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Title: Regulatory mechanisms of innate immune signaling in zebrafish embryos
Date: 2012-12-12
Chapter 4

MicroRNA-146 function in the innate immune response of zebrafish embryos to bacterial infection

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Abstract

MicroRNAs (miRNAs) have recently been shown to play important roles in development of the immune system and in fine-tuning of immune responses. Human miR-146 is known as an inflammation-inducible miRNA involved in negative feedback regulation of Toll-like receptor (TLR) signaling. Dysregulation of miR-146 has often been linked to inflammatory diseases and malignancies. In this study, we used zebrafish embryos, in which adaptive immunity is not yet active, as an in vivo system to investigate the role of miR-146 in the innate immune response to bacterial infections. Both members of the miR-146 family, miR-146a and miR-146b, were induced upon infection of embryos with Salmonella typhimurium or Mycobacterium marinum. Knockdown of traf6 and use of myd88 knockout mutants showed that this induction is partially dependent on the MyD88-Traf6 pathway that mediates transduction of TLR signals. RNA-Seq analysis of miR-146 knockdown embryos showed increased induction of the matrix metalloproteinase 9 (mmp9) gene upon S. typhimurium infection, but no general hyperinduction of other pro-inflammatory markers was observed. While the overall knockdown effect was relatively minor, apolipoprotein-mediated lipid transport emerged as an infection-inducible pathway under miR-146 knockdown conditions, suggesting a function of miR-146 regulating lipid metabolism during inflammation. Bacterial burden in infection experiments with the attenuated S. typhimurium Ra strain was not significantly affected in miR-146 knockdown embryos compared to control embryos. In contrast, chronic infection with M. marinum under miR-146 knockdown conditions led to increased bacterial burden. This effect was primarily due to knockdown of miR-146b, suggesting a protective function of miR-146b-mediated control during M. marinum infection.

Introduction

Timely activation as well as termination of inflammatory responses is vital for proper functioning of the immune system. A balanced output of the vertebrate immune response is dependent on several regulatory mechanisms, in which microRNAs (miRNAs) have recently emerged as novel players with critical importance (O’Neill et al., 2011). Several human diseases, including autoimmune diseases and chronic inflammations, have been associated with dysregulation of miRNA expression, and oncogenic and tumor suppressor functions of miRNAs have also been reported (O’Neill et al., 2011; Sonkoly and Pivarcsi, 2009; Schetter et al., 2009, 2010; Persengiev, 2012; Visone and Croce, 2009; Sassen et al., 2008). MiRNAs are evolutionary conserved, genome-encoded small RNAs (~22 nucleotides) involved in post-transcriptional gene repression (Fire, 1999; Grishok et al., 2001). They down-regulate gene expression at the post-transcriptional level by either translational repression or by mRNA degradation through binding to the 3’-UTR of their target mRNAs (Ambros, 2004). Over recent years, miRNAs were found to play roles in diverse processes, ranging from development, cellular differentiation, hematopoiesis, apoptosis, and growth, to
regulation of the immune system (Belver et al., 2011; O’Connell et al., 2011; Schulte et al. 2011; Ambros, 2004; Nahid et al., 2011; Liu et al., 2011; Quinn et al. 2012).

