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Cancer and inflammation studies using zebrafish cell lines

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Chapter 3

Toll-like Receptor signaling in zebrafish cell lines

Toll-like receptors (TLRs) are a family of key pathogen recognition receptors which recognize microbial molecules and transduce intracellular signaling via recruitment and activation of adapter molecules e.g. TRAF6, which in turn deliver the signal to activate the transcription factor NF κ B and the mitogen-activated protein kinases (MAPKs), which subsequently regulate transcription of many genes involved in inflammation and the immune response. Reverse transcription PCR revealed unique expression profiles of a set of immune-related genes in three zebrafish cell lines (ZF4, PAC2 and ZFL), including TLRs, their downstream signaling molecules, transcription factors and cytokines. The transcription factor NF κ B can be activated by either TRAF6 over-expression or by stimulation with TLR ligands, in a cell-line dependent manner. Stimulation with flagellin, which is recognized by TLR5, activated NF κ B in all cell lines. Microarray analysis revealed distinct transcriptome response in different cell lines upon flagellin stimulation, suggesting that zebrafish cell lines can be used to study specific signaling events involved in pathogen recognition at cellular level.

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Introduction

The innate immune system of vertebrates acts as the first line of host defense against microbial pathogens by detecting and responding to a broad range of cues of invading pathogens directly after infection [1]. Toll-like receptors (TLRs) form a family of key pathogen recognition receptors of the innate immunity and each member recognizes a restricted subset of molecules produced by microbes [2]. The TLR family can be divided into two subfamilies: one subfamily of cell surface TLRs recognizes microbial membrane components such as lipids, lipoproteins and proteins, and the other subfamily, localized on endosomal compartments, mainly recognizes microbial nucleic acid species [3]. After recognition, transduction of intracellular signaling depends on the recruitment of adapter molecules such as MyD88, TIRAP and TRIF and the activation of IRAK family kinases and TRAF6. In turn, activated TRAF6 can deliver the signal to activate the transcription factor NF κ B and the mitogen-activated protein kinases (MAPKs), which regulate transcription of many genes involved in inflammation and the immune response [2, 4]. It is known that different TLRs can exert distinct but overlapping sets of biological effects, which may attributed to both common and unique aspects of the signaling mechanism [4]. However, there is limited understanding of how specificity in the activation of downstream targets is achieved.

The zebrafish, *Danio rerio*, has been recognized as an attractive experimental model for infectious disease and immunity studies because of the small size, rapid generation time, vertebrate biology and the optically transparent embryos [5]. The primary defense mechanisms against microbial agents of zebrafish are similar to those of mammals and TLR signaling mechanisms are already functional in early embryos [5-7]. Especially the transparency of zebrafish embryos allows imaging of cellular behavior related to pathogen recognition and defense, which is impossible or very hard to achieve in mammalian systems.

The 19 putative TLRs have been found in the zebrafish genome, including orthologs of mammalian TLRs 2-5, 7-9 and a group of fish specific TLRs [8]. Previous work in our laboratory characterized the human TLR homologue genes in zebrafish and found up-regulated expression in response to the tuberculosis-inducing pathogen *Mycobacterium marinum* infection [8]. Analysis of the zebrafish embryonic innate immune response to *Salmonella* infection showed that *Salmonella* flagellin was recognized by the zebrafish homologs (tlr5a/b) of the mammalian flagellin receptor TLR5 [7]. Together with fact that exposure of zebrafish to snakehead rhabdovirus or *Edwardsiella tarda* led to up-regulation of mRNA expression of TLR3 and TRAF6 [6], it demonstrated that zebrafish possess TLR-signaling pathways conserved to the mammals. It was also found that multiple TIR domain-containing adaptors, including MYD88, MAL, TRIF and SARM, were expressed in myeloid leukocytes which contributed to wound healing and bacteria phagocytosis in zebrafish embryos [9]. These findings underscore the suitability of analysis of the vertebrate innate immune system in fish. In addition, zebrafish TRIF exhibits sequence divergence from its mammalian orthologs and did not interact with TRAF6 [10]. Together with the fact that zebrafish also lacks the homologue of mammalian TIR domain-containing adaptor TRAM [10], it suggested that evolution of vertebrate TLR pathway and zebrafish can be used as an important model for understanding the evolution of the vertebrate immune system.

One of the current bottlenecks of using zebrafish for biomedical research to supplement studies in other vertebrate models is the lack of *in vitro* zebrafish cell models. As in mammalian model organisms, *in vitro* cell models can bridge the knowledge gained from different organisms to the research in zebrafish, and can be used to dissect the findings in zebrafish embryos or adult fish at detailed molecular and cellular level. Owing to the transparency of zebrafish embryos and versatile cell implantation protocols, zebrafish cell cultures can also be used for *in vivo* studies after cell implantation into embryos, to fully explore the power of zebrafish system.

As zebrafish is a relatively new model organism, only a few zebrafish cell lines have been generated. Methods were developed to culture cells isolated from early stage zebrafish embryos and organs of adult fish such as caudal fin and liver [11]. A few laboratories have tried to isolate zebrafish hematopoietic cells, but the establishment of continuous cell cultures has not been reported yet. Most of the established zebrafish cell lines were non-professional antigen presenting cells, described as fibroblast cell lines or fibroblast-like cell lines [12]. Previous reports have shown that signaling pathways involved in innate immunity can be activated in these cell lines [6, 13]. However, their genetic and physiological properties are still not well known, which limits their application.

In order to better characterize the existing zebrafish cell lines and to evaluate their application for immunological research, we analyzed three previously described cell lines, ZF4, PAC2 and ZFL for their response to external bacterial stimuli. The ZF4 and PAC2 cell lines were established from 24 hour post fertilization (hpf) embryos [14, 15]. The ZFL cell line was derived from adult fish liver as the first zebrafish cell line showing a typical epithelial morphology [16]. These three cell lines can be easily maintained for at least 50 passages in conventional medium containing mammalian serum. Transfection and stable cell lines can be achieved in all three cell lines following lipid-mediated methods or by nucleofection. In this work, these three zebrafish cell lines were further characterized for their expression of TLR-signaling compounds and their function in the pathogen recognition. The stimulation with flagellin, recognized by TLR5 in mammals, activated NF κ B in all three zebrafish cells lines.

