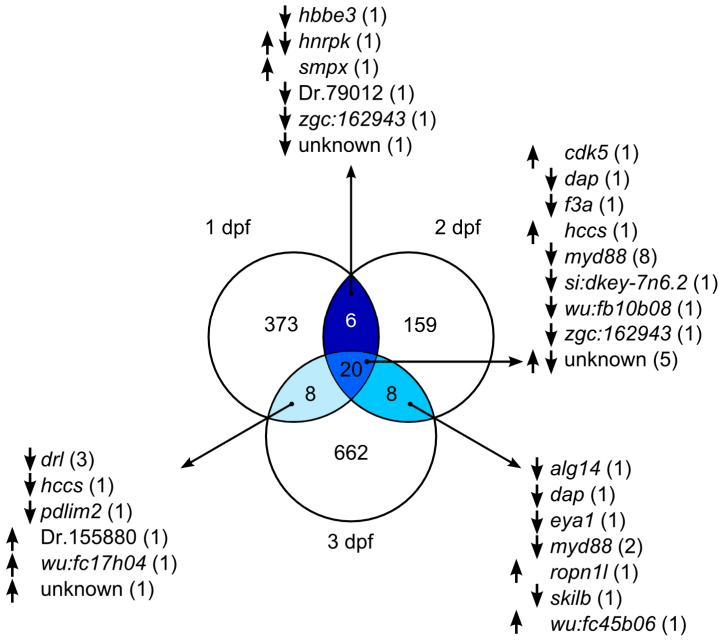


Figure 2. Comparison of *myd88*$^{-/-}$ transcriptomes at different development stages. RNA isolated from single embryos at 1, 2 and 3 dpf was analyzed by microarray. For each stage samples from three individual WT or *myd88*$^{-/-}$ embryos were hybridized and averaged. The Venn diagram indicates the genes that were significantly up- or down-regulated in *myd88*$^{-/-}$ embryos compared to WT siblings in samples of at least two developmental stages. The arrows in front of the gene names indicate whether the gene was up- or down-regulated. The numbers behind the gene name indicates the number of different probes sequences for that gene that showed up- or down-regulation.

Only 20 probes in total were consistently changed (P<0.00001) between *myd88*$^{-/-}$ and wild type embryos at all three developmental time points, and only 22 additional probes were changed at 2 out of 3 time points (figure 2, supplementary table S1). The probes changed at all time points included 8 probes for *myd88* itself, all showing down-regulated expression in *myd88*$^{-/-}$ embryos in comparison to wild type siblings. Two other probes for *myd88* additionally showed down-regulation at 2 out of 3 time points (2 and 3 dpf). None of the other probes that were differently expressed between wild type and *myd88*$^{-/-}$ at 2 or 3 time points corresponded to TLR signalling components. Besides the probes for *myd88*, 5 other probes corresponded to genes associated with the immune response and were down-regulated in *myd88-/-* embryos compared to wild type, including: *death associated protein* (*dap*, identified by 2 probes), identified in HeLa cells as a positive mediator of interferon-γ induced programmed cell death [44]; *coagulation factor 3a* (*f3a*); and *pdz and lim domain 2* (*pdlim2*), which has been shown to function as a negative regulator of transcription factor NF-κB [45].

Analysis of the immune response of myd88$^{-/-}$ embryos infected with *E. tarda*

To study the effect of the *myd88-/-* mutation on the immune response to infection, we injected *E. tarda* bacteria into the bloodstream of 28 hpf embryos from MyD88$^{+/-}$ parents. The inoculum was approximately 200 CFU per embryo embryo and to avoid clumping of the bacteria we used a carrier solution of

