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Transcriptome profiling and functional analyses of the zebrafish embryonic innate immune response to *Salmonella* infection

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Abstract

Due to the clear separation of innate immunity from adaptive responses, the externally developing zebrafish embryo represents a useful in vivo model for identification of innate host determinants of the response to bacterial infection. Here we performed a time-course transcriptome profiling study and gene ontology analysis of the embryonic innate immune response to infection with two model Salmonella strains that elicit either a lethal infection or an attenuated response. The transcriptional response to infection with both the lethal strain and the avirulent LPS O-antigen mutant strain showed clear conservation with host responses detected in other vertebrate models and human cells, including induction of genes encoding cell surface receptors, signalling intermediates, transcription factors and inflammatory mediators. Furthermore, our study led to the identification of a large set of novel immune response genes and infection markers, the future functional characterization of which will support vertebrate genome annotation. From the time series and bacterial strain comparisons, matrix metalloproteinase genes, including \textit{mmp9}, were among the most consistent infection-responsive genes. Purified Salmonella flagellin also strongly induced \textit{mmp9} expression. Using knockdown analysis we showed that this gene was downstream of the zebrafish homologs of the flagellin receptor TLR5 and the adaptor MyD88. In addition, flagellin-mediated induction of other inflammation markers, including \textit{il1b}, \textit{il8} and \textit{cxcl-C1c}, was reduced upon TLR5 knockdown as well as expression of \textit{irak3}, a putative negative TLR pathway regulator. Finally, we showed that induction of \textit{il1b}, \textit{mmp9} and \textit{irak3} requires MyD88-dependent signalling, while \textit{ifn1} and \textit{il8} were induced MyD88-independently during Salmonella infection.

Introduction

The innate immune system represents the evolutionary ancient part of vertebrate immunity and relies on germline encoded receptors, commonly referred to as pattern recognition receptors (PRRs), to mediate immune responses to pathogenic microorganisms. Triggering of these receptors activates a variety of signal transduction pathways ultimately resulting in large alterations of the host transcriptome profile, of which the dynamic complexity and underlying mechanisms are still poorly understood, especially at the whole organism level.

An essential class among the investigated PRRs are the Toll-like receptors. Detection of microorganisms by TLRs is facilitated through specific interaction of the members of this family with evolutionary conserved microbial molecules that are not found in higher eukaryotes, e.g. lipopolisaccharide (LPS) from the outer membrane of gram-negative bacteria (TLR4), flagellin from bacterial flagella (TLR5) or unmethylated CpG dinucleotides commonly found in bacterial DNA (TLR9) (1-3). Stimulation of TLRs by their ligands leads to the recruitment of adaptor pro-
teins to the receptors. Differential utilization of the adaptor molecules by the TLRs causes specific activation of a range of transcription factors such as nuclear factor-κB (NF-κB), activator protein 1 (AP-1) and interferon regulatory factor (IRF) 3, 5 and 7 through distinct signalling pathways eventually leading to the downstream activation of pro-inflammatory cytokines (4). To date five adaptor proteins involved in TLR signalling have been described in human: myeloid differentiation factor-88 (MyD88), MyD88 adaptor-like protein (Mal/TIRAP), Toll/IL1 receptor (TIR) domain-containing adaptor protein inducing IFNβ (TRIF/TICAM1), TRIF-related adaptor molecule (TRAM/TICAM2) and sterile α- and armadillo motif-containing protein (SARM) (4). Among all adaptors, MyD88 is the most commonly used adaptor and signalling through MyD88 has been implicated for all human TLRs with the exception of TLR3 that was shown to signal in a TRIF-dependent and MyD88-independent fashion (4, 5). Furthermore, MyD88 is involved in signal transduction of the interleukin 1 receptor (IL-1R) and is associated with interferon-γ receptor signalling (IFN-γR) leading to p38 activation (6, 7).

In recent years the zebrafish (*Danio rerio*) embryo system has emerged as a new model to study vertebrate innate immunity, offering several advantages that complement mammalian model systems. The transparent character of the externally fertilized zebrafish embryo in combination with fluorescently labelled immune cells and bacteria facilitate the study of host microbe interaction and inflammation processes in the living organism (8-14). The efficiency at which infections and chemical treatments in zebrafish can be performed at a large-scale allows identification of novel microbial virulence factors and high throughput compound screens to investigate disease mechanisms (15, 16). Moreover, the zebrafish system is particularly suitable for large-scale forward and reverse genetic screens aimed at the identification of genes with novel functions in development of the immune system or in the immune response (17-19).

Analysis of the immune system of the zebrafish revealed a fully developed adaptive and innate immune system showing notable similarities to the mammalian equivalent (20-25). An active innate immune system is detectable already at day one of zebrafish embryogenesis demonstrated by the appearance of macrophages originating from the lateral plate mesoderm (26). Phagocytic activity of these cells was demonstrated upon bacterial encounter (8, 26, 27). By contrast, a functionally mature adaptive immune system is not active during the first three weeks of zebrafish development (28-30). This clear temporal separation in zebrafish embryos provides a convenient system for in vivo study of the vertebrate innate immune response to infection independently from the adaptive immune response. To validate this model, we previously demonstrated that zebrafish embryos express a broad range of TLRs and adaptors. Furthermore, we demonstrated a conserved immune function for the TLR adaptor Myd88 by showing elevated susceptibility of the zebrafish embryo to an avirulent strain of *Salmonella enterica* serovar Typhimurium (hereafter referred to as S. typhimurium) upon morpholino mediated knockdown (31). Zebrafish embryos
also release conserved cytokines and chemokines, such as TNFα, IL-1β and IL-8, in response to bacterial infections (32, 33). In addition, zebrafish embryos express a virus-induced interferon system ancestral to that of other vertebrates (34). However, knowledge of infection markers is still limited and the global transcriptional response to an infection has not been previously investigated in zebrafish embryos.

In recent years many infection systems for zebrafish have been developed (35-37). In this paper we used *S. typhimurium* as a case study for gram-negative infections. An infection model for this well studied human pathogen in zebrafish embryos has previously been established and it was demonstrated that an LPS-mutant that shows an attenuated pathogenesis in mouse studies shows also attenuated infection in zebrafish embryos (9). Here we report on a time resolved transcriptome profiling study of the zebrafish embryonic host immune response to *S. typhimurium* wild type and LPS-mutant infection. Gene ontology comparisons of expression signatures in infections of zebrafish embryos and human and other vertebrate systems revealed a substantial overlap, further underscoring the validity of the zebrafish model. Furthermore, we identified a large set of novel immune response genes and infection markers, providing a strong basis for future research. Finally, we used infection markers resulting from this study to investigate the functions of Tlr5 and MyD88 by knockdown analysis using morpholino antisense oligonucleotides.