It has been well established that miRNAs have diverse roles in pathogenic infections, including responses to viral and bacterial pathogens. The anti-viral function of miRNAs was first discovered in plants, and subsequent plant research also demonstrated a miRNA-mediated resistance mechanism to bacterial infection via down-regulation of auxin signaling (Ding and Voinnet, 2007; Navarro et al., 2006). It has been reported that miRNAs produced by the host can stimulate viral replication (Jopling et al., 2005; Jopling, 2008; Umbach and Cullen, 2009). However, host miRNAs, such as miR-29a, miR-32, miR-24, and miR-93, have anti-viral activities by suppressing viral mRNAs (Lecellier et al., 2005; Pederson et al., 2007; Nathans et al., 2009; Otsuka et al., 2007). In addition, several viruses encode their own miRNAs that affect host mRNA expression, or produce virulence factors that interfere with host miRNA functions (Sullivan et al., 2005, 2009; Aparicio et al., 2006; Hussain et al., 2008; Seo et al., 2008, 2009). The strong connections between miRNAs and viral infections, together with evidence for substantial roles of miRNAs in immune cell differentiation, inflammation, and immune-related diseases, stimulated research into the function of miRNAs in bacterial infections (Baltimore et al., 2008; Lindsay, 2008). MiRNAs were observed to be differentially regulated by Toll-like receptor (TLR)-mediated recognition of bacterial molecules; for instance, lipopolysaccharide (LPS) recognition by TLR4 and downstream NF-κB activity induced expression of miR-146a and miR-146b (miR-146a/b) and miR-155 (Taganov et al., 2006; O’Connell et al., 2007; Tili et al., 2007; Androulidaki et al., 2009; Ceppi et al., 2009; Liu et al., 2009). More recently, Salmonella typhimurium was found to induce a highly specific change in the expression of a subset of host miRNAs in macrophages. This study implicated the let-7 miRNA gene family in anti-bacterial defense by showing that the down-regulation of let-7 miRNAs promotes the expression of the key cytokines, IL-6 and IL-10, in cells invaded by S. typhimurium (Schulte et al., 2011). IL-10 production was also found to be regulated by miR-98 (Liu et al., 2011). Furthermore, miR-29 was found to suppress immune responses to Listeria monocytogenes and Mycobacterium tuberculosis by targeting interferon γ (IFN-γ) (Ma et al., 2011).

MiRNA-146 is expressed as a family with two members, miR-146a and miR-146b, which have the same seed sequence (i.e. the sequence essential for the binding of the miRNA to the mRNA) and are located on chromosome 5 and 10 respectively, within different genes (Labbaye and Testa, 2012). The structural differences of both miRNAs are limited, since they differ in their mature sequence by only two nucleotides at the 3’end. The first indication of the role of miR-146a/b in innate immunity came from work of Taganov et al. (2006), showing increased expression of these miRNAs in the human monocytic THP-1 cell line when triggered by LPS. Promoter analysis revealed that induction of the miR-146a gene by LPS, TNFα, and IL-1 is mediated by the NF-κB transcriptional factor (Taganov et al., 2006). In addition, 3’UTR luciferase reporter assays demonstrated that the TLR signaling intermediators IRAK1 and TRAF6 are potential targets of miR-146a/b (Taganov et al., 2006). These data suggested that miR-
146a/b function in a negative feedback pathway of TLR and cytokine signaling by targeting IRAK1 and TRAF6 mRNAs for down-regulation, a conclusion supported by recent analysis of miR-146a knockout mice (Boldin et al., 2011). MiR-146a/b were also reported to be expressed highly in synovial tissues of rheumatoid arthritis patients compared to normal individuals, and this induction was stimulated by inflammatory cytokines, such as TNFα and IL-1 (Nakasa et al., 2008). MiR-146a has also been proposed to function as a negative regulator of IFN signaling by targeting the IRF5 and STAT-1 transcription factors (Tang et al., 2009).

Like several other miRNAs that have been implicated in regulation of the immune system, MiR-146 has also been linked to cancer processes. Dysregulation of miR-146a/b has been observed in many types of malignant tumors (Rusca and Monticelli, 2011; Labbye and Testa, 2012). Overexpression of miR-146a/b in a highly metastatic human breast cancer cell line was shown to significantly reduce NF-κB activity by negatively regulating IRAK1 and TRAF6 (Bhaumik et al., 2008). The up-regulation of miR-146a/b in breast cancer was associated with reduced metastasis (Bhaumik et al., 2008; Hurst et al., 2009). Similarly, miR-146b was found to inhibit glioma cell migration and invasion by targeting matrix metalloproteinases (MMPs) (Xia et al, 2009). Thus, modulating the levels of miR-146 could have a therapeutic potential to suppress cancer metastasis.