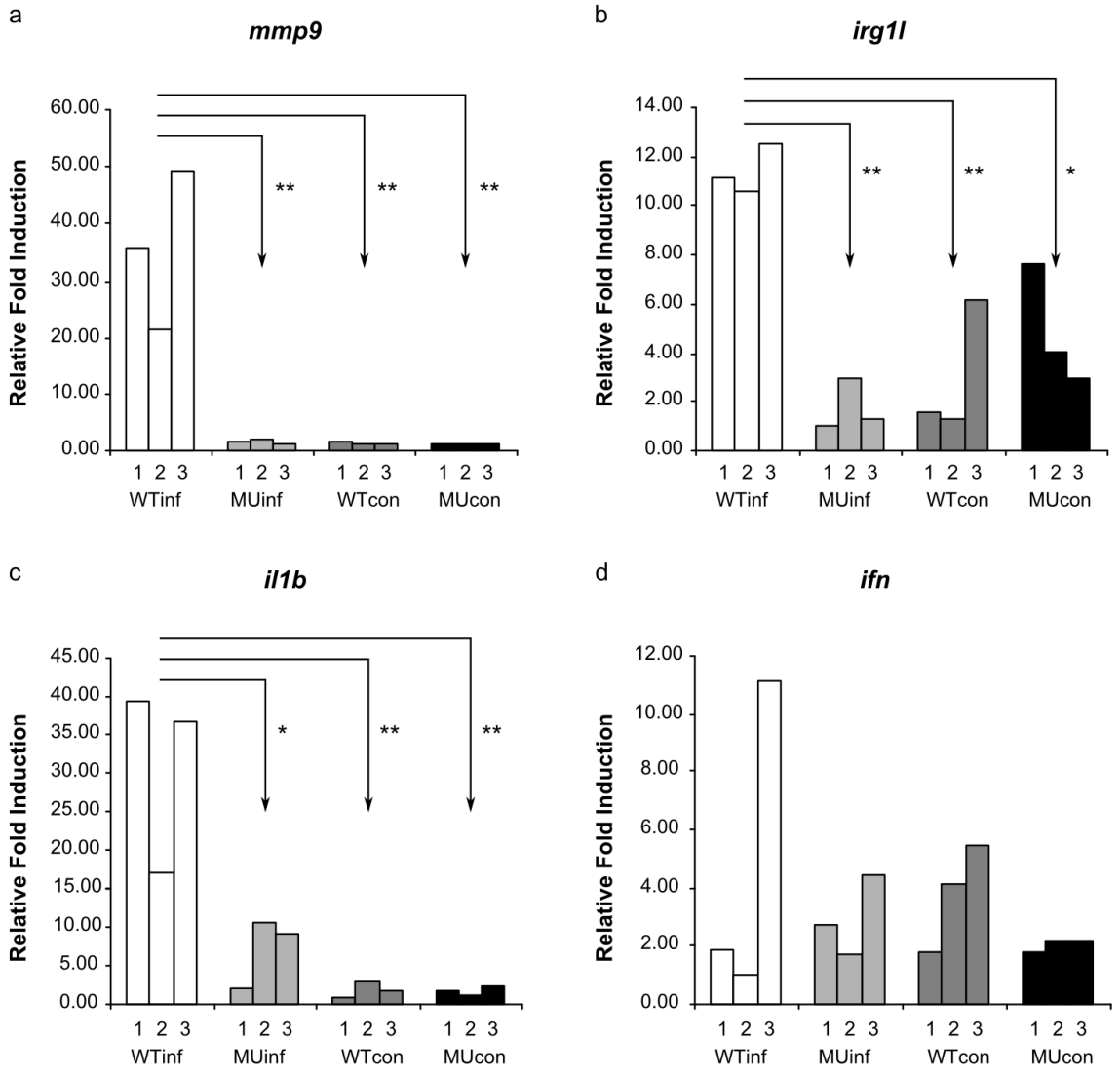


Figure 3. qPCR analysis of the response of wild type and *myd88*$^{-/-}$ embryos to *E. tarda*. Expression of *mmp9*, *irg1l*, *il1b* and *ifnphi1* was analysed by qPCR in uninfected (con) and infected (inf) wild type (WT) and mutant (MU) embryos. The bars represent the relative expression level with the lowest expression set at 1. Each bar represents a single embryo. Lines with * indicate a significant difference of $P < 0.05$. Lines with ** indicate a significant difference of $P < 0.01$ (tested by two-way ANOVA analysis with the Bonferroni method as post-hoc test).

polyvinylpyrrolidone (PVP) in PBS for the injections [49]. As a control, embryos from the same parents were injected with the carrier solution alone. DNA and RNA from control and infected embryos was isolated at 8 hpi and the DNA was used for genotyping. RNA from three individual embryos per group was then used for quantitative PCR (qPCR) analysis of three previously identified *E. tarda*-responsive genes, *matrix metalloproteinase 9* (*mmp9*), *interleukin 1b* (*il1b*), and *immunoresponsive gene 1 like* (*irg1l*) (Chapter 3). In addition, we analyzed the interferon *ifnphi1*gene, since the pathway to interferon induction is expected to be independent of *myd88*. *E. tarda* infection did not induce *mmp9* expression in *myd88*$^{-/-}$ embryos, while a 20 to 50 fold induction was observed in infected wild type siblings (Figure 3a). Expression of *il1b* was induced by *E. tarda* infection both in wild type and mutant embryos, but induction levels were significantly lower in the mutants (Figure 3b). Expression levels of *irg1l* showed a large variation between individual uninfected embryos, but clear induction by *E. tarda* infection was observed only in wild type embryos (Figure 3c). It was not possible to draw any conclusion on the *myd88*-dependence or -independence of *ifnphi1,* since the expression of this gene was increased in only one out of three wild type embryos compared to the controls. In summary, qPCR analysis showed that a functional *myd88* gene is required for the induction of *mmp9* and *irg1l* expression during *E. tarda* infection, and that *il1b* induction is partially dependent on *myd88*.

Expression profiling of the *E. tarda* response in myd88$^{-/-}$ embryos

To further characterize the immune response of *myd88*$^{-/-}$ embryos, the same samples as used for qPCR were subjected to microarray analysis. RNA samples from the individual embryos were hybridized against a common reference using the custom designed 44k Agilent microarray platform, as above. In the data analysis, the triplicate measurements of the *myd88*$^{-/-}$ embryos were combined and compared to the combined measurement of the wild type siblings. Next, four expression ratios were derived from re-ratio analysis of the sample data against the common reference: wild type control vs. wild type infected, *myd88*$^{-/-}$ control vs. *myd88*$^{-/-}$ infected, wild type infected vs. *myd88*$^{-/-}$ infected, and wild type control vs. *myd88*$^{-/-}$ control. Expression differences of the wild type control vs. *myd88*$^{-/-}$ control ratio were in most cases not significant, consistent with the earlier microarray analysis of uninfected embryos at three

developmental time points. In contrast, clear differences were observed between the infected wild type and mutant embryos.

Probes that showed a significant expression signature of at least 2-fold ($P < 0.00001$) in the wild type control vs. wild type infected ratio (663 probes) and/or in the *myd88*$^{-/-}$ control vs. *myd88*$^{-/-}$ infected ratio (355 probes) were selected (912 different probes in total, supplementary table S2). The genes corresponding to these infection-responsive probes were then grouped into three annotation categories for further analysis. Category 1 (433 probes) contains annotated genes previously implicated in the vertebrate immune response based on GO annotations of the zebrafish genes and their human homologues and on searching of PubMed abstracts. Novel/hypothetical genes with sequence similarity to these immune response genes were also included in category 1. Category 2 (266 probes) contains all annotated or novel/hypothetical genes whose known or predicted functions could not be linked to the vertebrate immune response based on the same criteria. Category 3 (213 probes) contains genes with unknown functions or without any similarity to genes with known functions. A hierarchical cluster analysis was performed separately for each category (figure 4). For each annotation category two main clusters could be identified: the first containing probes that were mostly higher expressed in the *myd88*$^{-/-}$ infected embryos than in the wild type infected embryos (blue in figure 4), and the second (yellow in figure 4) showing the opposite response, i.e. a higher expression upon infection of wild type than of *myd88*$^{-/-}$ embryos. In category 1, this second (yellow) cluster was the largest, containing 81% of the total number of probes (figure 4a), while in categories 2 (figure 4b) and 3 (not shown) the two clusters were approximately equal in size. This difference is partially explained by the fact that the larger cluster of category 1 contains many immune response genes that are represented by multiple probes for the same gene. However, also at the gene level, the yellow cluster of category 1 was still larger than the first, i.e.73% of the total number of genes in category 1 showed higher expression in infected wild type than in *myd88*$^{-/-}$ embryos. This group of genes included substantial subgroups of complement and acute phase response genes, cytokine and chemokine genes, defence response genes, lectin genes, proteolytic genes, signal transduction genes and transcription factor genes. Smaller subgroups of each, except for the transcription factor gene subgroup, were present in the other (blue) cluster,