### Results

**Global changes in gene expression upon *Salmonella typhimurium* infection**

To characterize the host response of zebrafish embryos to bacterial infection we performed a time-resolved transcriptome analysis of zebrafish embryos infected with either the *Salmonella typhimurium* wild type strain TL2 (wt) or an isogenic LPS mutant (Ra) (9). Zebrafish embryos were systemically infected at the onset of blood circulation (27 hpf) by microinjection of 250 cfu of DsRed-labelled bacteria into the caudal vein or were mock injected with PBS. Injected embryos were sacrificed after incubation periods of 2, 5, 8 and 24 h and microarray experiments were performed using a custom designed 4x44k Agilent zebrafish platform. Datasets of three independent experiments revealed a strong response of the embryo to systemic infection with *S. typhimurium* compared to the mock injected control group (Fig. 1, Supplementary table II). The response to *S. typhimurium* wt was characterized by a gradually increasing number of responsive genes over the time course of the experiment, peaking at 24 hours post infection (hpi). A similar but attenuated response was visible for the Ra infection over the first 8 h. Furthermore, in contrast to the wt infection, a clear decline of responsive genes was observed at 24 hpi (Fig. 1). The temporal expression profiles correspond well to the course of *S. typhimurium* wt and Ra mutant infections in the zebrafish embryo as observed by fluorescence imaging.
As shown in supplementary figure 1, the transient infection caused by the Ra mutant was nearly eliminated at 24 hpi, while a strong accumulation of DsRed-labelled wt bacteria was observed at 24 hpi resulting in lethality around 30 hpi.

To further analyse the response to the wt and Ra strain over the first 8 h of the infection we performed two dimensional hierarchical cluster analysis. In the first dimension, the *S. typhimurium* wt and Ra infection profiles clustered together according to the experimental time points (Fig. 2A), indicating that zebrafish embryos respond similarly to infections with both strains. In the second dimension, we could distinguish three major clusters of genes showing different trends in expression over the time course (Fig. 2A-C, Supplementary table V). Genes in cluster 1 exhibited a successive down-regulation over the first 8 h of the infection whereas genes grouped in cluster 2 were generally induced over time. This cluster was further partitioned into four sub-clusters termed 2a-2d (Fig 2A and 2D-E). Genes in cluster 2a showed induction over all time points. However, induction at 5 and 8 hpi was more pronounced after *S. typhimurium* wt infection. In contrast genes in cluster 2b exhibited a broad down-regulation at 2 hpi followed by a successive up-regulation peaking at 8 hpi. In general down-regulation of genes in cluster 2b was stronger for the Ra infection. A transient response was observed for genes in cluster 2c, showing an induction peak at 5 hpi followed by a declined at 8 hpi. Genes in cluster 2d showed a
Zebrafish embryonic transcriptome response to infection

significant down-regulation at 2 hpi, followed by relatively low induction at 5 and/or 8 hpi. Finally, genes in cluster 3 showed a strong induction at 2 hpi with a subsequent decrease at 5 and 8 hpi.
In conclusion, we observed a strong response to *S. typhimurium* infection in the zebrafish embryo with largely similar temporal expression profiles upon wt or Ra infection during the first 8 h after inoculation. Even though the Ra strain elicits only a transient infection and the wt infection becomes lethal, no gene groups with clearly anti-correlated behaviour were revealed by hierarchical cluster analysis. Instead, differences in the host response to wt and Ra infection appeared to be limited to differences in trend at a single time point and to quantitative differences in up- or down-regulation.

Validation of microarray data by qRT-PCR and in situ hybridisation

To validate the microarray data quantitative real time PCR (qRT-PCR) was performed on six genes that were significantly induced upon *S. typhimurium* infection (Fig. 3). The expression levels for *mmp9* (matrix metalloproteinase 9, NM_213123), *il1b* (interleukin 1, beta, NM_212844), *LOC100002946* (similar to interleukin 8, XM_001342570) hereafter referred to as *il8*, *tnfa* (tumor necrosis factor a, NM_212859), *ifn1* (interferon 1, NM_207640) and *irak3* (interleukin-1 receptor-associated kinase 3, NM_001099421) were evaluated on the RNA samples of the 8 hpi time point previously used for the microarray study. The expression levels were normalized to *ppial* (peptidylprolyl isomerase A like, NM_199957) (44), which showed no changes over the infection time course. Expression levels are presented as the relative induction in the infected group compared to the control group (Fig. 3A). In agreement with the
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Microarray data, all genes tested in the qRT-PCR assay showed clear induction upon *S. typhimurium* wt infection and showed a lower response (*mmp9, il1b, il8* and *irak3*) or no significant response (*tnfa, ifni*) after infection with the Ra mutant (Fig. 3B).

In addition to the quantitative analysis by qRT-PCR we also tested several genes by whole mount in situ hybridization to further validate the microarray data and to add spatial information to the expression of infection-induced genes. Embryos were challenged with *S. typhimurium* wt by injection into the caudal vein close to the urogenital opening (I,J,K,L), or mock-injected with PBS at the same location (E,F,G,H). All embryos are oriented anterior to the left and dorsal to the top. cht, caudal hematopoietic tissue; cvs, cranial vascular system; i, location of injection into somite tissue; ppd, posterior pronephric duct; ve, vessels of the eye.