The zebrafish provides a useful model to study innate immunity, which is the primary line of defence against infections during the first few weeks of development, when there is no functional adaptive immunity present (Stockhammer et al., 2009; Meijer and Spaink, 2011). The zebrafish miRNA family is well characterized (Chen et al., 2005; Soares et al., 2009) and previous microarray analysis showed that infection-responsive miRNAs are well conserved between human and zebrafish (Ordas, 2010). As in human, the zebrafish miR-146 family has two members, named dre-miR-146a and dre-miR-146b, which are present within genes located on chromosome 13 and 21 respectively. The IRAK1 and TRAF6 homologs of both zebrafish and human contain putative target sites for miR-146 in their 3'UTRs, suggesting that miR-146 feedback control of TLR signaling is evolutionary conserved (Ordas, 2010).

Here we have used zebrafish embryos as an in vivo model for a functional study of the role of miR-146a and miR-146b towards S. typhimurium and Mycobacterium marinum infection. The pathologies caused by these two pathogens have been well characterized in zebrafish. S. typhimurium is known to cause an acute infection after intravenous injection into one-day old embryos. This infection is accompanied by a strong pro-inflammatory response and is lethal within approximately one day, while an LPS mutant (Ra) of S. typhimurium is non-pathogenic (van der Sar et al., 2003; Stockhammer et al., 2009). Infection of zebrafish embryos or adults with M. marinum causes a different pathology accompanied by the formation of tissue aggregates of infected and uninfected immune cells that resemble the human tuberculous granuloma in which mycobacteria can persist chronically (Davis et al., 2002; Swaim et al., 2006). We demonstrate the requirement of the Myd88-Traf6 pathway for the infection-triggered induction of miR-146a/b in zebrafish. Furthermore, we used morpholino
knockdown to suppress the function of miR-146a/b and analyzed the effects of this down-regulation on the transcriptome and bacterial burden. While the effect of miR-146a/b knockdown on pro-inflammatory gene expression in the immediate response to acute *S. typhimurium* infection was minor, miR-146b deficiency increased bacterial burden in chronic *M. marinum* infection.

**Materials and Methods**

**Zebrafish husbandry**

Zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). Embryos from the zebrafish AB/TL line were used for the infection experiments. In addition, an infection experiment was performed using embryos from a *myd88* knockout mutant line and wild type siblings as a control (van Soest, 2012; van der Vaart et al., unpublished). Embryos were grown at 28–30°C in egg water (60 µg/ml Instant Ocean sea salts). For the duration of bacterial injections embryos were kept under anesthesia in egg water containing 200 µg/mL tricaine (Sigma-Aldrich). Embryos used for immunostaining and Myeloperoxidase (Mpx) assay were kept in egg water containing 0.003% 1-phenyl-2-thiourea (Sigma-Aldrich) to prevent melanization.

**Morpholino knockdown**

Morpholino oligonucleotides (GeneTools) were diluted to the desired concentration in 1× Danieau 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-Aldrich) and approximately 1 nl was injected at the 1-2 cell stage using a Femtojet injector (Eppendorf). For knockdown of miR-146a and miR-146b two morpholinos were used against each of them. The first morpholino for miR-146a (146aMO1: 5’ACCATCTATGGAATTCAGTTCTCAG3’) targets the miRNA guide strand and the second morpholino (146aMO2: 5’GAGCCCAUAGAUGAACUUUUCAUGA3’) overlaps with the star strand and the dicer cleavage site on the star strand (Supplementary Fig. 1A,B). For miR-146b, the first morpholino (146bMO1: 5’GACACCCTTGGAATTCAGTTCA3’) also targets the guide strand, and the second morpholino (146bMO2: 5’CGTGGGCTGAATATAAAGCAGACAC3’) overlaps with both dicer cleavage sites and part of the star strand (Supplementary Fig. 1B). All miR-146 morpholinos could be used at a concentration of 0.75 mM without causing morphological defects, except 146b-MO2, which was highly toxic. Another morpholino design for miR-146b was not recommended by GeneTools. For *traf6* knockdown we used a previously described morpholino (Stockhammer et al., 2010). As a control the standard control morpholino (scMO) from GeneTools was used as previously described (Chapter 2).
Infection experiments
S. typhimurium infections were performed using strain SL1027 and its isogenic LPS Ra mutant derivative SF1592, carrying the DsRed expression vector, pGMDs3 (van der Sar et al., 2003). For Mycobacterium marinum infection experiments, the Mma20 strain was used expressing mCherry in pSMT3 vector (van der Sar et al., 2004). Bacteria were grown and prepared for injections as described in Cui et al., (2011) and microinjected into the caudal vein of embryos at 28 hours post fertilization (hpf), using a dose of 200-250 CFUs of S. typhimurium or 100 CFU of M. marinum per embryo. As a control, embryos were mock-injected with phosphate-buffered saline (PBS). After injections, embryos were transferred to fresh egg water and incubated at 28°C. M. marinum-infected zebrafish adults (Mma20 strain, 6 dpi) were from a previous study (van der Sar et al., 2009).