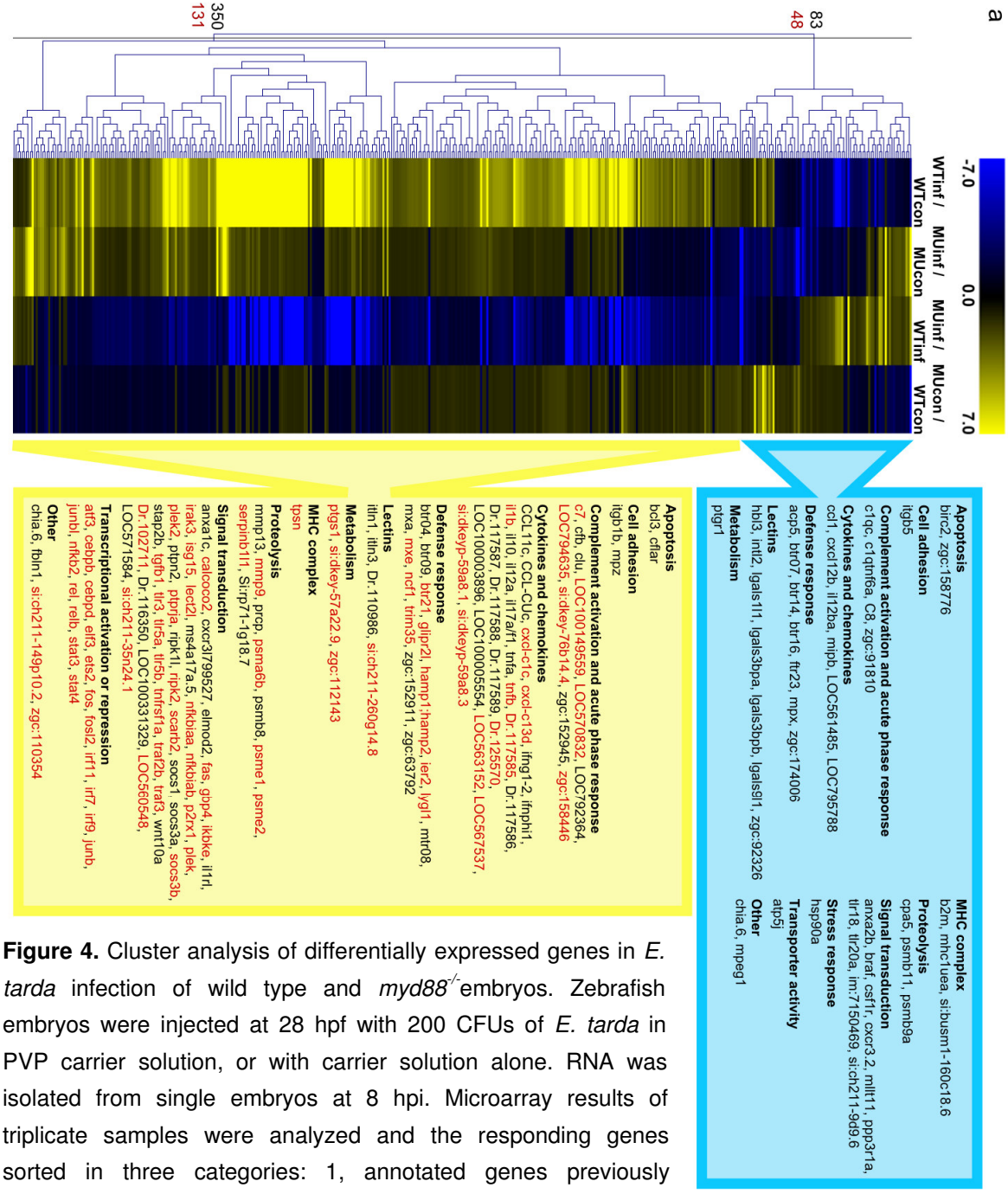
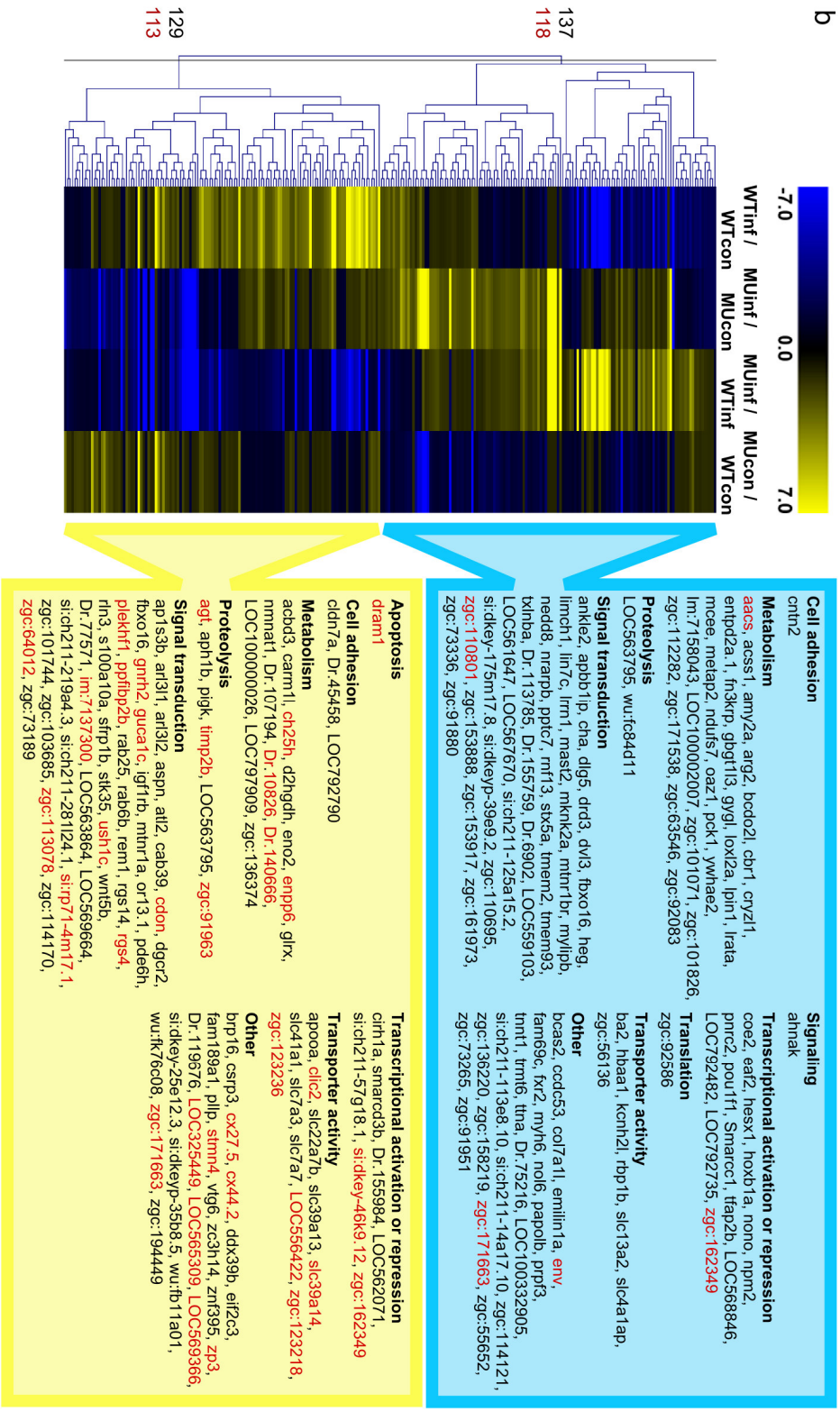