Results

Expression of genes involved in the TLR signaling

Expression of a set of immune-related genes was examined by semi-quantitative reverse transcriptional PCR in three zebrafish cell lines: ZF4, PAC2 and ZFL (Figure 1). In mammals, TLR1, TLR2, TLR4, TLR5 and TLR6 are mainly expressed on the plasma membrane and recognize microbial membrane components [3]. Homologs of TLR1, TLR2, TLR4, TLR5 and TLR6 have been identified in the zebrafish genome [8] and their expression was checked in the zebrafish cell lines. The results showed that TLR1, TLR4a, TLR4b, TLR5a and TLR5b are commonly expressed in all three cell lines. TLR2 had highest expression level in the ZFL cell line and the lowest level in the PAC2 cell line. The expression of TLR18 (a second homologue of human TLR1) was not detected in the PAC2 cell line. Among the TIR-domain containing adaptors downstream of TLRs, MYD88 and TRIF were expressed in all cell lines, whereas the expression of TIRAP was only detected in the ZFL cells. Some other important molecules involved in the TLR signaling, such as TRAF6, ERK1 and ERK2, were also expressed in the three cell lines.

Five transcription factors of the NF κ B family have been found in the zebrafish genome. Four of them, NF κ B2, RelA, RelB and cRel are detected in all three cell lines. NF κ B1 was only detected in the ZFL cell line. Several cytokines known to be downstream of the TLR signaling in human and mouse were also expressed in all cell lines, including IL1 β , IL8, IL12 and TNF α . In general, most of the genes are commonly expressed in all three zebrafish cell lines, with only variations in their expression level.

Over-expression of TRAF6 activates NF κ B

TRAF6 is an important signaling molecule in the TLR signaling, downstream of the TLRs and adaptors such as MYD88, TIRAP and TRIF. It can be activated by TLR-mediated signal transduction and in turn activate the TAK/TAB-I κ B kinase cascade, which leads to activation of NF κ Bs. Activation of TRAF6 can be mimicked by its over-expression in cells [17]. It was reported that over-expression of TRAF6 activated NF κ B in the ZFL cell line [6]. In this study we over-expressed TRAF6 by nucleofection in the three zebrafish cell lines to mimic its activation by TLR signaling and measured the downstream NF κ B activation using a luciferase reporter assay (Figure 2). Independent experiments were performed using two different NF κ B-Luc reporters that were widely used in mammalian cell cultures. No response after TRAF6 over-expression was detected using the reporter NF κ B-Luc-A (containing 5x GGGGACTTTC repeats of κ B enhancer) in any cell line (data not shown). Using the reporter NF κ B-Luc-B (containing 4x GGGAATTTC repeats of κ B enhancer), hyperactivation of NF κ B induced by the over-expression of TRAF6 was detected in the ZFL cell line, but not in the ZF4 and PAC2 cell lines (Figure 2B), strongly indicating the variation in the signal transduction downstream TLR and TRAF6 activation in the three zebrafish cell lines.

Figure 1. Expression of a set of immune-related genes in three zebrafish cell lines.

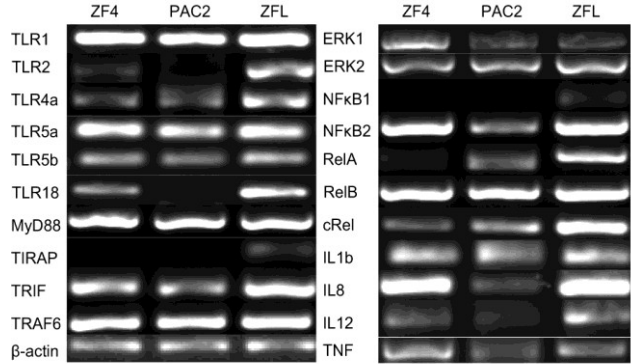


Figure 2. Over-expression of TRAF6 activated NFκB in the ZFL cell line.

A: TRAF6 was over-expressed in zebrafish cell lines by nucleofection. Semi-quantitative RT-PCR was performed to check the TRAF6 expression after nucleofection in the ZFL cells. Data of ZF4 and PAC2 cell lines are not shown.

B: Over-expression of TRAF6 activated NFκB in the ZFL cell line, not detected in the ZF4 and PAC2 cell lines. These results are representative of at least 3 independent experiments with at least 3 biological controls in each cell line.

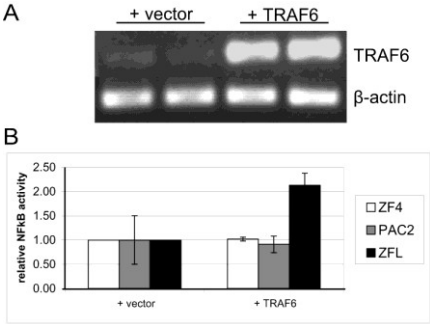
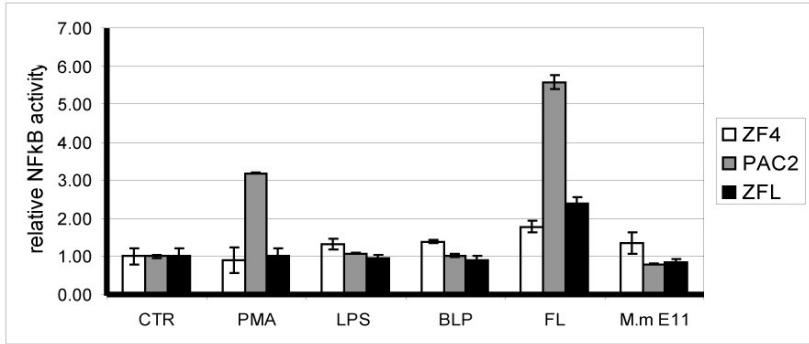


Figure 3. Stimulation with flagellin activated NFκB in zebrafish cell lines.