RNA isolation and quality check
Embryos were snap frozen in liquid nitrogen and kept at -80°C. Total RNA was isolated using the miRNeasy Mini kit (Qiagen) with an on-column DNA purification with RNase Free DNase set (Qiagen). RNA from adult zebrafish was isolated as previously described (Ordas, 2010). RNA quality of samples for deep sequencing was analyzed with an Agilent Bioanalyzer 2100 using the RNA 6000 Nano series Kit (Agilent, Santa Clara, CA, USA). All samples had a RNA integrity value (RIN) of 10.

Quantitative RT-PCR analysis
For quantification of miR-146a/b expression levels, RT-PCR reactions were performed using a TaqMan microRNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s instructions. Briefly, 10 ng of total RNA was reverse transcribed using 5x RT primers (Custom TaqMan small RNA Assay-Applied Biosystems) in a total reaction volume of 15 μl. Reactions were kept on ice for 5 min and then were transferred to the thermal cycler for incubations at 16°C and 42°C for 30 min each, followed by an incubation at 85°C for 5 min. Quantitative RT-PCR with 0.665 μl cDNA input per reaction was performed using a Custom TaqMan small RNA Assay for each miRNA and a TaqMan Universal PCR Master Mix (Applied Biosystems) in a total of 10 μl per reaction. Cycle threshold values were calculated under the parameters of 40 cycles of 10 min at 95°C, 15 sec at 95°C and 60 sec at 60°C. All reactions were performed in at least two technical replicates. For normalization, miR-222, which showed no changes in response to bacterial challenge, was taken as reference. Results were analyzed using the ΔΔCt method. Quantification of mmp9 expression was performed as previously described (Stockhammer et al., 2009).

RNA-Seq analysis
For RNA-Seq analysis, embryos were injected with a combination of 146aMO1 and 146bMO1, or with the scMO. Subsequently, at 28 hpf they were infected with S. typhimurium or mock-injected with PBS, and RNA was isolated at 8 hours post injection.
Two independent experiments were performed for RNA-Seq analysis of biological duplicates. A total of 3 μg of RNA was used to make RNA-Seq libraries using the Illumina TruSeq RNA Sample Preparation Kit v2 (Illumina Inc., San Diego, USA). In the manufacturer's instructions two modifications were made. In the adapter ligation step 1 μl instead of 2.5 μl adapter was used. In the library size selection step the library fragments were isolated with a double Ampure XP purification with a 0.7x beads to library ratio. The resulting mRNA-Seq library was sequenced using an Illumina HiSeq2000 instrument according to the manufacturer's description with a read length of 2 x 50 nucleotides. Image analysis and base calling was done by the Illumina HCS version 1.15.1. Sequence reads were quality trimmed using the quality_trim module in the CLCbio Assembly Cell v4.0.6. Filtered reads were mapped to Ensembl transcripts (Zv9_63) using the ref_assemble_short module in the CLCbio Assembly Cell v4.0.6. Accumulation of transcripts to Ensembl genes was done by first converting the mapping files to a table with the assembly_table module in the CLCbio Assembly Cell v4.0.6. Secondly, a custom script was used that sums all reads belonging to the same gene. Non-uniquely mapped reads were divided between genes according to their ratio of uniquely mapped reads. Finally, read counts of transcripts belonging to the same gene were summed to obtain count data at Ensembl gene level. Fold-change and differential expression significance values were calculated from gene level read counts using the DESeq package (version 1.8.3) available in Bioconductor (version 2.10). DESeq utilizes a negative binomial distribution for modeling read counts per gene and implements a method for normalizing the counts (Anders and Huber, 2010). KEGG pathway analysis of RNA-Seq data was performed using DAVID (Huang et al., 2009).