Figure 4. Cluster analysis of differentially expressed genes in *E. tarda* infection of wild type and *myd88*$^{-/-}$ embryos. Zebrafish embryos were injected at 28 hpf with 200 CFUs of *E. tarda* in PVP carrier solution, or with carrier solution alone. RNA was isolated from single embryos at 8 hpi. Microarray results of triplicate samples were analyzed and the responding genes sorted in three categories: 1, annotated genes previously implicated in the immune response or novel genes with strong sequence similarity to those genes; 2, genes with known or predicted functions not previously linked to the immune response; 3, genes with unknown function. Hierarchical cluster analysis was performed separately for genes in category 1 (a) and in category 2 (b). In both cases two main clusters are distinguished: the first containing probes that show lower expression levels in wild type infected embryos than in mutant infected embryos, and the second containing probes that show higher expression levels in wild type infected embryos than in mutant infected embryos. The genes in these main clusters are indicated in the blue and yellow boxes and are sorted in functional annotation categories. The genes indicated in red are induced in a *myd88*-dependent manner based on the microarray data, and additional qPCR data for selected genes (Fig. 3). The black numbers in front of the clusters are the number of different probes in a cluster and the red numbers are the number of different genes represented by



these probes. Increasingly brighter shades of yellow indicate a higher up-regulation of the probe. Increasingly brighter shades of blue indicate a higher down-regulation.

WTinf/WTcon: infected vs uninfected (control) wild type embryos; MUinf/MUcon: infected vs uninfected (control) *myd88* knockout mutants; MUinf/WTinf: infected *myd88* knockout mutant vs infected wild type embryos; MUcon/WTcon: uninfected *myd88* knockout mutant vs uninfected wild type embryos.

containing the genes with higher expression in infected mutants than wild type. In category 2 (figure 3b), the majority of genes in both the blue and yellow clusters fitted in subgroups of metabolic genes, signal transduction genes, transcription factor genes, and genes for proteins with transporter activity.

A closer look at the blue and yellow clusters of categories 1 and 2 revealed further differences. In the blue cluster of category 1 most genes were down-regulated by infection both in wild type and in *myd88*$^{-/-}$ embryos, but to a lesser extent in the mutants than in the wild type. In the blue cluster of category 2 many genes are slightly up-regulated in the *myd88*$^{-/-}$ embryos, while down-regulated in the wild type embryos. In addition, approximately half of the blue cluster of category 2 represents a group of genes with weakly up-regulated expression in infected wild type embryos, but much stronger up-regulation in *myd88*$^{-/-}$ embryos. Such a group of genes is not observed in category 1. Most of the genes in the yellow cluster of category 1 were higher induced in wild type infected embryos than in *myd88*$^{-/-}$ infected embryos. Again there is a clear difference between the genes in category 1 and 2. In category 1, most genes are up-regulated both in wild type and *myd88*$^{-/-}$ embryos, while only a small number of genes is up-regulated in the wild type embryos and down-regulated in the mutant embryos. In category 2, however, there is a larger group of genes that is up-regulated by infection in wild type and down-regulated in *myd88*$^{-/-}$ embryos.

In conclusion, microarray analysis showed that the infection-induction of a large group of immune response genes was reduced in *myd88*$^{-/-}$ embryos compared to the induction in wild type siblings, as expected based on its role as a signalling adaptor of the TLR and IL1R pathways. In addition, the microarray data also indicated a positive influence of *myd88*-deficiency on other *E. tarda*-induced gene groups.

Myd88-dependency of transcription factor and signal transduction gene induction during *E. tarda* infection

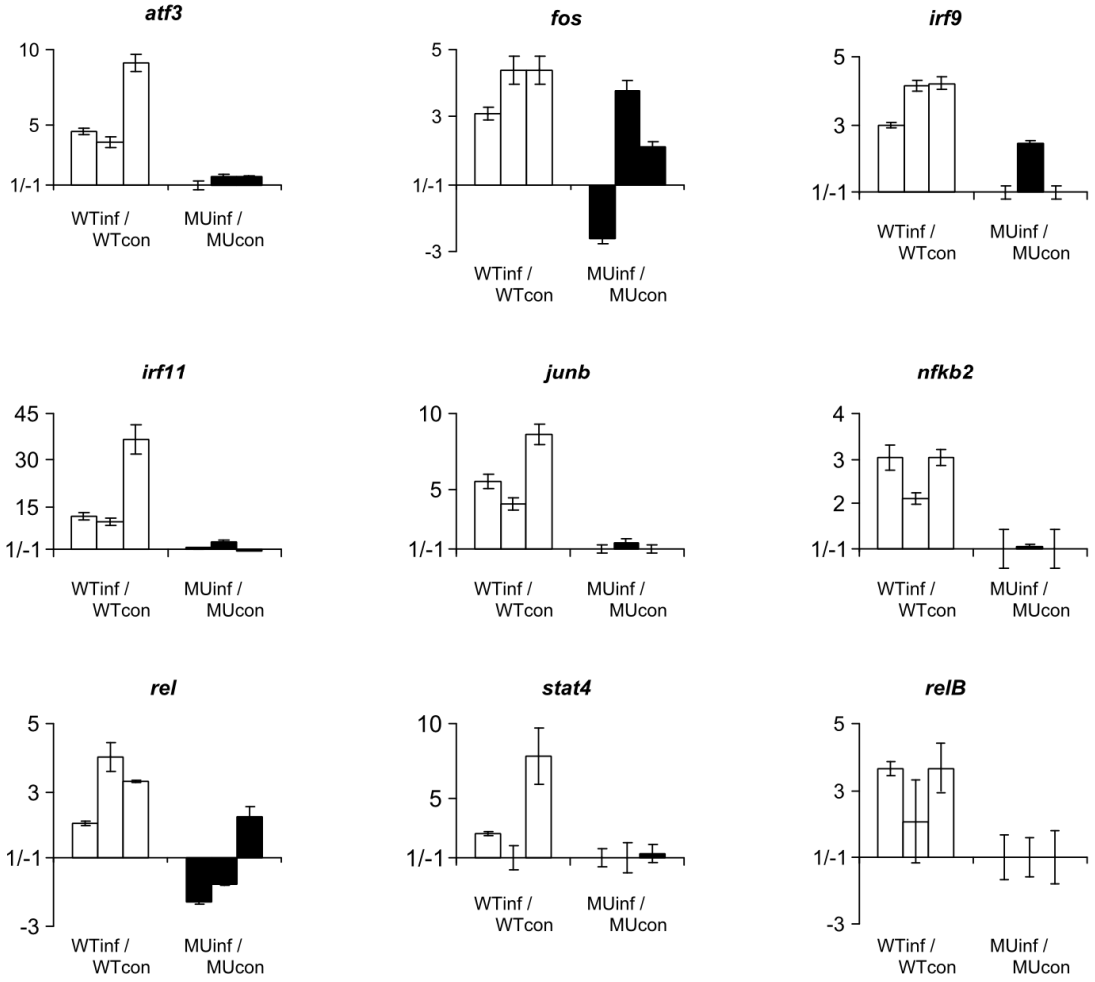
As shown in the cluster analysis of figure 4a, a number of known immune-related transcription factor and signal transduction genes were induced by *E. tarda* infection. Several of these genes are represented by more than one probe on our custom microarray. We selected these genes for further analysis of