**FIGURE 4.** Analysis of spatial expression patterns of *S. typhimurium*-induced genes. Expression patterns of the indicated genes were analysed at 8 hpi by whole mount in-situ hybridization. Embryos were injected at 27 hpf with *S. typhimurium* wt bacteria into the caudal vein (A,B,C,D), or injected with *S. typhimurium* wt bacteria into the somite tissue of the tail above the urogenital opening (I,J,K,L), or mock-injected with PBS at the same location (E,F,G,H). All embryos are oriented anterior to the left and dorsal to the top. cht, caudal hematopoietic tissue; cvs, cranial vascular system; i, location of injection into somite tissue; ppd, posterior pronephric duct; ve, vessels of the eye.
phric duct (Fig. 4A). Expression of cxcl-C1c (Fig. 4B) was predominantly visible in single cells located in the vascular system of the embryonic eyes. A second area of elevated expression was observed around the caudal vein, also defined to single cells. Like cxcl-C1c, also the zebrafish homolog of irak3 (Fig. 4C) showed an expression pattern restricted to the cranial vascular system of the embryo. However, a small number of embryos displayed a diverse pattern with a strong expression restricted to an area in the tail that corresponds with the site of the bacterial injection. To further investigate this observation we repeated the assay with a revised infection strategy. Bacteria were now locally injected into the somite tissue of the tail at 27 hpf leading to a confined accumulation of the bacteria at that site. Analysis of the expression pattern at 8 hpi confirmed the observed expression of irak3 (Fig. 4K) in the tissue closely surrounding the infection site. Similarly, socs3 also showed locally increased expression upon injection of bacteria into somite tissue (Fig. 4L). Local infection increased ccl-C5a (Fig. 4I) in the pronephric duct similar as observed upon blood infection (Fig. 4A), however, ccl-C5a expression did not accumulate around the infection site. In contrast, cxcl-C1c (Fig. 4J) did exhibit localized expression around the infection site, apparently restricted to single cells. To exclude the possibility that the observed expression patterns were provoked by local damage of the tissue that occurs upon bacterial injection we also investigated the expression of all four genes in mock (PBS) injected embryos (Fig. 4E-H). No signal was detected in the controls (n=5 per gene) validating the specific host response towards the bacterial infection. Furthermore, the in situ hybridization results show that transcriptome profiling at the whole embryo level is highly suited to identify gene expression changes that are restricted to specific tissues or cell types.

Statistical testing for enrichment of gene ontology groups
To perform an unbiased functional annotation of the genes identified by microarray analysis we used eGOn, a web-based gene ontology (GO) tool (40). The eGOn software classifies user input gene lists by GO criteria for Biological Process (BP), Molecular Function (MF) and Cellular Component (CC), it produces hierarchical trees of GO-terms in these three categories, and it allows statistical testing for enrichment or under representation of specific GO-terms in the input lists. First we used the eGOn software to perform master-target statistical tests on the clusters of genes described above (Fig. 2), comparing the UniGene identifiers of each cluster (targets) versus all UniGene identifiers present on the chip (master). Master-target testing of the three main clusters (clusters 1-3 in Fig. 2) showed that the GO-terms “immune system process” and “response to stimulus”, at level 2 in the hierarchical tree for BP, were significantly enriched in cluster 2 that contained the majority of the up-regulated genes. Next we analysed the responses to the wt and Ra strains separately and at each individual time point of the infection. In agreement with the result obtained for cluster 2, master-target tests on the up-regulated UniGene sets showed that the BP GO-terms “immune system process” and “response to stimulus”
A master-target statistical test using eGOn software was performed with input gene lists of zebrafish UniGene identifiers or the UniGene identifiers of their human homologs. The master input lists contained all UniGene identifiers present on the microarray (19122 zebrafish UniGenes, for 10620 of which the human homologs could be identified). The target lists contained the UniGene identifiers that were ≥ 1.5-fold up-regulated (p ≤ 0.0001) at different time points of S. typhimurium wild type or Ra mutant infection. The table indicates the number of genes in each list that are associated with the indicated GO-terms. Highlighted numbers are significantly enriched in the target list compared to the master (p < 0.01). It should be noted that genes can be associated with more than one GO-term. For example, enrichment of the GO-terms “development” and “growth” in the target list of S. typhimurium wild type 2h infection is due to the presence of genes such as socs1 and 3, c7, il10, mmp9, fos, pim2 and nfkb2, which also fall under the GO-terms “immune response” and/or “response to stimulus.”
were significantly enriched at multiple time points of both the wt and Ra infections (Table I). In the MF tree, the level 2 GO-term “enzyme regulator activity” was enriched (Supplementary table VI), and in the CC tree there was enrichment of GO-terms associated with extracellular compartments (Supplementary table VII).

Although master-target testing of the up-regulated UniGenes identified several enriched GO-terms with obvious relevance to an infection study, we noted that the number of UniGene identifiers associated with each of these GO-terms was relatively low. We considered that this might be due to poor annotation of the zebrafish genome and that we might strengthen the results by performing a GO analysis of the human homologs of the zebrafish genes, thereby taking advantage of the much better annotated human genome. To this extent we developed a software tool with which the human homologs of the zebrafish UniGene list could be automatically retrieved from the NCBI HomoloGene database. Using this approach we could identify the human homologs of 56 % of the zebrafish UniGene clusters present on the chip. As we had anticipated the total number of associated GO-terms was 1.6 fold higher. Furthermore, the relevant GO-terms “immune system process” and “response to stimulus” were associated with 230 and 817 of all human homologs compared to only 72 and 213 of all zebrafish UniGenes. Repeating the master-target tests at the level of the human homologs showed that a larger number of genes was now associated with each of the enriched GO-terms in the up-regulated signature sets of the infection time course. For example, in the *S. typhimurium* wt infection signature of 24 hpi there were 73 human homologs with the GO-term “immune system process” compared to 19 zebrafish UniGenes, and 225 human homologs with the GO-term “response to stimulus” compared to 44 zebrafish UniGenes (Table I). Additionally, the GO-terms “biological regulation” (BP) and “transporter activity” (MF) were significantly enriched at multiple time points in the analysis of the human homologs, while not in the analysis of the zebrafish UniGenes.

For a more detailed GO analysis of the up- and down-regulated gene groups at the different time points of the infection study, we used the DAVID tools for Functional Classification and Functional Annotation Clustering (41). The results of DAVID analyses are described in the supplementary material 1. In summary, DAVID analyses of the infections with both strains demonstrated rapid induction over the first 8h of the time course of an increasing number of gene groups encoding transcription factors, signalling molecules, complement and acute phase response proteins, proteinases and proteinase inhibitors, and solute carriers. At 24 hpi, the near-lethal wild type infection led to the additional induction of apoptotic and anti-apoptotic genes as well as negative regulators of cell cycle and proliferation, whereas gene groups involved in primary metabolic processes and DNA replication were down-regulated.