The zebrafish cell lines were transfected with NFκB-Luc reporters and stimulated for 24 hours with PMA (25 ng/ml), LPS (1 μg/ml), BLP (1 μg/ml), flagellin (100 ng/ml) or extracts of *M. marinum* (5 μg/ml). These results are representative of at least 3 independent experiments with at least 3 biological controls in each condition.



Flagellin stimulation leads to NFκB activation

It is known that the NFκB transcription factors can be activated by TLR signaling upon pathogen recognition [2]. To characterize the functionality of the zebrafish TLRs in zebrafish cell lines, PAC2, ZF4 and ZFL cell lines were stimulated with compounds that commonly function as TLR ligands in mammalian cell cultures. The subsequent NFκB activation was measured after 24 hours of stimulation, using the luciferase reporter assay (Figure 3). Independent experiments were performed using the two NFκB-Luc reporters and experiments using the reporter NFκB-Luc-B did not show a response upon any stimulation in each of the listed cell lines (data not shown).

Using the reporter NFκB-Luc-A, stimulation with PMA, a known activator of human NFκB, resulted in elevated NFκB activity in the PAC2 cell line. The other two cell lines did not respond to PMA, suggesting cell-line specificity of intracellular signal transduction. Despite the expression of TLR2 in all three cell lines, the stimulation with bacterial lipoprotein (BLP), the ligand of human and mouse TLR2, did not induce NFκB activity in any of these lines. In addition, no NFκB activation was detected upon stimulation with lipopolysaccharide (LPS), the ligand of mammalian TLR4. The cells were also stimulated with extracts of *Mycobacterium marinum* (strain E11), a known pathogen of zebrafish [8], but no significant response was detected in the zebrafish cell lines (Figure 3), whereas the same extracts induced NFκB activation in a HEK293 cell line stably expressing human TLR2 (data not shown). In contrast, stimulation with flagellin, recognized by TLR5 in mammals, activated NFκB in all three zebrafish cells lines which was detected by the reporter NFκB-Luc-A.

Distinct transcriptional programs activated by flagellin stimulation

The cell-line dependent activation pattern of NFκB strongly suggested that although many genes involved in the TLR signaling are commonly expressed, the cellular responses towards extracellular stimulation and intracellular signaling downstream of TLR activation are highly cell-line specific.

To further characterize the cellular response upon activated TLR signaling of each cell line, the cells were stimulated with flagellin, which activated NFκB in all cell lines (Figure 3), and a microarray approach was taken to investigate the responses at the transcriptome level after one hour of stimulation. Comparing with non-stimulated cells, expression of 2668 Unigene clusters were significantly up- or down-regulated in the ZF4 cell line, 1224 Unigene clusters regulated in the ZFL cell line and 855 Unigene clusters regulated in the PAC2 cell line ($P \leq 1.0 \times 10^{-5}$, Figure 4A). The overlap of up- or down-regulated Unigene clusters between the three cell lines was limited (Figure 4B). This result indicates that although the NFκB transcription factors were commonly activated in all cell lines after flagellin stimulation, the transcriptome responses of these cell lines were very distinct. 2D-hierarchical cluster analysis showed that the ZFL and PAC2 cell lines had more similarity in their transcriptional response to flagellin than the ZF4 cell line (Figure 5). Many genes involved in immune and wounding responses were regulated in the zebrafish cell lines upon flagellin stimulation, but the common signatures in the three cell lines were very limited (Figure 6). The ZF4 cell line had more immune

and wounding related signatures than the ZFL cell line, whereas the PAC2 cell line had the least signatures (Figure 6). This difference between cell lines was also reflected by the significance of expression level changes in the flagellin-signature sets that more percentage of signatures was regulated ≥ 1.5 fold in the ZF4 cell line than the other two cell lines (Figure 4).

Flagellin stimulation in the three zebrafish cell lines activated distinct genes involved in TLR signaling

To examine the transcriptional responses upon flagellin stimulation at the level of the TLR signaling pathway, map-based pathway analysis was performed using the GenMAPP software [7]. GenMAPP analysis showed that numerous genes involved in the TLR signaling were differentially regulated in the three zebrafish cell lines (Figure 7). In the ZF4 cell line, several genes involved in signal transduction downstream of TLR activation were up-regulated, such as *trif*, *ikbkg*, *rel*, *rela*, *relb*, *nfkbb2*, *jun* and *atf3*, whereas *irf5* was down-regulated. Several negative regulators of the TLR signaling were also up-regulated, including *traf1*, *socs3a*, *socs3b*, *nfkbiaa*, *nfkbiab*, *dusp1* and *dusp2*. Up-regulation of the negative regulators of the TLR signaling was also observed in the ZFL cell line, where *pik3cg*, *irak3*, *dusp1* and *dusp2* were up-regulated. Different than in the ZF4 cell line, the ZFL cell line showed more genes involved in the TLR signal transduction to be down-regulated (*trif*, *tab3*, *irf5*, *rel*, *relb* and *atf3*) than up-regulated (*tpl2*). Compared to the other two cell lines, less genes involved in the TLR signaling were transcriptionally regulated in the PAC2 cell line: *trif* and *mapk1* was down-regulated and *nfkbiaa* was up-regulated.

It is known that activation of the TLR signaling leads to transcription of pro-inflammatory genes [4], which was also found in the three zebrafish cell lines upon flagellin stimulation. The expression of *il15l*, *ifn* and *tnfβ* was up-regulated in the ZF4 cell line. The expression of *il8*, *ifn*, *cox2a* and the two homologs of human TNFα (*tnfa*, *tnfb*) were up-regulated in the ZFL cell line. In the PAC2 cell line, *ifng1.2* and *tnfβ* were up-regulated.

Among all these genes involved in the TLR signaling, *tnfb* was the only commonly regulated gene in all three cell lines.