myd88 function in the infection response. To this end, we re-analyzed the microarray data, this time comparing each of the individual *myd88*$^{-/-}$ embryos separately to a wild type sibling in order to show the biological variation (supplementary table S2). Subsequently, the induction levels (control vs. infected) of different probes for the same gene were averaged and compared between *myd88*$^{-/-}$ and wild type embryos (figure 5). This analysis showed that *E. tarda*-induction of immune-related transcription factor genes, including members of the ATF, AP-1, IRF, NFκB, and STAT families, was largely dependent on *myd88* function. In addition, this analysis showed *myd88*-dependency of the induction of *irak3*, a negative regulator of TLR signalling, *isg15*, encoding an interferon-activated ubiquitin-like protein, the receptor-interacting serine-threonine kinase gene *ripk2*, and the TLR family genes, *tlr3*, *tlr5a*, and *tlr5b*.

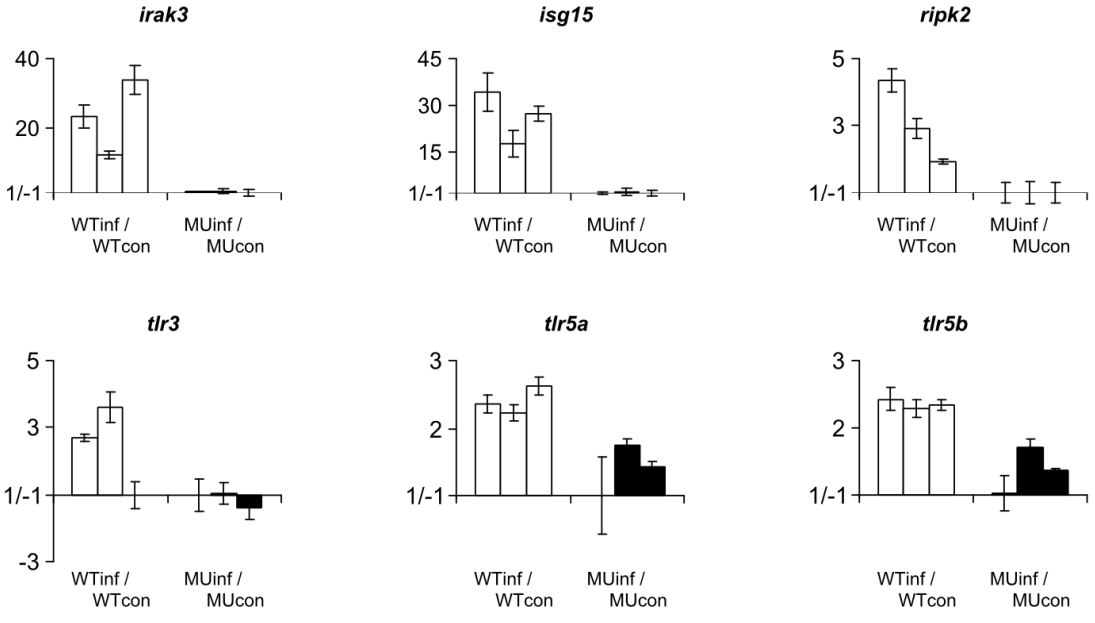
Role of myd88 in induction of other immune response genes

The same analysis as performed for the transcription factor and signal transduction genes was applied to other immune response genes that were induced by *E. tarda* infection in wild type embryos and represented by multiple probes with a significant expression signature on the microarray (figure 6, supplementary table S2). These included seven cytokine/chemokine genes and the genes for matrix metalloproteinase 9 (*mmp9*), neutrophil cytosolic factor 1 (*ncf1*) and the homologue of the human metalloreductase STEAP4, also known as TNFα-induced protein9 (*zgc:112143*). Seven of the ten investigated genes, including two CXCL chemokine genes (*cxcl-C1c*, *cxcl-C13d*), a CC chemokine gene (*si:dkeyp-59a8.1*), *tnfb*, *mmp9, ncf1* and *zgc:112143*, were not or barely induced in *myd88*$^{-/-}$ embryos, indicating their dependency on the *myd88* pathway during *E. tarda* infection. Consistent with the qPCR analysis described above, the *il1b* gene showed lower induction in *myd88*$^{-/-}$ embryos than in WT embryos, suggesting a partial dependence on *myd88*. The expression of *interleukin 10* (*il10*) also seemed to be partially dependent on *myd88*, but this result remains inconclusive since one of the wild type infected embryos showed a very low induction of this gene. In zebrafish, two genes, *tnfa* and *tnfb*, are homologous to the mammalian *TNF* gene, which encodes tumor necrosis factor alpha and is a known target of *MYD88*-dependent signalling. While our microarray data showed that *E. tarda*-induction of *tnfb* was *myd88*-dependent,