**Comparison with expression data from infection studies using human cell lines**

To compare the *S. typhimurium*-induced gene profiles of zebrafish with gene profiles
of infected human cell lines, we took advantage of a study by Jenner and Young (42) who systematically compared transcription profiling data from 32 studies that involved 77 different host-pathogen interactions. By cluster analysis of these data the authors identified an expression signature of 511 genes, which they designated 'the common host response' as most of these genes were induced in many different human cell types upon exposure to several different pathogens. We sought to identify the zebrafish homologs of the genes in this common host response cluster using the ZFIN and NCBI Gene and Homologene databases, and found that 322 out of the 511 genes were represented on our zebrafish microarray. Of these 39% were up-regulated (≥ 1.5-fold, \( p < 0.0001 \)) at one or more time points of \( S. \) typhimurium wild type and/or Ra infection (Supplementary table III). The overlap included genes encoding matrix metalloproteinases (e.g. \( \text{mmp9, mmp13} \)), adhesion molecules (e.g. \( \text{itga5, lgals9} \)), co-stimulatory molecules (e.g. CD83), antigen processing molecules (e.g. \( \text{tap2, psmb} \) family members), prostaglandin biosynthetic enzymes (e.g. \( \text{ptgs2} \)), signalling intermediates (e.g. \( \text{tradd, myd88, traf6, dusp} \) family members), apoptotic and anti-apoptotic molecules (e.g. \( \text{casp} \) family members, \( \text{mcl1, birc2, cflar} \)), transcription factors (e.g. members of the \( \text{NFkB, Jun, Fos, ATF, IRF and STAT} \) families, BCL6, Cebpg, xbp1), interferons and interferon-stimulated genes (e.g. \( \text{isg15(gp12), mx1/2 homologs} \)) and various chemotactic, proinflammatory and other cytokines (e.g. ho-
mologs of \( IL1\beta, IL8, IL10, TNF, TNFSF10, ccl \) and \( cxcl \) chemokines). In conclusion, many important immune markers respond in a similar way in human cell cultures and in the early embryonic zebrafish system, thus allowing further functional \textit{in vivo} studies of these genes.

Identification of novel putative immune response genes

As the next step we sought to determine which proportion of the genes regulated by \textit{S. typhimurium} infection had not been previously linked to a function in the immune response. To this extent we grouped all genes that were differentially expressed over the first 8 h of the infection time course (i.e. all genes included in the cluster analysis of Fig. 2) into four distinct categories (Fig. 5, Supplementary table VIII). The first category contained all genes that were identified as immune specific by means of GO annotation and by overlap with the common host response gene set as described above (wt 169 genes, Ra 119 genes). In the second category we gathered those genes that have a described immune function in vertebrates but were missed in category 1, e.g. \textit{cebpb} and \textit{socs3a} (wt 34 genes, Ra 28 genes). The third category consists of genes that were functionally annotated in zebrafish but were not yet linked to immune defence processes (wt 223 genes, Ra 152 genes). The remaining genes, grouped in category 4, still lack any functional annotation (wt 1079 genes and Ra 763 genes). The distribution of genes over the four categories (Fig. 5) shows that a substantial proportion (50%) of all annotated genes that we identified upon \textit{S. typhimurium} wt or Ra infection were correlated to an immune function (categories 1 and 2). Still, considering all genes that were regulated over the first 8 h upon infection, the majority of genes (approximately 70%) have not yet been functionally characterized in the context of immunological processes and therefore represent novel putative immune response genes.

GenMapp-based Toll-like receptor pathway analysis reveals specific regulation of zebrafish TLR5 and downstream signalling components

To further examine the immune response profiles of \textit{S. typhimurium} wt and Ra infection at the level of a single signal transduction pathway we performed a map based

\textbf{FIGURE 6.} GenMapp analysis of gene expression responses in the TLR pathway upon \textit{S. typhimurium} wt infection. Expression profiles of the 2, 5, 8 and 24 hpi time points (infected versus control, \( p \leq 0.0001 \) and fold changes \( \geq 1.5 \) and \( \leq -1.5 \)) were simultaneously mapped on the TLR pathway. Gene boxes are colour coded from left to right with the 2, 5, 8 and 24 hpi expression data. Up-regulation is indicated in yellow, down-regulation in blue and unchanged expression in grey. White denotes genes that were not represented on the array platform. The pathway is based on knowledge of TLR signalling in mammalian species and it should be noted that most interactions remain to be experimentally confirmed in zebrafish. GenMapp analysis of infection with the Ra mutant strain is shown in supplementary Fig. 2.
pathway analysis using the GenMapp software package (Gene Map Annotator and Pathway Profiler, http://www.genmapp.org) (43). GenMapp provides a platform to visualize gene expression datasets on customised maps of a desired signalling cascade and thereby allows the interpretation of gene expression changes in the context of a biological pathway. We used GenMapp for the analysis of the Toll-like receptor pathway, one of the most important pathways of the innate immune system (Fig. 6 and Supplementary figure 2). Zebrafish homologs of the TLR pathway components used to create the GenMapp were identified by searching the database of ZFIN (http://www.zfin.org) or the Gene and HomoloGene databases at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) (Supplementary

**FIGURE 7.** Response to *S. typhimurium* flagellin challenge in Tlr5a/b knock down embryos. Embryos were injected at the single cell stage with tlr5a/b or control morpholinos (mo) or were untreated (wt). The three groups were challenged at 27 hpf by flagellin injection or injection of toxin-free water as a control. Gene expression levels of *mmp9* (A), *cxcl-C1c* (B), *irak3* (C), *il8* (D), *il1b* (E) at 1 hpi were determined by qRT-PCR and are shown as the fold induction of flagellin stimulation versus the water control. Values are the mean ± S.D. of three independent experiments. The untreated and control mo groups did not behave significantly different. Differences between the tlr5a/b mo and control mo groups were significant by two way ANOVA analysis (p<0.01).
The responses upon *S. typhimurium* wt (Fig. 6) and Ra (Supplementary figure 2) infection showed a similar trend between 2 to 8 hpi with a strong initial response at 2 hpi (14 genes wt, 11 genes Ra), a decrease at 5 hpi (6 genes wt, 2 genes Ra) followed by a new increase at 8 hpi (14 genes wt, 8 genes Ra). At 24 hpi the wt infection peaked with 38 regulated genes whereas the Ra infection showed a decline (4 genes), similar to what was observed for the global changes in gene expression described above (Fig. 1). GenMapp analysis of the TLR pathway revealed a conserved pattern between wt and Ra mediated infection. Both bacterial strains likewise induced the expression levels of genes encoding members of the NF-κB protein complex (*rel, rela, relb, and nfkβ2*) that plays a key role in the transcriptional activa-
At the same time also *nfkbiaa* and *nfkbiab* genes that encode the zebrafish homologs of the NF-κB transcription factor inhibitor NFKBIA are induced. Other transcription factor genes responsive to both strains include the *fos, jun, atf3, atf4* and *atf5* genes encoding components of the AP1 transcription factor complex. Genes that have been implicated in the negative regulation of the TLR-pathway in higher vertebrates like *socs1, socs3a* and *socs3b, irak3, trafi* and *pik3cg* (phosphatidylinositol 3-kinase gamma) showed a clear activation from the earliest time point on. Finally, among the members of the Toll-like receptors that we previously identified in zebrafish (23), only *tlr5a* and *tlr5b* (the counterparts of the human *tlr5* gene) show a broad up-regulation over the course of the infection.