Detection of leukocytes
Embryos were fixed in 4% paraformaldehyde (PFA) in PBS. Immunofluorescence detection of leukocytes was performed with a 1:500 dilution of polyclonal rabbit Ab against L-plastin (Mathias et al 2007) and Alexa Fluor 488 goat anti-rabbit IgG secondary Ab (Molecular Probes), as described in Cui et al., (2011). Histochemical detection of neutrophils was performed by Mpx activity staining using the Peroxidase Leukocyte Kit (Sigma-Aldrich) as described in Cui et al., (2011).

Microscopy and image analysis
Fluorescence images were taken with a Leica MZ16FA stereo fluorescence microscope equipped with a DFC420C digital color camera. Composite images of different focal planes were made using Adobe Photoshop. Pixel counts on stereo fluorescence images were performed as described in Stoop et al (2011).
Results

miR-146a/b are induced during zebrafish infection with *S. typhimurium* and *M. marinum*

Previous microarray data suggested that miR-146a/b are infection-inducible miRNAs in zebrafish embryos and adult fish (Ordas, 2010). To confirm these results we analyzed miR-146a/b expression by TaqMan qPCR analysis using miR-222 as a control for normalization, since it showed unaltered expression in the microarray study. In agreement with the microarray data, miR-146a/b were specifically induced in embryos at 8 hours post injection (hpi) with *S. typhimurium* wild type or LPS mutant (Ra) strains (Fig. 1A, B). This infection-dependent increase in miR-146a/b levels could be blocked by injection of morpholinos targeting these miRNAs (Fig. 1A, B). Induction of miR-146a/b was also detected in zebrafish larvae containing a granulomatous *M. marinum* infection (Fig. 1C) as well as in adult fish infected with *M. marinum* (Fig. 1D).
Figure 1. Expression of miR-146a/b is enhanced upon bacterial infections. (A) MiR-146a/b induction in zebrafish embryos by *S. typhimurium*. Embryos were infected with *S. typhimurium* wild type bacteria (inf) or injected with PBS (mock) at 28 hpf and expression of miR-146a/b was analyzed at 8 hpi. Infection-dependent induction of miR-146a/b was blocked by morpholinos (MO) targeting miR-146a (146aMO1) or miR-146b (146bMO1). As a control, a standard control morpholino (scMO) was injected. (B) MiR-146a/b induction in zebrafish embryos by the attenuated *S. typhimurium* Ra strain. Experimental conditions were the same as for infection with wild type *S. typhimurium* (A). (C) MiR-146a/b induction in zebrafish larvae with a granulomatous *M. marinum* infection. Embryos were infected with *M. marinum* Mma20 bacteria (inf) or injected with PBS (mock) at 8 hpf, and expression of miR-146a/b was analyzed in larvae at 6 dpi. Infection-dependent induction of miR-146a was reduced by a morpholino (MO) targeting this miRNA (146aMO1), but a morpholino against miR-146b (146bMO1) had lost its effect during this late stage of larval development. (D) MiR-146a/b induction in *M. marinum*-infected zebrafish adults. Adult zebrafish were injected intraperitoneally with *M. marinum* Mma20 or mock-injected with PBS and RNA was collected at 6 dpi (van der Sar et al., 2009). Expression levels in all experiments were determined by TaqMan qPCR and relative expression levels are shown with the mock control set at 1. Data are the mean ± SEM of two or three independent experiments, except for the miR-146a *S. typhimurium* Ra infection where a single experiment is shown. Asterisks indicate significant differences (*, P < 0.05; **, P <0.01; ***, P <0.001) tested by one-way ANOVA analysis with Tukey’s method as post-hoc test (A-C) or by an unpaired t-test (D).

Infection-inducible expression of miR-146a and miR-146b is partially dependent on signaling via the MyD88-Traf6 pathway

We used the *S. typhimurium* embryo infection model to investigate the dependency of miR-146a/b induction on TLR pathway genes. First, we used a previously described morpholino knockdown model for *traf6*, a central intermediate in TLR and TNF receptor signaling (Stockhammer et al., 2010). The *S. typhimurium*-induced expression levels of miR-146a/b were significantly lower in *traf6* knockdown embryos compared to controls (Fig. 2A).