Transcription factor genes



Signal transduction genes



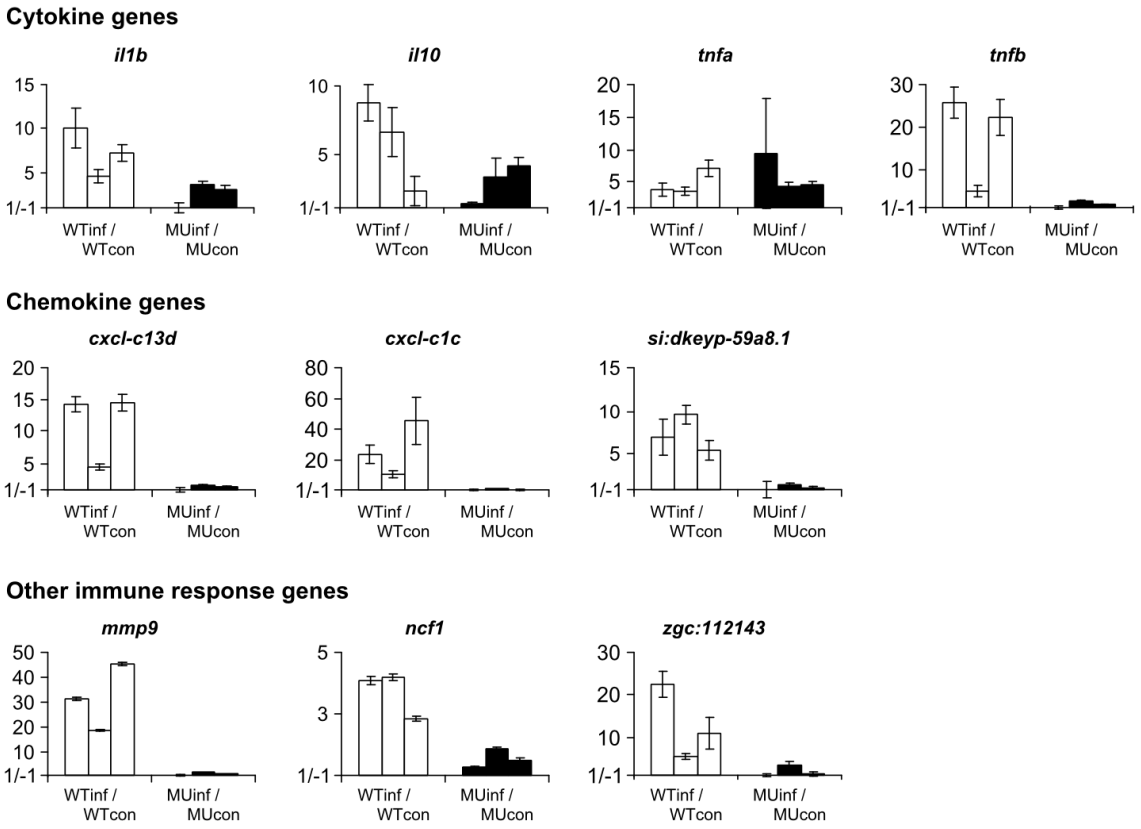


Figure 6. Induction of effector genes of the immune response by *E. tarda* infection of wild type and mutant embryos. Microarray induction levels of cytokine genes, chemokine genes and other immune response genes were compared for three individual wild type (WT) embryos and three individual *myd88* knockout mutant (MU) embryos infected with 200 CFUs of *E. tarda*. Gene expression levels of each infected embryo (WTinf and MUinf) are shown relative to a corresponding uninfected control embryo (WTcon and MUcon). For all genes shown in the figure at least three different probes on the microarray had a significant signature (*il1b*: 11 probes, *il10*: 3 probes, *tnfa*: 10 probes, *tnfb*: 11 probes, *cxcl-c13d* (*LOC100003911*): 7 probes, *cxcl-c1c* (*si:ch73-6k14.1*): 6 probes, *si:dkeyp-59a8.1*: 3 probes, *mmp9*: 15 probes, *ncf1*: 12 probes, *zgc:112143*: 11 probes). Induction levels of the different probes are averaged and the errorbars indicate the standard error.

Figure 5. Induction of transcription factor and signal transduction genes by *E. tarda* infection of wild type and mutant embryos. Microarray induction levels of transcription factor and signal transduction genes were compared for three individual wild type (WT) embryos and three individual *myd88* knockout mutant (MU) embryos infected with 200 CFUs of *E. tarda*. Gene expression levels of each infected embryo (WTinf and MUinf) are shown relative to a corresponding uninfected control embryo (WTcon and MUcon). For all genes shown in the figure at least three different probes on the microarray had a significant signature (*atf3*: 8 probes, *fos*: 14 probes, *irf9*: 13 probes, *irf11*: 3 probes, *junb*: 15 probes, *nfkb2*: 5 probes, *rel*: 10 probes, *relB* (*Wu:fj85b02*): 4 probes, *stat4*: 5 probes, *irak3*: 5 probes, *isg15*: 6 probes, *ripk2*: 10 probes, *tlr3*: 9 probes, *tlr5a*: 4 probes, *tlr5b*: 9 probes). Induction levels of the different probes are averaged and the error bars indicate the standard error.

surprisingly, *tnfa* was induced to similar levels in wild type and *myd88*$^{-/-}$ embryos, suggesting that expression of the two zebrafish *tnf* genes is controlled by different pathways. The complete *myd88*-dependency of *mmp9* induction and partial *myd88*-dependency of *il1b* induction was consistent between the results of qPCR (figure 3) and microarray analysis (figure 6).

Discussion

Many zebrafish models for infectious diseases have recently been developed and these have proved useful additions to mammalian models [27]. However, with the zebrafish still being a relative newcomer in immunological studies, there is a strong need to expand the toolbox of immunological reagents and mutants. Targeted knockout approaches in zebrafish, known as TILLING, allow the detection of point mutations in genes of interest in chemically mutagenized genomes. The success of this approach was first demonstrated in 2002 with the targeted inactivation of a gene central to the adaptive immune system, the recombination activating gene 1 (*rag1*) [47]. However, mutants disrupted in central pathways of the innate immune system, have not been available thus far. Here we describe a knockout mutant of the zebrafish homolog of human *MYD88*, a gene with a pivotal function in TLR and IL1R signalling. By transcriptome profiling analysis we show that zebrafish *myd88* is critically involved in the innate immune response of zebrafish embryos infected with the natural fish pathogen *E. tarda*.

An early stop codon in the *myd88*^{hu3568} mutant allele leads to deletion of the conserved C-terminal TIR domain, which is indispensible for interaction with the cytoplasmic face of the TLR and IL1R receptors. In addition, the mutation disrupts the N-terminal death domain of Myd88, required for interaction with downstream IRAK family kinases. We cannot exclude a possible effect of the presence of a partial death domain in the truncated Myd88 mutant protein. However, this is unlikely to play a major role, because we observed that *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 or by a possible feed-back mechanism by which wild type Myd88 might regulate its own expression level. In any case, based on the deletion of the TIR domain,

the truncation of the death domain and reduced mRNA expression level, the *myd88*^{hu3568} mutation can be considered a null allele.