**TLR5a/b is required for the activation of distinct host defence genes upon flagellin stimulation**

Zebrafish *tlr5a* and *tlr5b* are two highly homologous genes located in tandem on chromosome 20 (Zv7_scaffold2018.4). To elucidate their role in the embryonic immune response we performed knockdown experiments simultaneously using two morpholinos that specifically target the *tlr5a* and *tlr5b* mRNAs. Preliminary results indicated that Tlr5a/b was not required for the induction of inflammation markers such as *il1b, il8* and *mmp9* upon *S. typhimurium* infection, which is not surprising since many other TLRs are involved in sensing of gram-negative bacteria.

To further investigate Tlr5a/b function, we next addressed the question if the presumed ligand flagellin is able to elicit an immune response in the zebrafish embryo. Embryos were challenged by injection of 4nl (100μg/ml) purified *S. typhimurium* flagellin (Invivogen) or solvent (toxin-free water) into the caudal vein at 27 hpf and the effect on expression of infection marker genes was investigated by qRT-PCR. Expression levels of *tlr5a, tlr5b, ifn1, irak3, il1b, il8, cxcl-C1c* and *mmp9* were used as readout for qRT-PCR analyses. In wild type embryos the expression of *il1b, il8, cxcl-C1c, mmp9* and *irak3* was induced within 1 h of flagellin stimulation, while the expression of *tlr5a, tlr5b* and *ifn1* was not induced over a time course of 5 h (Fig. 7 and Supplementary figure 3). Results of three independent experiments showed that knockdown of Tlr5a/b led to an almost complete block of gene regulation of *mmp9, cxcl-C1c* and *irak3* upon flagellin stimulation (Fig. 7A-C). Furthermore *il8* and *il1b* showed a reduced response to the flagellin challenge after *tlr5a/b* knockdown (Fig. 7D-E). These data demonstrate that flagellin is a bona fide ligand of the zebrafish Tlr5a/b receptor and that Tlr5a/b plays a pivotal role in the activation of specific host defence genes upon stimulation.

**Myd88 knockdown reveals Myd88-dependent and -independent gene activation upon bacterial infection**

Signal transduction upon TLR stimulation is dependent on a group of five TIR domain containing adaptor molecules. Among these MyD88 is the most commonly used adaptor participating in signal transduction processes with all TLRs except
TLR3 in mammals (4). To further investigate the role of the TLR pathway during bacterial infection we performed myd88 knockdown studies followed by S. typhimurium wt challenge and analyzed expression of the same infection marker set as used in the tlr5 knockdown analysis. Due to the stochastic nature of bacterial infections, the absolute levels of gene induction that were observed upon S. typhimurium challenge varied considerably between different experiments. However, in three independent experiments, the induction levels of mmp9, il1b and irak3 expression were significantly reduced in the myd88 morphants compared to non treated embryos and embryos injected with the control morpholino (Fig. 8A-C). Induction of cxc1c was reduced in both mismatch control and myd88 morphants, suggesting a currently unexplainable aspecific morpholino effect on the expression of this gene under infection conditions (Fig. 6F). Notably no changes were observed for ifn1 and il8 expression, indicating a MyD88-independent activation of these genes (Fig. 6D-E). Therefore, we conclude that the innate immune response of zebrafish embryos to S. typhimurium infection involves both MyD88-dependent and MyD88-independent signalling pathways.

Discussion

Here we report the first time-resolved characterization of the immune response of a vertebrate embryo to a systemic bacterial infection at the transcriptome level. Aiming specifically at the analysis of innate host determinants, we took advantage of the clear temporal separation of the innate from the adaptive immune system in the externally developing zebrafish embryos (22, 28-30). In addition to the identification of a large set of genes that had not been previously linked to the immune response, we found a substantial overlap between the embryonic host response and immune responses measured in human and other vertebrate systems, indicating that the embryo model has a good predictive value for the vertebrate immunity. Furthermore, we present here the first demonstration of a conserved Toll-like receptor ligand specificity and the presence of MyD88-dependent and -independent signalling pathways in the zebrafish embryo.

The bacterial infection model used in this study is a previously described Salmonella typhimurium system where a wild type (wt) and an attenuated LPS O-antigen mutant strain (Ra) are utilized (9). Our transcriptome analysis of S. typhimurium wt and Ra mutant challenge demonstrated that both strains elicit a distinct temporal expression profile, correlated to the symptoms of disease progression. Comparison of the host response over the first 8 hours revealed similar expression trends for both strains in spite of the fact that the wt strain elicits a fatal infection, and the Ra mutant infection is cleared within a day. The main difference between the strains was observed at 24 hpi, when the transcriptome response to the wt strain further increased and the response to the attenuated Ra mutant strain was on its
return. The pathological differences are most likely due to a higher susceptibility of the Ra mutant to the embryonic immune response. As previously suggested, the complement system might be responsible for extracellular lysis of *S. typhimurium* LPS mutants observed in infected zebrafish embryos (9). In agreement, *S. typhimurium* O-antigen mutants showed higher susceptibility to complement lysis in *in vitro* studies (47, 48). The rapid induction of complement components like *c3b*, *c3c*, *c6* and *cfb* (Supplementary table II) upon *S. typhimurium* challenge that we observed in our microarray study further supports the role of the complement system. Although there was a large overlap in the expression signatures of *S. typhimurium* wt and Ra infection during the first 8h of the infection, the majority of the affected genes showed quantitative differences in expression levels, the functional implications of which are not yet understood. The transcriptome study reported here provides a useful reference for future studies aimed to advance the understanding of host-pathogen interactions.

Unbiased analysis of the infection datasets by two complementary annotation tools, eGOn and DAVID, clearly demonstrated an immune specific host response of the zebrafish embryo to the *S. typhimurium* wt and Ra infections. Master-target testing performed by eGOn revealed the GO-terms “immune system process” and “response to stimulus” as significantly enriched in the infection-upregulated signature sets over the microarray background. However, fairly low numbers of genes were associated with these GO-terms, which could be attributed to the still poor annotation of the zebrafish genome. We found that use of the NCBI HomoloGene database to convert the zebrafish UniGene identifiers to their predicted human homologs was a powerful tool to overcome this problem. Through their human homologs, over 3-fold more zebrafish genes could be associated with the immune-specific GO-terms. In addition, gene groups with a clear correlation to immune processes (i.e. “complement pathway” and “acute phase response”) were identified by DAVID analysis taking advantage of the human homolog conversion dataset. It remains to be experimentally confirmed if there is functional conservation between these unannotated zebrafish genes and their predicted human homologs, however their up-regulation in response to *S. typhimurium* infection supports that they play a role in the host immune response.