Genes transcriptionally regulated by flagellin stimulation are predicted to be involved in different biological processes

To further characterize the transcriptional responses upon flagellin stimulation in the three zebrafish cell lines, GO term analysis was performed to analyze the functionality of the signatures in an unbiased manner. This revealed cell-line-specific distributions of the signatures over different functional categories (Figure 8). In the ZF4 cell line, the up-regulated signatures were enriched in biological processes such as apoptosis, protein kinase cascade, response to stress, negative regulation of cellular protein metabolic process and regulation of immune system process. The down-regulated signatures were enriched in biological processes including receptor linked signal transduction, cell proliferation, cell projection organization and negative regulation of transcription. In the ZFL cell line, many

Figure 4. Expression profiles of genes responsive to flagellin stimulation in zebrafish cell lines.

A: Total numbers of Unigene clusters that were significantly up- or down-regulated compared with un-stimulated cells ($P \leq 1.0 \times 10^{-5}$).
 B: Venn diagrams showing the over-lap of Unigene clusters up- or down-regulated in the three cell lines ($P \leq 1.0 \times 10^{-5}$).

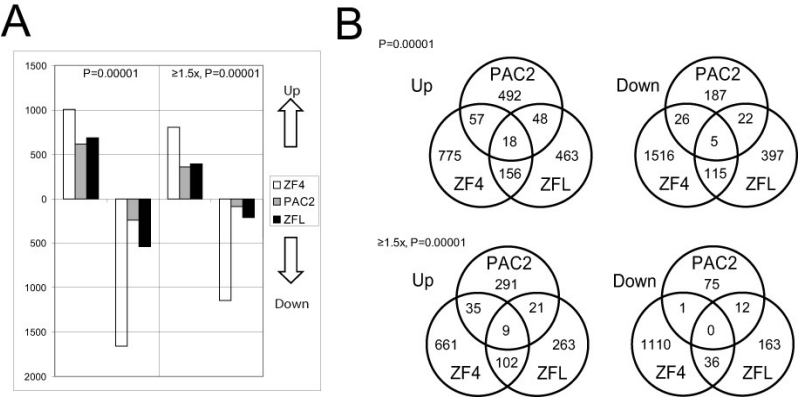
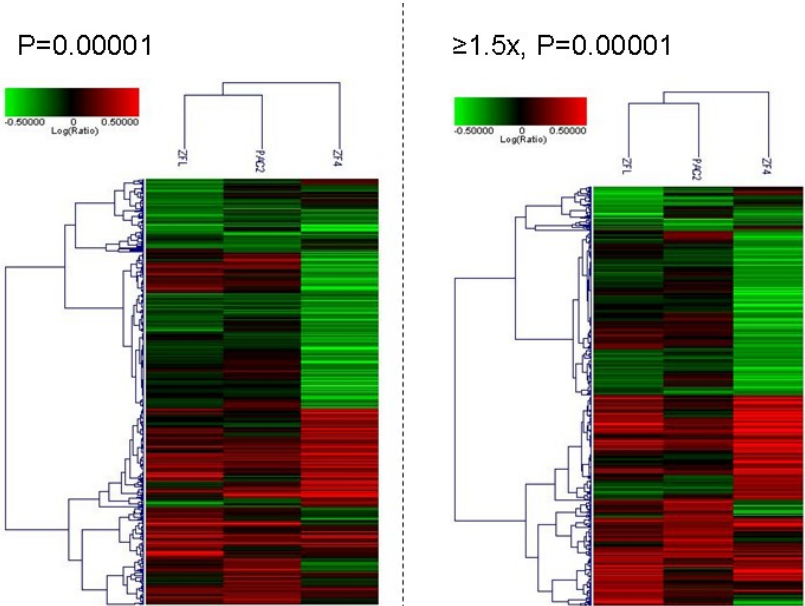


Figure 5. 2D- hierarchical cluster analysis of genes significantly regulated upon flagellin stimulation in the zebrafish cell lines.

Only the Unigene clusters that were significantly regulated by flagellin in at least one cell line ($P \leq 1.0 \times 10^{-5}$) were included for the clustering.



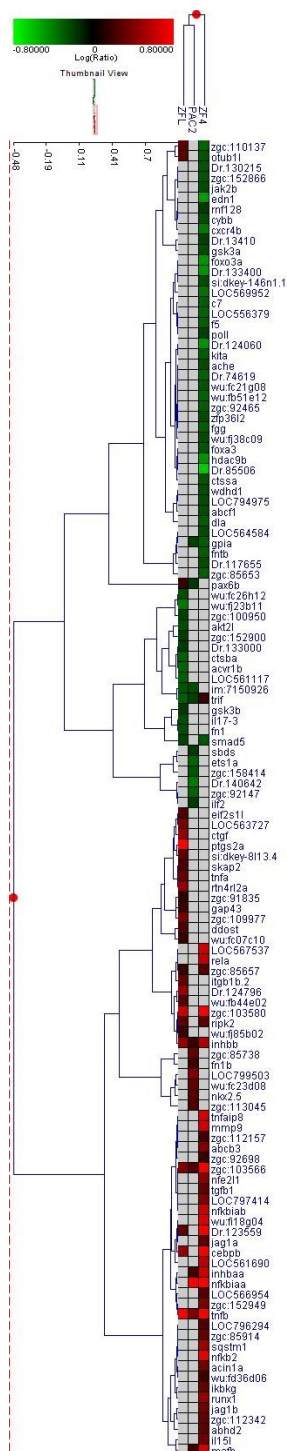
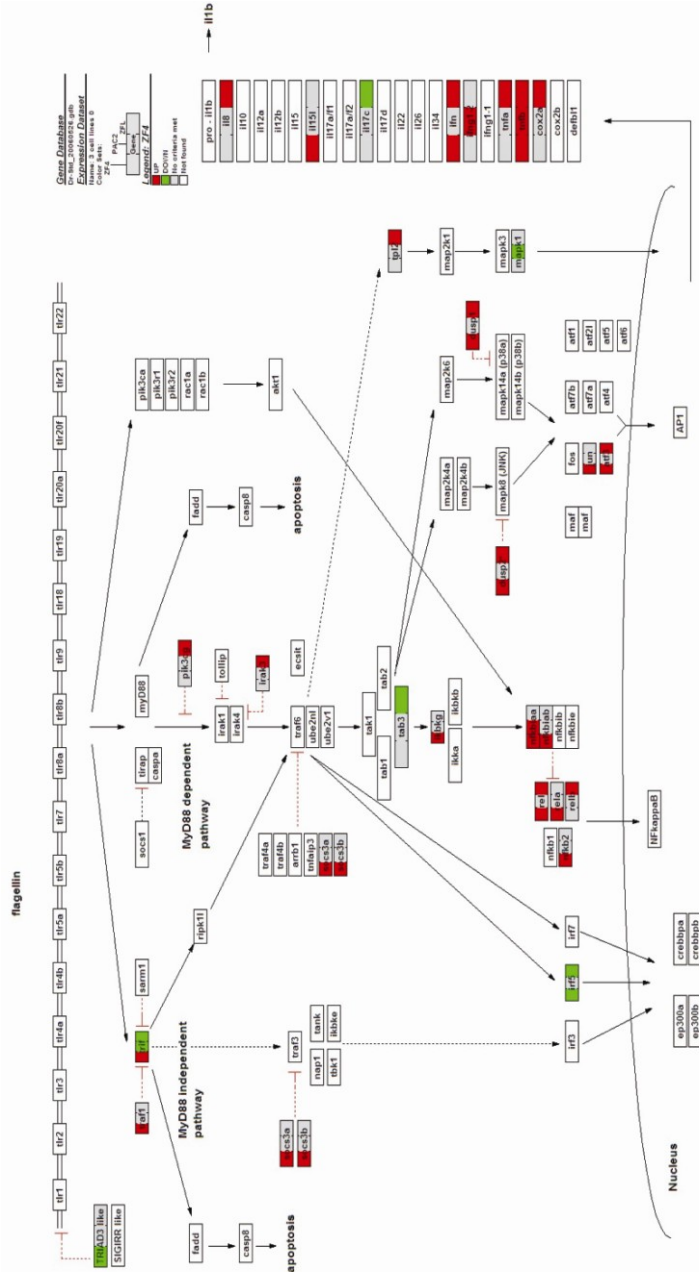