While *myd88*$^{-/-}$ embryos and larvae showed no developmental abnormalities, it has been difficult to rear them to adulthood. We suspect that this problem can be attributed to an increased susceptibility to infections, since the few *myd88*$^{-/-}$ adults that we obtained appeared normal but died after fin clipping, similar to what has been reported for *rag1*$^{-/-}$ fish [42, 43]. Therefore, we kept heterozygous fish and analyzed individual wild type and mutant siblings from their offspring. To this end, we used a procedure for simultaneous DNA and RNA isolation from single embryos [48] and compared the gene expression profiles of embryos at 1, 2 and 3 dpf, i.e. before intestinal colonization might induce microbe-dependent differences. Besides the observed down-regulation of *myd88* mutant transcript levels, discussed above, our microarray analysis identified only a few other genes that showed consistent differences between wild type and mutant siblings at all developmental time points. Genes changed at only one developmental time point probably reflect individual variation rather than effects of the mutation. In previous studies we used triplicate pools of embryos to level out such variation (e.g. [33]), but here triplicate analyses were performed on single embryo level as our study required individual genotyping. Only four of the genes, other than *myd88*, that were differentially expressed between wild type and mutant siblings had an association with the immune system. Therefore, microarray analysis showed that, in the absence of infection, wild type and mutant siblings display largely similar basal levels of immune-related genes. In contrast, following *E. tarda* infection, the transcriptome profiles of mutants and wild type siblings were significantly different.

As expected, the induction of a large set of immune-related genes by *E. tarda* infection was found to be dependent on the presence of a functional *myd88* gene. For several of these genes, including a group of transcriptional regulators of the immune response and several effector genes of the innate immune system, the *myd88*-dependency was supported by multiple probes on our microarray platform that showed consistent expression differences between infected mutants and wild type embryos. In addition, we confirmed the *myd88*-dependency of *mmp9*, *il1b* and *irg1l* by qPCR. Of these three genes, *mmp9* and *il1b* show the highest induction levels upon *E. tarda* infection of wild embryos, in both cases in the range of 15-50-fold induction compared to uninfected controls.

Interestingly, the induction profiles of these two proinflammatory markers in mutants were different: *il1b* induction showed a partial dependence on *myd88,* while *mmp9* induction was completely absent in *myd88*$^{-/-}$ embryos. Several other genes also showed complete *myd88*-dependency, for example chemokine genes such as *cxcl-c1c* and *cxcl-c13d*, and *irak3*, a negative regulator of TLR signalling.

The *myd88*-dependency of *E. tarda* induced *mmp9*, *il1b,* and *irak3* 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*-dependent. However, in the previous studies it was not possible to discriminate between complete or partial *myd88*-dependency because a morpholino knockdown effect in itself may be incomplete. Furthermore, for other genes analyzed in the previous study, including *cxcl-c1c* and *tnfa*, we could not draw conclusions due to non-specific morpholino effects on the expression of these genes. Therefore, we have not subjected *myd88* morpholino knockdown embryos to microarray analysis. The availability of a knockout mutant now solves these problems. Microarray analysis of *myd88*$^{-/-}$ embryos clearly demonstrates the *myd88* dependency of *cxcl-c1c*, whereas *tnfa* induction by *E. tarda* infection appears *myd88* independent. For the first time, we also show that induction of the flagellin receptor homologues, *tlr5a* and *tlr5b*, is directly dependent on *myd88*-mediated signalling. Several genes identified as (partially) dependent on *myd88* in the *E. tarda* infection study overlap with genes identified as *traf6*-dependent based on morpholino knockdown in the context of *S. typhimurium* infection, for example *mmp9*, *il1b*, *tnfb*, *ncf1*, zgc:*114032* (complement factor B), zgc:*112143* (TNFα-induced protein 9), and *tlr5b*. Therefore, these genes can now be placed as targets of a *myd88-traf6* signalling pathway.

In multiple studies investigating macrophages and spleen cells from *MyD88* knockout mice the expression of *Tnfα* in response to different stimuli (IL-1, LPS, *S. aureus* infection, Staphylococcal enterotoxin A) was found to be critically dependent on *MyD88* [5, 7, 10, 49-51]. Zebrafish contain two copies of the gene encoding Tnfα, designated *tnfa* and *tnfb*, both of which are induced by *E. tarda* infection in wild type embryos. Interestingly, we observed that only the induction of *tnfb* was dependent on *myd88*, whereas *tnfa* induction levels where similar between wild type and mutant siblings. The difference in Myd88 dependency of

zebrafish *tnfa* and mouse *Tnfα* may be linked to functional divergence between these cytokines in fish and mammals. Recombinant zebrafish Tnfa protein and recombinant Tnfα proteins from seabream and carp failed to exert classical proinflammatory effects on phagocytes [52, 53]. Instead, fish Tnfα is thought to be predominantly involved in the recruitment of phagocytes rather than in their activation and its main proinflammatory effects are thought to be mediated through the activation of endothelial cells [53].

In agreement with studies of cells and organs from *MyD88*-deficient mice the induction of several interleukin and chemokine genes was *Myd88* dependent in zebrafish embryos [5, 7, 10, 50, 51, 54]. In some cases induction was not completely abrogated in mutant embryos. For example, as mentioned above, *il1b* induction appeared to be mediated both by *Myd88*-dependent and *MyD88*-independent pathways. It is currently unknown if these pathways operate simultaneously or sequentially in the same cell type, or that distinct cell types or organs in the embryo induce *il1b* either in a *Myd88*-dependent or *Myd88*-independent manner. In this respect, it is of interest to note that microarray analysis of spleen and liver from septic wild-type and *Myd88*-deficient mice revealed striking differences. Whereas sepsis-induced gene expression in the liver was strongly dependent on Myd88, responses in the spleen were largely independent of *Myd88*, thus indicating an organ-specific contribution of *Myd88* to gene expression [54].

In previous *S. typhimurium* infection studies, we concluded that *ifnphi1* and *il8* were induced independently of *myd88* morpholino knockdown. In the present study we observed no induction of *il8* upon *E. tarda* infection and *ifnphi1* was induced only in one out of three wild type embryos, thus not permitting conclusions about the relationship to *myd88*. *E. tarda* infection also did not induce zebrafish homologues of interferon-responsive genes that were shown to be induced through a TRIF-dependent pathway in mice [50]. However, our microarray analysis indicated that approximately 61% of the genes induced by *E. tarda* infection are induced in a *myd88*-independent manner. This is much less than in a study of LPS stimulation, where approximately 75% of the transcriptional response was similar between macrophages taken from *Myd88* null mice and wild type mice [49].

In addition to the large set of genes whose induction during *E. tarda* infection was impaired by *myd88* deficiency, we also observed gene groups that

were repressed to a larger extent in *myd88* mutants than in wild type embryos and observed a positively influence of *myd88* on the expression of other gene groups. Similar observations were made in mice knockout mutants [49, 54, 55]. In particular, interferon-regulated genes were found to be overexpressed in septic *Myd88*-deficient organs compared to wild type organs [54]. In another study, overexpression of genes involved in post-transcriptional processes, including mRNA splicing and translation, was observed in LPS-stimulated macrophages from *Myd88* knockout mice [55]. The overexpression of these genes was suggested to act as a compensatory mechanism for the reduced transcriptional activation of many immune response genes. In our study, we did not observe increased expression of interferon-regulated genes or of a gene set involved in post-transcriptional processes, but many metabolic genes and genes involved in signal transduction were induced to higher levels in *E. tarda*-infected mutant embryos than in wild type siblings. These observations are of great interest for follow up studies.