The relevance of the zebrafish embryo model to study vertebrate immunity was further investigated by comparison of our transcriptome data to a meta analysis of microarray data of various human cell lines challenged by different pathogens (42). We found a substantial overlap between the zebrafish host response to *S. typhimurium* and a set of genes that was commonly induced in all cell lines upon pathogen challenge referred to as “the common host response”. The overlap included genes for well known immune responsive transcription factors, cell surface receptors, signal transduction intermediates, adhesion factors and proteins involved in tissue remodelling. Even though adaptive immunity is not yet developed in the zebrafish embryo, we also observed induction of genes encoding predicted zebrafish homologs of
molecules involved in antigen processing and co-stimulation. Furthermore, various interferon, chemokine, proinflammatory cytokine and anti-inflammatory cytokine genes were shared between the gene sets of the zebrafish embryonic host response and the human common host response. Taken together, these observations further underscore the predictive value of the zebrafish embryo model.

Toll-like receptors are key players in the recognition of pathogens during host defence. We previously analysed the zebrafish genome for genes encoding members of TLR signalling pathway leading to the identification of the zebrafish counterparts of the human TLRs and adapter proteins (23). GenMapp based analysis of the TLR pathway in this study demonstrated that various TLR signalling intermediates at different levels in the pathway are induced upon *S. typhimurium* wt and Ra infection in vivo. Genes implicated in the negative regulation of the pathway, e.g. *socs1*, *socs3* and *pik3cg*, were among the earliest regulated genes (49-51). In addition, we observed induction of genes encoding NF-κB inhibitors and the *irak3* gene, whose human homolog functions as a negative regulator of the TLR pathway (52). These observations suggest a tight regulation of the TLR pathway most likely to limit the potential negative consequences of excessive cytokine production. Furthermore the zebrafish homologs of the human TLR5, Tlr5a and Tlr5b, were specifically up-regulated upon *S. typhimurium* wt and Ra challenge. It has previously been demonstrated that the mammalian TLR5 is mediating the immune response to bacterial flagellin (53) specifically recognizing conserved domains of monomeric flagellin crucial for bacterial motility and protofilament assembly (54). Our results showed that challenge of the zebrafish embryo with purified *S. typhimurium* flagellin elicited a strong activation of zebrafish homologs of the pro-inflammatory cytokines *il1b*, *il8* and *tnfa*. Flagellin challenge also induced expression of the chemokine gene *cxcl-C1c*, the matrix metalloproteinase gene *mmp9*, and the putative negative regulator *irak3* discussed above. Functional assessment of *tlr5a* and *tlr5b* by morpholino-mediated knockdown followed by flagellin stimulation clearly demonstrated tlr5-dependent gene activation of *mmp9*, *cxcl-C1c* and *irak3* in the zebrafish embryo. Therefore, Tlr5 pathway activation appears to induce the expression of inflammatory mediators as well as feedback control of the innate immune response.

Although we found that flagellin induction of immune response genes was mediated by Tlr5, the *tlr5a* and *tlr5b* genes themselves were not induced upon flagellin stimulation. Furthermore, induction of *tlr5a* and *tlr5b* was still observed upon challenge with a non-flagellated derivative of the *S. typhimurium* wt strain (data not shown), demonstrating a flagellin-independent transcriptional activation of *tlr5a* and *tlr5b* during infection in the zebrafish embryo. Furthermore, preliminary results of knockdown experiments suggest that Tlr5 expression is not required for the induction of inflammatory mediators during *S. typhimurium* infection. This is consistent with observations in mice showing that TLR4 is able to compensate for the function of TLR5 upon *Salmonella* infection (55).

MyD88 functions as adaptor protein of TLR5 in mammalian systems and is also
an important adaptor of other TLRs, including TLR4. In previous work we demonstrated the significance of MyD88 in the zebrafish embryo host defence by showing that accumulation of \textit{S. typhimurium} Ra bacteria was increased upon MyD88 morpholino knockdown (31). Here, we extended this work and assessed transcriptional effects of MyD88 on downstream target genes of innate immunity signalling. Our results demonstrated a clear dependency of \textit{mmp9}, \textit{il1b} and \textit{irak3} on MyD88 for transcriptional activation upon \textit{S. typhimurium} wt challenge. In contrast, \textit{ifn1} and \textit{il8} did not show changes in their induction upon bacterial challenge, demonstrating MyD88-independent activation of these genes. Differential use of the MyD88 adaptor in the TLR-pathway is well documented in mammalian systems with \textit{IL1b} as the primary example of a MyD88-dependent target gene and \textit{IFN} as a target of both MyD88-dependent and independent routes (3, 4, 56). Our observations suggest a conserved mechanism in the zebrafish embryo. Furthermore, we have identified \textit{mmp9} and \textit{irak3} as novel MyD88-dependent immune response genes. MyD88-dependent as well as MyD88-independent induction of \textit{il8} has been observed in mammalian systems (57, 58). However, regarding the MyD88-independent \textit{il8} induction that we observed in zebrafish embryos it should be noted that the orthology of this gene with mammalian \textit{il8} is ambiguous and that zebrafish express other closely related putative \textit{il8} homologs.

In this study we found that the matrix metalloprotease genes \textit{mmp9} and \textit{mmp13} were among the strongest infection responsive genes, with induction levels around 10 to 20-fold at 8 hpi for the \textit{S. typhimurium} Ra and wt strains and over 100-fold at 24 hpi for the \textit{S. typhimurium} wt strain. Proteins of the mammalian MMP family degrade extracellular matrices thereby facilitating cell migration. Additionally, they are thought to affect the activity of inflammatory molecules (59). \textit{S. typhimurium} and other bacterial species have been reported to secrete proteases that activate inactive proenzyme forms of MMPs, which may promote bacterial spreading through the host tissues (60). Here we have shown that \textit{mmp9} expression is a target of the TLR5 pathway that is induced by \textit{Salmonella} flagellin. Therefore, it appears that \textit{Salmonella}-derived molecules can stimulate MMP activity both at the transcriptional and the post-translational level. We have observed that induction of \textit{mmp} genes is a common characteristic also of other types of bacterial infections in zebrafish, including \textit{Edwardsiella}, \textit{Pseudomonas} and \textit{Mycobacterium} infections (unpublished results). Likewise, these genes are induced during pulmonary \textit{Mycobacterium} infection in mice (61). Another metalloprotease gene that we found induced upon \textit{S. typhimurium} infection is \textit{adam8} (a disintegrin and metalloproteinase domain 8), which belongs to a family of membrane-anchored glycoproteins that have been implicated in a variety of biological processes involving cell-cell and cell-matrix interactions. Recently, up-regulation of ADAM8 surface expression in human neutrophils was correlated with joint inflammation (62) and increased ADAM8 mRNA expression was also associated with allergic inflammation (63). The transparency of zebrafish embryos offers good possibilities to further investigate how gene expression of met-
alloproteinases such as the MMPs and ADAM8 contribute to tissue remodelling, inflammation and bacterial dissemination.