Next, we analyzed miR-146a/b induction in a knockout mutant of *myd88* (van Soest 2012, van der Vaart et al., unpublished). Similar as under Traf6 knockdown conditions, miR-146a/b were still infection-inducible in *myd88* knockout embryos, but their induction levels were significantly higher in wild type siblings (Fig. 2B). Therefore, we conclude that miR-146a/b induction is partially dependent on Myd88 and Traf6, but also induced via Myd88 and Traf6-independent signaling.
Figure 2. The Traf6-MyD88 pathway is involved in up-regulation of miR-146a/b expression levels upon infection. (A) Traf6-dependent miR-146a/b induction. Embryos were injected with traf6 morpholino (MO) or a mismatch morpholino (MM) as a control. Embryos were infected at 28 hpf with S. typhimurium (inf) or mock-injected with PBS and samples were collected at 8 hpi. (B) Myd88-dependent miR-146a/b induction. Mutant (myd88-/−) and wild type siblings (myd88+/+) were infected with S. typhimurium at 28 hpf followed by sample collection at 8 hpi. Expression levels in both experiments were determined by TaqMan qPCR and relative expression levels are shown with the mock control set at 1. Data are the mean ± SEM of two or three independent experiments. Asterisks indicate significant differences (*, P < 0.05; **, P < 0.01) tested by one-way ANOVA analysis with Tukey’s method as post-hoc test.

MiR-146a/b do not affect leukocyte development in zebrafish embryos

Loss of function studies in mice and zebrafish suggested a possible role of miR-146a in the development of myeloid cells, in addition to its proposed inhibitory effect on pro-inflammatory signaling (Ghani et al., 2011). To investigate the possible requirement of miR-146a/b for leukocyte development in zebrafish embryos, we designed two different morpholinos for each miRNA (Supplementary Fig. 1). The efficiency of the knockdown was confirmed by TaqMan qPCR analysis, showing an approximately 10-fold decrease in the expression levels of miR-146a and miR-146b at 2 dpf with each morpholino (Fig. 3G). Immunostaining for L-plastin, a pan-leukocytic marker, was performed to determine the number of immune cells over a time course of 26, 28, 30, and 32 hpf. During this period primitive myeloid cells first appear over the yolk sac, and subsequently invade the head. This first wave of primitive myeloid cells is rapidly followed by differentiation of the first erythro-myeloid precursor cells in the caudal blood island region. The total number of L-plastin-positive leukocytes showed a similar
Figure. 3 Knockdown of miR-146a/b does not affect leukocyte development. Embryos were injected at the 1-cell stage with morpholinos targeting miR-146a (146aMO1 and 146aMO2) or miR-146b (146bMO1 and 146bMO2) or were injected with standard control morpholino (scMO). (A–F) Representative images of L-plastin immunostaining of 32 hpf embryos injected with the indicated morpholinos. The pattern of L-plastin-positive immune cells was comparable between embryos injected with scMO (A), 146aMO1 (B), 146aMO2 (C), and 146bMO1 (D). 146bMO2 gave non-specific phenotypes and the number of immune cells was more variable dependent upon the severity of phenotype (E,F). (G) Confirmation of morpholino knockdown. Embryos were injected with the indicated morpholinos and RNA was collected at 2dpf. Knockdown of miR-146a and miR-146b was confirmed by TaqMan qPCR. (H) Quantification of L-plastin-positive leukocytes at 26, 28, 30 and 32 hpf. Embryos were injected with the indicated morpholinos. L-plastin-labeled cells were counted manually on the left side
of each embryo and the numbers present in the head, on the yolk sac, and in the caudal blood island were accumulated (n ≥ 16 embryos per time point). (I) Quantification of L-plastin-positive leukocytes at 2 dpf. Embryos were injected with the control morpholino (scMO) or with a combination of 146a/bMOs. L-plastin-labeled cells were counted manually as described above (n ≥ 26 embryos per group). (J) Quantification of Mpx-positive neutrophils at 2dpf. Embryos were injected with the indicated morpholinos. Neutrophils stained for Mpx activity were counted manually as described above (n ≥ 13 embryos per group).