Figure 6. 2D- hierarchical cluster analysis of immune and wounding related genes significantly regulated upon flagellin stimulation in the zebrafish cell lines.

Only the Unigene clusters that were significantly regulated by flagellin in at least one cell line ($P \leq 1.0 \times 10^{-5}$) were included for the clustering. The data points with $P > 1.0 \times 10^{-5}$ are displayed in gray.

Figure 7. GenMAPP analysis of transcriptome responses in the TLR signaling pathway upon flagellin stimulation in the zebrafish cell lines.

Expression profiles of the three zebrafish cell lines upon flagellin stimulation ($P \leq 1.0 \times 10^{-5}$) were simultaneously mapped on the TLR pathway. Gene boxes are colored from left to right with expression data of the ZF4, PAC2 and ZFL cell lines. Up-regulated genes are indicated in red and down-regulated genes are indicated in green. The map is based on knowledge of the TLR signaling in mammalian specie. It should be noted that not all interactions have been experimentally confirmed in zebrafish.



deregulated genes were involved in processes related to cellular transport and localization. For example, up-regulated signatures were enriched in transport, endocytosis, protein targeting, regulation of cellular localization, neurotransmitter transport and regulation of vesicle-mediated transport, and down-regulated signatures were enriched in intracellular protein transport, nucleocytoplasmic transport and RNA localization. In the PAC2 cell line, many genes involved in transcription regulation were up-regulated. Other up-regulated signatures were enriched in biological processes such as signal transduction, cell differentiation and cell adhesion. The down-regulated signatures were mainly enriched in metabolic related process, including oxidation reduction, generation of precursor metabolites and energy, protein and cofactor metabolic processes.

In addition to the GO term analysis, enrichment analysis of KEGG pathways was also performed to sort the flagellin-signatures depending on which biological pathways they belong. The three zebrafish cell lines showed cell-line-specific KEGG pathway enrichment patterns (Figure 9). In the ZF4 cell line, the top-three pathways with enriched up-regulated signatures were the MAPK signaling pathway, apoptosis and small cell lung cancer. The top-three pathways with enriched down-regulated signatures were basal cell carcinoma, long-term depression and DNA replication. In the ZFL cell line, many deregulated genes were associated in pathways related to cancer, especially the small cell lung cancer which was enriched by both up- and down-regulated signatures. The focal adhesion pathway was also deregulated in both directions in the ZFL cell line, and many genes involved in the Wnt signaling pathway were down-regulated. In contrast, many genes involved in the Wnt signaling pathway were up-regulated in the PAC2 cell line. TGF β and Hedgehog signaling pathways were also enriched by up-regulated signatures in PAC2 cells, as well as pathways of focal adhesion and adherence junction. It indicates that the cellular inflammation responses in these zebrafish cell lines also involve alterations in signaling pathways associated with cancer.

Figure 8. GO analysis of transcriptome responses upon flagellin stimulation in the three zebrafish cell lines.

Flagellin-signatures ($P \leq 1.0 \times 10^{-5}$) in each cell line were categorized based on GO term analysis at the level 6 of 'biological process'. The functional categories with highest enrichment of regulated transcripts are listed.