Providing a case study for future immunity research in the zebrafish embryo model, our transcriptome analysis of the host response to S. typhimurium has linked a large set of zebrafish genes to the process of bacterial infection. Among these are a number of chemokine genes that have previously been annotated in the zebrafish genome, but that have not been functionally studied (64). Infection responsive chemokine genes that were more than 3-fold induced over multiple time points of our in vivo infection study include cxcl-C1c, cxcl-C5c, ccl-C5a, ccl-C24a, and similar to ccl-CUb (Dr.125570). Another example of a putative immune response gene that we found to be strongly induced by S. typhimurium infection is the zgc:65788 gene that encodes a homolog of the mammalian acidic chitinase family (5-10 fold at 8 hpi by the wt and Ra strains, and over 30-fold at 24 hpi by the wt strain). Increased chitinase gene expression was also observed in our previous Mycobacterium-infection study of adult zebrafish (65). There is increasing evidence for a role of acidic mammalian chitinases in Th2 inflammation and asthma (66, 67). The strong induction of zgc:65788 expression during infection of zebrafish embryos suggests that chitinases may also play a role in the innate immune response. Finally, we found that approximately 70% of all genes that were specifically regulated during the first hours of S. typhimurium infection had not been previously linked to immunological processes. Many of these genes include transcribed loci that have unknown functions and for which the functions of their predicted human homologs are also unknown. With the possibility of performing rapid gene knockdown studies, the zebrafish embryo provides a useful model for the future functional characterization of these genes that will also support the further annotation of the human genome.

Materials and Methods

Bacterial Strains and Growth Conditions

S. typhimurium wild type (wt) strain SL1027 and its isogenic LPS derivative SF1592 (Ra), both containing the DsRed expression vector pGMDs3, were used for the infection of zebrafish embryos (9). A non-flagellated derivative of this strain (flhC-) contained an flhC::Mu insertion generously provided by K. Hughes (University of Utah) (38). Bacteria were freshly grown overnight on LB agar plates supplemented with 100μg/ml carbenicillin (wt and Ra mutant strain) and 50μg/ml kanamycin (flhC-) and resuspended in phosphate-buffered saline (PBS) prior to injection.

Zebrafish husbandry

Zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (http://ZFIN.org). Embryos were grown at 28,5 -30 °C in egg water (60μg/ml Instant Ocean sea salts). For the duration of bac-
terial injections embryos were kept under anaesthesia in egg water containing 0.02% buffered 3-aminobenzoic acid ethyl ester (tricaine, Sigma). Embryos used for whole mount in situ staining were kept in egg water containing 0.003% 1-phenyl-2-thiourea (Sigma) to prevent melanization.

**Morpholino knock-down experiments**

For morpholino knockdown experiments, morpholino oligonucleotides (Gene Tools) were diluted to desired concentrations in 1x Danieu's buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5.0 mM HEPES; pH 7.6] containing 1% Phenol red (Sigma). To block translation of the *tlr5a*, *tlr5b* or *myd88* mRNA we injected 0.85ng (0.1mM), 4.2ng (0.5mM) and 8.4ng (1mM) per embryo, respectively. To control for aspecific morpholino effects we used the standard control morpholino (0.6mM) for the Tlr5 knockdown experiments and a previously described 5 bp mismatch morpholino (1mM) (31) for the MyD88 knockdown studies. Morpholinos used target the 5' UTR region of the respective gene. Morpholino sequences are shown in supplementary table I.

**Experimental design of infection study**

All infection experiments were performed using mixed egg clutches from three tanks of AB strain zebrafish. Embryos were staged at 27 hours post fertilization (hpf) by morphological criteria (39) and approximately 250 cfu of DsRed expressing *S. typhimurium* wt and *Ra* mutant bacteria were injected into the caudal vein close to the urogenital opening. As a control an equal volume of PBS was likewise injected. Injections were controlled using a Leica MZ Fluo 3 stereomicroscope with epifluorescence attachment together with a Femtojet microinjector (Eppendorf) and a micromanipulator with pulled microcapillary pipettes. Pools of 20-40 embryos were collected at 2, 5, 8 and 24 hours post infection (hpi). For the microarray analysis, the whole infection procedure was performed in triplicate on separate days. The order of injecting wt bacteria, *Ra* bacteria and PBS control was randomized in the different experiments. Microarray analysis was performed using custom-designed 44k Agilent chips. All *S. typhimurium* wt, *S. typhimurium Ra* and PBS control RNA samples were labelled with Cy5 and hybridized against a Cy3-labelled common reference, which consisted of a mixture of all samples from the infection study.

**Microarray design**

The micro-array slides were custom designed by Agilent Technologies. The slides contained in total 43,371 probes of a 60 oligonucleotide length. Of these probes a total of 21,496 probes were identical to the probes present on the Agilent probe set that is commercially available under catalogue number 013223_D. Most of the additional probes were designed using the eArray software from Agilent Technologies (https://earray.chem.agilent.com/earray/). Settings used were based on the following settings: base composition methodology, best probe methodology and design
with 3’ bias. The Agilent *D. rerio* transcriptome was used as a reference database. A small number of probes were manually designed based on knowledge of particular polymorphisms for genes encoding protein families such as 14-3-3 proteins, chitinase-like proteins and Toll-like receptors in order to obtain gene-specific probes. The micro-array design has been submitted to the GEO database under accession number GPL7735.

**RNA isolation, labelling and hybridization**

Embryos for RNA isolation were snap frozen in liquid nitrogen and subsequently stored at -80°C. Embryos were homogenized in 1 ml of TRIZOL® Reagent (Invitrogen) and subsequently total RNA was extracted according to the manufacturer’s instructions. The RNA samples were incubated for 20 min at 37° with 10 units of DNaseI (Roche Applied Science) to remove residual genomic DNA prior to purification using the RNeasy MinElute Cleanup kit (Qiagen) according to the RNA clean up protocol. The integrity of the RNA was confirmed by Lab-on-chip analysis using the 2100 Bioanalyzer (Agilent Technologies). Samples used for microarray analysis had an average RIN value of 9 and a minimum RIN value of 8.