increase over the time course between control embryos and embryos injected with 146aMO1, 146aMO2, or 146bMO1 (Fig. 3A-D, H). 146bMO2 could not be included in this quantitative analysis, because this morpholino resulted in non-specific phenotypes even at very low concentration (0.01mM) in comparison to the high inject-able doses of the other morpholinos (0.75mM) that had no overt effects on embryo development. Nevertheless, L-plastin positive immune cells were still present in 146bMO2 morphants with mild (Fig. 3E) or severe phenotypes (Fig. 3F). In addition, we analyzed a combination of morpholinos for miR-146a/b (146aMO1 and 146bMO1 (146a/bMOs)) at 2 dpf. As in the 28-32 hpf time course with the separate morpholinos, no difference was observed between controls and morphants in the numbers of L-plastin-stained immune cells at this stage (Fig. 3I). The number of neutrophils was also comparable between controls and morphants as visualized by histochemical staining for Mpx activity at 2dpf (Fig. 3J). Based on these results, we conclude that miR-146a/b are not required for leukocyte differentiation during zebrafish embryo development.

Combined knockdown of miR-146a/b does not have a major effect on pro-inflammatory gene expression during S. typhimurium infection
In previous work, we observed that knockdown of a negative regulator of the immune response (the ptpn6/shp1 phosphatase gene) resulted in a hyperinduction of pro-inflammatory gene expression during S. typhimurium infection (Chapter 2). Since miR-146 has also been proposed as a negative regulator of innate immunity (Taganov et al., 2006), we hypothesized that miR-146 knockdown might have a similar effect. To test this hypothesis, we used a combination of miR-146a/b morpholinos and analyzed the response to S. typhimurium infection by RNA deep sequencing (RNA-Seq) (Fig. 4A). First we analyzed the basal expression differences between uninfected miR-146a/b morphants and embryos injected with a control morpholino. Only 78 genes were affected by miR-146a/b knockdown, among which 5 genes in p53 signaling (Fig. 4B). This might reflect a non-specific effect of the miR-146 knockdown, since morpholino effects on the p53 pathway are relatively common (Robu et al., 2007). S. typhimurium infection resulted in differential expression of 726 genes in embryos injected with a control morpholino and 884 genes in miR-146 morphants. In agreement with previous studies (Chapter 2, Stockhammer et al., 2009, 2010, Ordas et al., 2011), S. typhimurium infection resulted in significant alteration of KEGG pathways related to the immune response and metabolism (Fig. 4B). While the total numbers of S. typhimurium up- and down-regulated genes in miR-146 morphants were somewhat higher than in control embryos (Fig. 4C), there was no general hyperinduction of pro-inflammatory genes in
miR-146 morphants. The only pro-inflammatory marker that was up-regulated to higher levels in infected miR-146 morphants compared with the infected controls was the *matrix metalloproteinase 9 (mmp9)* gene (Supplementary Table 1). RNA-Seq showed a 1.5-fold higher up-regulation of this gene in miR-146 morphants, which was confirmed by qPCR analysis (Supplementary Fig. 2). We did not observe effects of miR-146 knockdown on the expression of *traf6* and *irak1*, genes known to be targeted by miR-146 in human. Instead of an effect on innate immunity signaling, KEGG pathway analysis revealed a possible effect on lipid transport in *S. typhimurium*-infected miR-146 morphants. Six members of the apolipoprotein family (Fig. 4C) were significantly induced during *S. typhimurium* infection of miR-146 morphants but not in infected control embryos. In conclusion, miR-146a and miR-146b knockdown in zebrafish embryos did not have a strong effect on innate immunity signaling in the first 8 hours of the response to *S. typhimurium* infection, despite the increased expression of these miRNAs during this phase. Furthermore, bacterial burden in *S. typhimurium* infection (analyzed with an attenuated Ra strain; data not shown), was not significantly affected by miR-146a/b knockdown.