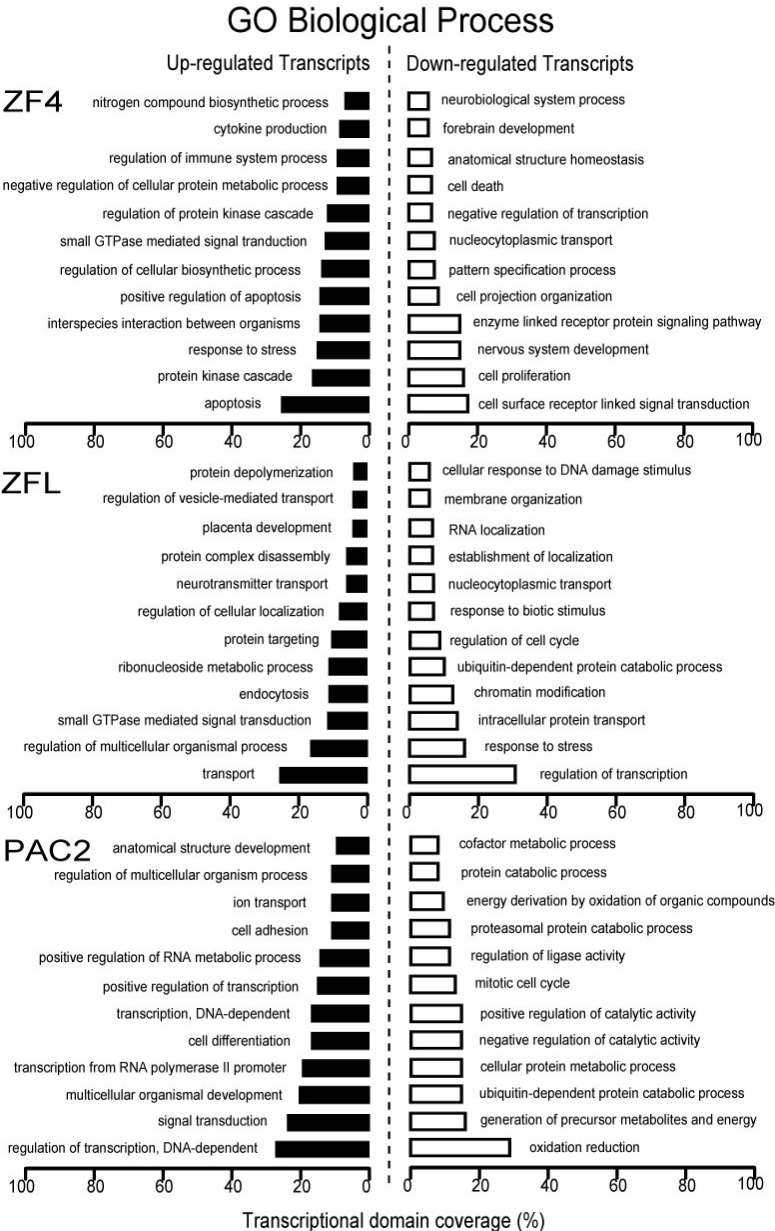
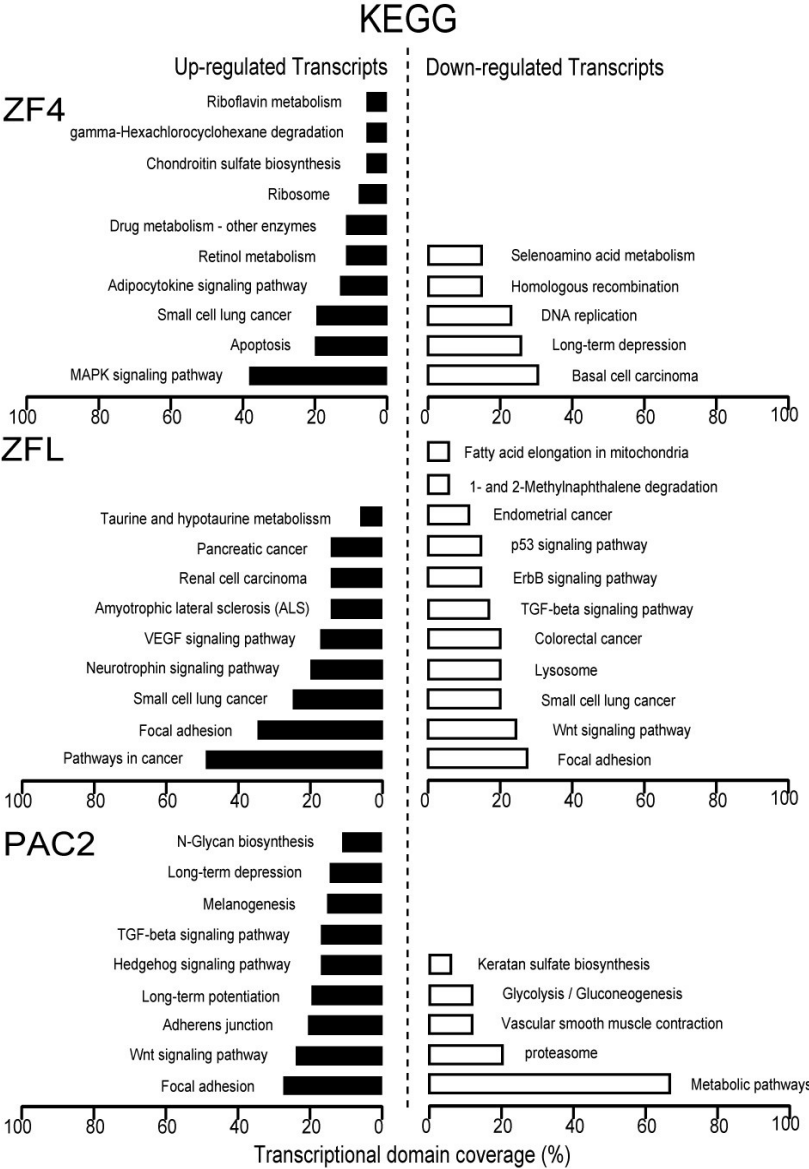


Figure 9. KEGG pathway enrichment analysis of transcriptome responses upon flagellin stimulation in the three zebrafish cell lines.

Flagellin-signatures ($P \leq 1.0 \times 10^{-5}$) in each cell line were analyzed for their enrichment in the KEGG pathways. The KEGG pathways with highest enrichment of regulated transcripts are listed.



Discussion

To explore the possible application of three zebrafish cell lines to study pathogen recognition and innate immunity, the ZF4, PAC2 and ZFL cell lines were first characterized for expression of genes involved in the TLR signaling pathways, such as TLRs, their downstream adaptors and cytokines. Most of the examined genes were expressed in all three cell lines, including homologs of human TLRs that recognize microbial membrane components (zTLR1, TLR2, TLR4a/b, TLR5a/b and TLR18).