Amino Allyl modified amplified RNA (aRNA) was synthesized in one amplification round from 1 μg of total RNA using the Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion). Subsequently, 6 μg of Amino Allyl modified aRNA was used for coupling of monoreactive Cy3 and Cy5 dyes (GE Healthcare) and column purified. The dual colour hybridization of the microarray chips was performed at ServiceXS (ServiceXS, Leiden, The Netherlands) according to Agilent protocol G4140-90050 v.5.7 (www.Agilent.com) for Two-Color Microarray-Based Gene Expression Analysis.

**Data analysis**

Microarray data was 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, Seattle, Washington) and subjected to default ratio error modelling. The raw data were submitted to the GEO database under accession number GSE13994. In order to compare *S. typhimurium* wild type and *S. typhimurium Ra* treated samples to the PBS injected control samples a re-ratio experiment was performed using the Rosetta build in re-ratio with common reference application. Data were analyzed at the level of UniGene clusters (UniGene build #105). Significance cut-offs for the ratios of wt versus PBS and Ra versus PBS were set at 1.5 fold change at *P* <10^-4 for UniGene clusters. Two-dimensional hierarchical cluster analyses were performed with Rosetta Resolver settings for agglomerative algorithm (average link) with Cosine correlation.

Gene ontology (GO) analysis was performed using the GeneTools eGOn V2.0 web-based gene ontology analysis software (http://www.genetools.microarray.ntnu.no/) (40) and using DAVID software tools (http://david.abcc.ncifcrf.gov/home.jsp)
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(41). GO analysis using eGOn was done at the level of the UniGene clusters (D. rerio UniGene build #105). GO analyses using DAVID tools were performed at the level of the Entrez Gene codes, because the DAVID database was not updated to a recent D. rerio UniGene build. To take advantage of the much better GO annotation of the human genome, we developed a software tool with which the UniGene and Entrez Gene records of the functionally related human homologs of our zebrafish UniGene list could be automatically retrieved from the NCBI HomoloGene database. Subsequently, eGOn and DAVID GO analyses were repeated using the UniGene (H. sapiens UniGene build #202) and Entrez Gene lists of human orthologs, respectively. UniGene and Entrez Gene lists subjected to eGOn and DAVID analyses are included in supplementary table II. In addition, for comparison with human microarray data, the (putative) zebrafish homologs of the set of 511 human common host response genes described by Jenner and Young (42) were manually identified by searching ZFIN (http://zfin.org) and the Gene and HomoloGene databases of the National Center for Biotechnology Information (NCBI) (Supplementary table III). Homologs of human cytokines were identified based on phylogeny reconstructions to be reported elsewhere. Direct or putative homologs could be identified for 397 out of the 511 human common host response genes (78%) and 322 of these (63%) were represented on our zebrafish microarray. Since some genes are duplicated in zebrafish and since sometimes there was more than one putative homolog, there were 473 zebrafish UniGenes corresponding to the 322 human genes represented on the array.

Pathway analysis was performed using the GenMapp software package (www.genmap.org) (43). GenMapp analysis was done at the level of UniGene clusters (D. rerio UniGene build #105). Significance cut-off was set at 1.5 fold change at P <10^{-4}. Zebrafish homologs of the genes contributing to the TLR pathway were identified by either searching the ZFIN (http://zfin.org) database or the Gene and HomoloGene database of NCBI (Supplementary table IV).

cDNA synthesis and quantitative Real-time PCR

cDNA synthesis reactions were performed in a 20 μl mixture of 500 ng RNA, 4 μl of 5x iScript Reaction mix (Bio-Rad) and 1 μl of iScript Reverse Transcriptase (Bio-Rad). 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, Hercules, CA) according to the manufacturer’s instructions. Each reaction was performed in a 25 μl volume comprised of 1 μl cDNA, 12.5 μl of 2x iQ SYBR Green Supermix (Bio-Rad) 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 sec and 59 °C for 45 sec. 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 (44). Results were analysed using the ΔΔCt method. Sequences of forward and reverse primers are described in supplementary table I.

Whole-mount in situ hybridization
Embryos were fixed overnight in 4% paraformaldehyde in PBS at 4 °C and whole mount in situ hybridization using alkaline phosphatase detection with BM Purple substrate (Roche) was performed according to Thisse et al. (45). Genomic DNA was used to generate templates for riboprobes synthesis by PCR using gene specific primers sets including the binding site for T7 RNA polymerase in the reverse primer. Sequences of forward and reverse primers are described in supplementary table I. Digoxigenin-labeled riboprobes were synthesised using the labelling mixes from Roche and Ambion MEGAscript reagents for in vitro transcription.

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Supplementary data

Supplementary tables and supplementary material 1 can be found online at: http://www.jimmunol.org/cgi/content/full/182/9/5641/DC1

SUPPLEMENTARY FIGURE 1. Imaging analysis of *S. typhimurium* wt and Ra infection over the experimental time course. Embryos were injected at 27 hpf with approximately 250 cfu of *S. typhimurium* Ra (A-D) or wt (E-H) into the caudal vein and imaged at 2, 5, 8 and 24 hpi. Overlay pictures of bright field and DsRed fluorescent images are shown. Anterior side is to the left and dorsal is to the top.

SUPPLEMENTARY FIGURE 2. GenMapp analysis of the TLR-pathway upon *S. typhimurium* Ra infection. Expression profiles (infected versus control, *p*<0.0001 and fold changes >1.5 and <-1.5) of the 2, 5, 8 and 24 hpi time points were simultaneously mapped on the TLR pathway. Gene boxes are colour coded from left to right with the 2, 5, 8 and 24 hpi expression data. Up-regulation is indicated in yellow, down-regulation in blue and unchanged expression in grey. White denotes genes that were not represented on the array platform. The pathway is based on knowledge of TLR signalling in mammalian species and it should be noted that most interactions remain to be experimentally confirmed in zebrafish.
SUPPLEMENTARY FIGURE 3. Temporal expression profiles of immune response genes upon challenge with purified *S. typhimurium* flagellin. Embryos were challenged with flagellin by injection of 4nl (100ug/ml) into the caudal vein at 27 hpf. Gene induction levels upon stimulation compared to the mock-injected controls were analyzed by qRT-PCR at 1, 2 and 5 hpi for *tlr5a*, *tlr5b*, *ifn1* and *il1b*. Values are the mean ± S.D. of at least 2 independent experiments. **p<0.01 (tested by one way ANOVA).
References

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Zebrafish embryonic transcriptome response to infection