Because *E. tarda* infection of zebrafish embryos is lethal within one to two days, this infection model is less useful to analyze the effect of *myd88* knockdown on bacterial proliferation or host survival. In future work, infection models using attenuated mutants of *E. tarda* or pathogens causing chronic infection, such as *Mycobacterium* marinum, will be more suitable to further study the *myd88* knockout effect. Preliminary results already point to a critical role of *myd88* in control of *M. marinum* infection. Currently, we are using this model to investigate the link between the *myd88*-dependent innate immune response and autophagy, a process recently shown to play a significant role in control of mycobacterial and other pathogenic infections by the host immune system. The *myd88* knockout mutant will also be highly useful to study the interplay with other components of TLR and IL1R signalling and for application as an immune-compromised zebrafish model in cancer studies.

Materials and methods

Zebrafish husbandry

Zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). Embryos were grown at 28.5-30℃ in egg water (60 µg/ml Instant Ocean salts). For the duration of bacterial injections embryos were kept under anaesthesia in egg water containing 0.02 % buffered 3-aminobezoic acid ethyl ester (tricaine; Sigma-Aldrich).

Myd88 mutant and Genotyping

The *myd88*^{hu3568} mutant line was isolated in the TILLING programme of the Hubrecht Institute led by Dr. Edwin Cuppen. Heterozygous carriers of the mutation were outcrossed twice against wild type (AB strain), and subsequently were incrossed twice. Heterozygous fish of the resulting family were used to produce embryos for the infection experiments. Genotyping zebrafish embryos was done by restriction analysis after PCR. 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 (New England Biolabs). Fragments were analysed on 2% agarose (Difco) gel. 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 two fragments of 200 and 100 bp.

Infection conditions

Edwardsiella tarda labelled with mCherry [56] was grown over night in tryptic soy broth (TSB, Difco) supplemented with 8 µg/ml tetracycline at 28 ˚C. For caudal vein injection experiments, *E. tarda* was washed and subsequently suspended in PBS amended with 2% polyvinylpyrrolidone (PVP, Sigma) to a final concentration of 10^8 CFU/ml. Embryos were manually dechorionated at 24 hpf. Approximately 200 CFUs of *E. tarda* were injected into the blood island after the onset of bloodflow, or PBS amended with 2% PVP was injected as a control. After injection, embryos were kept at 28 ℃ and snap-frozen in liquid nitrogen at the required time points.

DNA-isolation, RNA isolation, labelling, and hybridization

The single embryo RNA isolation procedure was according to de Jong *et al.* [48]. Embryos for RNA isolation were snap-frozen in liquid nitrogen and subsequently stored at -80°C. Embryos were homogenized in 300 µl TRI reagent (Ambion). After chloroform extraction the aqueous phase was stored at -80°C until the embryos were genotyped.

DNA isolation from the trizol phase was done according to the alternate DNA isolation protocol of the TRI reagent DNA/protein isolation protocol (Ambion), using glycogen as a co-precipitant.

RNA from the aqueous phase was purified using the RNeasy MinElute Cleanup kit (Qiagen) according to the RNA clean-up protocol.

Amino-allyl-modified amplified RNA (aRNA) was synthesized in one amplification round from 500 ng of total RNA using the Amino Allyl MessageAmp II aRNA Amplification kit (Ambion). Subsequently 6µg of amino-allyl-modified aRNA was used for coupling of monoreactive Cy3 and Cy5 dyes (GE Healthcare) and column purified. The dual-color hybridization of the microarray chips was performed at the Microarray Department of the University of Amsterdam according to Agilent protocol G4140-90050 version 5.7 (www.agilent.com) for two-color microarray-based gene expression analysis.

cDNA synthesis and quantitative reverse transcriptase PCR

cDNA synthesis reactions were performed in a 10-µl mixture of 100 ng of RNA, 2 µl of 5x iScript reaction mix (Bio-Rad Laboratories), and 0.5 µl of iScript reverse transcriptase (Bio-Rad Laboratories). The reaction mixtures were incubated at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min.

Real-time PCR was performed using the Chromo4 Real-time PCR detection system (Bio-Rad Laboratories) according to the manufacturer's instructions. Each reaction was performed in a 25-µl volume comprised of 1 µl of cDNA, 12.5 µl of 2x iQ SYBR Green Supermix (Bio-Rad Laboratories), and 10 pmol of each primer. Cycling parameters were 95°C for 3 min to activate the polymerase, followed by 40 cycles of 95°C for 15 s and 59°C for 45 s. Fluorescence measurements were taken at the end of each cycle. Melting curve analysis was performed to verify that no primer dimers were amplified. All reactions were performed as technical duplicates. For normalization, peptidylprolyl isomerase

A-like (*ppial*), which showed no changes over the infection time course series, was taken as reference. Results were analyzed using the $\Delta\Delta C_t$ method. Sequences of forward and reverse primers are as in chapter 3.

Microarray analysis

The microarray slides were custom-designed by Agilent Technologies as previously described [33]. The slides contained in total 43,371 probes of a 60-oligonucleotide length.

Samples from *E. tarda* or control injections were labelled with Cy5 and hybridized against a Cy3-labeled common reference that consisted of a mixture of samples from the injection experiments. *E. tarda* and control injections were analyzed in triplicate. Microarray data were processed from raw data image files with Feature Extraction Software 9.5.3 (Agilent Technologies). Processed data were subsequently imported into Rosetta Resolver 7.0 (Rosetta Biosoftware) and subjected to default ratio error modelling. The raw data were submitted to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under accession no. …. 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. Significance cut-offs for the ratios were set at 2-fold change at $p < 10^{-5}$ for the probes.

One dimensional hierarchical cluster analysis was performed using MultiExperiment Viewer (MeV, v 4.5.1) [57] with the settings to Pearson correlation, average linkage and leaf order optimization.

Acknowledgements

We thank Dr. Edwin Cuppen for the *myd88*^{hu3568} mutant, Dr. Philip Klesius (USDA, Auburn, AL) for providing *E. tarda* strain FL6-60, and Davy de Wit and Ulrike Nehrdich for fish care. This work was supported by the European Commission 6th framework projects ZF-MODELS (LSHG-CT-2003-503496) and ZF-TOOLS (LSHG-CT-2006-037220).

Supplementary data

Supplementary tables can be found online at: http://tinyurl.com/ch4suptables

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