TRAF6, the crucial molecule mediating the signal transduction from TLRs to NFκBs, is an ubiquitin ligase that catalyzes its auto-ubiquitination when activated by the TLR signaling [20]. The over-expression of TRAF6 also leads to its auto-ubiquitination [21]. Therefore TRAF6 over-expression mimics its activation and in turn activates NFκBs [6, 21], which was observed in the ZFL cell line. However, it was recently discovered that TRAF6 can also activate NFκB in an autoubiquitination-independent manner [20]. This indicates that the over-expression of TRAF6 does not fully represent its activation via TLR signaling. Since the NFκB activity induced by flagellin stimulation and TRAF6 over-expression were detected by different NFκB-Luc reporters, it suggested that different intracellular signaling pathways were activated after TLR activation by flagellin recognition and over-expression of TRAF6, perhaps by activation of different members of the zebrafish NFκB family. Considering the facts that the expression of NFκB1 was only detected in ZFL cell line but not in ZF4 and PAC2 cells, it is possible that NFκB1 might be activated by TRAF6 over-expression and its activity can be recognized by the NFκB-Luc-B reporter in the ZFL cells.

To characterize the functionality of the TLRs in zebrafish cell lines, several bacterial ligands were used to stimulate the cells. Stimulation with flagellin induced activation of the transcription factor NFκB in all three cell lines. Flagellin was the only compound that induced NFκB activation in all three cell lines. Flagellin is the primary structural component of the bacterial flagellum and in mammals, it is known to be recognized by TLR5 [18]. In zebrafish, it was recently found that TLR5a and TLR5b are required for flagellin recognition in zebrafish embryos [7]. Both TLR5s are expressed in the zebrafish cell lines, suggesting that TLR5a and TLR5b are putative recognition receptors of flagellin in these cells.

Mycobacterium marinum is a fish pathogen genetically closely related to the human pathogen *Mycobacterial tuberculosis*, which causes tuberculosis in fish. In human, TLR1 and TLR2 have been associated with mycobacterial recognition. In zebrafish, it was found that TLR1, TLR2, TLR5a/b, TLR9, TLR18, TLR20a and TLR22 were expressed at higher levels in the adult fish infected by *M. marinum*, compared with un-infected controls [8], suggesting that some of these TLRs might contribute to the mycobacterium recognition by the zebrafish innate immunity system. However, when the cell lines were stimulated with extracts of *M. marinum*, no induced NFκB activation was detected, despite the expression of TLRs in the cell lines. One explanation is that the response towards the pathogen in the three tested *in vitro* cell lines might be different than the *in vivo* situation in adult fish. It also possible that the *M. marinum* was actually recognized but the downstream response was not detected due to lack of sensitivity of the luciferase reporter assay.

In this study two NF κ B-Luc reporters with different κ B enhancers were used. These two reporters were widely used in mammalian cell cultures and induced NF κ B activity was detected by both reporters upon BLP stimulation in HEK cells overexpressing TLR2 (unpublished data). However, only few responses were detected using these reporters in the zebrafish cells. For example, despite the expression of TLR2 and TLR4 in the cell lines, no induced NF κ B activation was detected in any cell line after stimulation with ligands of human TLR2 and TLR4. It is probably due to the specificity of the enhancer recognition by the NF κ B dimmers. The specificity of target gene transcription by NF κ B is determined by the dimerization of different NF κ B subunits and their interaction with other co-activators [19]. TLR activation can result in formation of various NF κ B dimers in the zebrafish cells and not all of the dimers might be able to recognize the enhancer sites in the two reporter constructs used in this work.

Cell-line specificity in responses to activated TLR signaling in the three zebrafish cell lines was suggested by their NF κ B activation pattern upon either ligand stimulation or TRAF6 overexpression. It was further confirmed by transcriptome analysis of the immediate transcriptional responses to flagellin stimulation. Although similar NF κ B activation was induced in all cell lines upon flagellin stimulation, the transcriptome alterations were very different, with limited overlapping gene signature sets. The different transcriptome responses might reflect the different origins of the cell lines. The ZFL cell line is an epithelial cell line derived from adult zebrafish liver [16]. The ZF4 and PAC2 cell lines were derived from 24 hour zebrafish embryos in different laboratories and their exact tissue origins are not known. ZF4 cells have typical fibroblast morphology [14]. Although the PAC2 cell line was originally described as a fibroblast cell line [22], it showed epithelial-like morphology in culture [12]. 2d-cluster analysis showed that the ZFL and PAC2 cell lines shared more common transcriptional responses to flagellin, suggesting that the PAC2 cell line might be closer to an epithelial cell type rather than a fibroblast line.

It has been found that TLRs are widely expressed in most cell types, not only in professional antigen presenting cells (APCs) such as macrophages, dendritic cells and B-cells, but also in non-professional APCs including fibroblasts and epithelium [1]. However, the pattern of their expression and functionality in the non-professional APCs remain poorly characterized. Recently it was found that in mice, TLR5 is not expressed on macrophages and conventional DCs, but on lamina propria cells (LPCs) in the small intestine [23]. These LPC cells induced immune responses and secreted pro-inflammatory cytokines upon flagellin recognition by TLR5 [23]. The fact that flagellin induced inflammatory responses in the three zebrafish epithelial or fibroblast cell lines strongly suggested that these cell lines can be used to understand TLR5 signaling and the biological relevance of TLRs in non-professional APC cells. However, additional cell lines of myeloid origin will also be needed for further immunological studies in the zebrafish model.

Enrichment analysis of KEGG pathways of the flagellin-signatures in the three cell lines has revealed cell-line specific KEGG pathway enrichment patterns. Although the patterns varied in the three cell lines, pathways associated with cancer were among those with highest enrichment of flagellin-signatures in all cell lines. In the ZF4 cell line, genes involved in basal cell carcinoma progression were down-

regulated. Many up-regulated genes were in the MAPK signaling pathway and apoptosis, which are essential events in tumor initiation and progression. In the PAC2 cells, many up-regulated signatures were enriched in focal adhesion, Wnt, Hedgehog and TGF β signaling pathways, the deregulation of which are frequently found in cancer. The association of flagellin-signatures with cancer is the most profound in the ZFL cell line, where the top pathways with enriched signatures were involved in many cancer types, as well as deregulation in the VEGF, Wnt, p53, ErbB and TGF β signaling pathways. These results are consistent with the well known association of inflammatory responses with cancer. It was reported that infection-driven inflammations are linked to 15-20% of human cancers [24]. Recent experimental studies have associated inflammatory pathways to tumor pathogenesis and identified key factors in cancer-related inflammation, such as cytokines and NF κ B transcription factors [24, 25]. A lot remains unclear in this field and more experimental and clinical studies are required. The zebrafish cell lines can be used to further study the connection between inflammation and cancer as *in vitro* models. They can be transformed into tumorigenic cell lines. After implantation into transparent zebrafish embryos or adult fish, the zebrafish cells can also be used to monitor the interactions of the inflammatory microenvironment and tumor development *in vivo* at molecular and cellular level.

Acknowledgement

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Material and Methods

Plasmids and compounds

The NFκB-Luc reporter A (NFκB-Luc-A, containing 5x GGGGACTTCC repeats) was purchased from Straatgene. The NFκB-Luc reporter B (NFκB-Luc-B, containing 4x GGGAATTCC repeats) was purchased from Clontech. Zebrafish TRAF6 coding sequence from ATG to stop codon was amplified from clone NM_199821 using primers TRAF6-ATG and TRAF6-STOP (5'-CGGGATCCTCACCATGGCTTGCAACGACGTGGACAAG-3', 5'-CGAGGAATTCTCAAATTGAAGGTCTGGTCCTCGAG-3', respectively), and subsequently cloned into BamHI-EcoRI-digested pCS2+ vector. Purified LPS from *Escherichia coli* and flagellin from *Salmonella typhimurium* were purchased from Invivogen. Synthetic bacterial lipoprotein (BLP, Pam3Cy-OH) was purchased from EMC Micro Collections. Phorbol 12-myristate 13 acetate (PMA) was purchased from Sigma. Extracts of *Mycobacterium marinum* (strain E11) was kindly provided by Dr. B.J. Appelmelk.

Cell culture

The ZF4 cell line (ATCC CRL-2050) was grown at 28 °C in a mixture of 90% 1:1 mixed Dulbecco's modified Eagle's medium and Ham's F12 medium (containing 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate, Invitrogen-Gibco) and 10% fetal calf serum (FCS, Invitrogen-Gibco). The PAC2 cell line (supplied by Nick Foulkes) was grown at 28 °C in Leibovitz L-15 medium supplemented with 15% FCS. The zebrafish liver cell line (ZFL; ATCC, CRL2643) was cultured in complete growth medium (CGM; 50% Leibovitz's L-15 medium, 35% Dulbecco's modified Eagle's medium, 15% Ham's F12 medium, supplemented with 15 mM HEPES, 0.01 mg/ml insulin, 50 ng/ml EGF and 5% fetal bovine serum; all purchased from Invitrogen) at 28°C without additional CO₂.

Nucleofection

DNA for nucleofection was prepared using the GenElute Endotoxin-free plasmid kit (Sigma-Aldrich) according to the manufacturer's instructions. 1×10^6 cells were harvested and resuspended in 100 µl Nucleofector Solution V (Amaxa, Cologne, Germany) containing 5 µg DNA for each nucleofection. The cell suspension was transferred into a kit-provided cuvette and positioned into a Nucleofector device. The nucleofections were performed with a single pulse using the preprogrammed nucleofection programs T27 according to the manufacturer's instructions.

Luciferase reporter assay

For the luciferase reporter assay, 1×10^6 cells were nucleofected with 2 µg of the NFκB-Luc reporter and 3.6 µg of TRAF6 (or 2.5 µg empty vector as control). After nucleofection the cells were divided into 4 wells of a 24-well plate and incubated for 40 hours. The lysis and luciferase measurement was performed using the Dual-Luciferase reporter assay system (Promega) and a luminescence counter (Wallac Jet 1450, PerkinElmer) according to the manufacturer's instructions.

RNA isolation

Total RNA was isolated from cells using Trizol reagent (Invitrogen). After DNase treatment (DNaseI, Roche) RNA samples were purified using MiniElute columns under the manufacturer's instruction (Qiagen).

RT-PCR

RT-PCR was performed using SuperScript™ III One-Step RT-PCR (Invitrogen).

RNA preparation and microarray hybridization

For RNA isolation, cells were prepared as described before [12]. Agarose gel electrophoresis showed the good quality of isolated and purified RNA. cDNA and amino allyl-modified aRNA were synthesized using Amino Allyl MessageAmpII aRNA Amplification Kit (Ambion). The aRNA was coupled with Cy3/Cy5 (GE Healthcare) and purified using NucleoSpin RNAII (Macherey-Nagel). RNA concentration and coupling efficiency was determined by NanoDrop (Thermo Scientific). 500 ng of labeled aRNA was used for each hybridization on the custom-designed Agilent 4x44k zebrafish microarray containing 43365 oligos (19122 unigene clusters, build 105). Hybridization and scanning were performed according to standard Agilent protocols. The feature extraction software version 9.5, protocol ge2_V5_95 from Agilent was used to generate the feature extraction data. For the background subtraction the option 'No background subtraction and spatial detrend' was used. The arrays were scanned twice with 10% PMT and 100% PMT laser power and the XDR function was used to extend the dynamic range with 10-fold.

Microarray data analysis

Microarray data was imported into Rosetta Resolver 7.0 (Rosetta Biosoftware) and subjected to default ratio error modeling. Data analysis was performed at $P \leq 1.0 \times 10^{-5}$ for unigene clusters. The Unigene and Entrez Gene records of the functionally related human homologs of our zebrafish unigene list of gene signatures were automatically retrieved from the NCBI HomoloGene database. GenMAPP analysis was performed as described before [7]. General Gene Ontology (GO) analysis at the level of 'biological process' and the KEGG pathway analysis were performed using the FunNet software (www.funnet.info) [26] using the Entrez Gene codes of human orthologs.

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