**Knockdown of miR-146a/b increases bacterial burden of *M. marinum* infection**

To see the effect of miR-146a/b knockdown under chronic infection conditions, we infected the embryos with *M. marinum*. First we analyzed the effect of combined miR-146a/b knockdown. Fluorescent pixel quantification of mCherry-labelled *M. marinum* bacteria at 3 days post infection demonstrated a significantly increased bacterial burden in miR-146a/b morphants in comparison with embryos injected with the control morpholino (Fig. 5A). Subsequently, two independent experiments were performed to elucidate whether this effect could be attributed to knockdown of miR-146a or miR-146b or both. These experiments consistently showed miR-146b knockdown to result in more bacterial proliferation as compared to miR-146a knockdown or control embryos (Fig. 5B, C). The bacterial burden was not significantly different between miR-146b knockdown embryos and embryos with combined miR-146a/b knockdown. Thus, miR-146b knockdown was the major contributor to the increased bacterial burden in *M. marinum* infection.
**Figure 4. Transcriptome response of miR-146a/b morphants to S. typhimurium infection.**

(A) Experimental set-up of the deep sequencing methodology. Embryos were injected at the 1-2 cell stage with a combination of 146aMO1 and 146bMO1 (146a/bMOs) or with the standard control morpholino (scMO). Approximately 250 CFU of *S. typhimurium* bacteria were injected into the caudal vein at 28 hpf after the onset of the blood circulation, or PBS was injected as a control. RNA-Seq analysis was performed on RNA samples extracted from pools of ≥50 embryos at 8 hpi. RNA samples from the four treatment groups (control/PBS (con), control/infected (con St), 146a/bMOs/PBS (146MO), and 146a/bMOs/infected (146MO St)) were obtained from two independent experiments. DESeq was used for statistical comparison of transcript count data. The significance cut-offs were set at an absolute fold change ≥1.5 and Padj ≤ 0.1. (B) Venn diagram showing the overlap between the effect of 146a/b knockdown on basal gene expression levels (con vs. 146MO) and the effect of *S. typhimurium* on gene expression in 146a/b morphants (146MO vs. 146MO St). The numbers of genes with significantly changed expression are shown in the Venn diagram and significantly enriched KEGG pathways for each comparison are indicated below. The 5 genes indicated for p53 signaling were up-regulated in 146a/b morphants compared with control embryos. (C) Venn diagrams showing comparisons of the numbers of genes that were up-regulated or down-regulated by *S. typhimurium* infection in control embryos (con vs. con St) or in miR-146a/b morphants (146MO vs 146MO St). KEGG pathways that were significantly enriched in the DESeq comparison of infected miR-146a/b morphants with infected controls (con St vs. 146MO St) are indicated below. The 6 genes indicated for p53 signaling and 6 genes for lipid transport were up-regulated in infected 146a/b morphants compared with infected control embryos.
**Discussion**

Recent studies have demonstrated the involvement of miRNAs in immune processes and inflammatory disorders, which has increased interest to find the molecular pathways responsible for miRNA action. MiR-146 has been recognized as a modulator of the innate and adaptive immune responses. Here, we exploited the zebrafish at the embryonic and larval stages, when adaptive immunity is not functional yet, to study the role of miR-146 in the innate immune response to bacterial infections. Both of the miR-146 family members, miR-146a and miR-146b, were found to be inducible by *S. typhimurium* and *M. marinum*, which are used as models for acute and chronic infections, respectively. The induction of miR-146a and miR-146b was in line with earlier microarray studies, which identified miR-146a and miR-146b as infection-inducible miRNAs along with some other miRNAs, like miR-9, miR-21, miR-29, miR-132, miR-155, and miR-147 (Bazzoni et al., 2009; Liu et al., 2009; Ordas, 2010; Sheedy et al., 2010; Boldin et al., 2011). The miR-146a and miR-146b sequences are conserved...
between zebrafish and human as well as target sites in the 3'UTR of mRNAs of innate immune pathway genes such as *IRAK1* and *TRAF6*, which are experimentally validated targets of miR-146 (Taganov et al., 2006, Boldin et al., 2011). To determine the pathway by which miR-146 expression is induced in zebrafish embryos upon infection we used *traf6* knockdown and *myd88* knockout models. The induction levels of miR-146a and miR-146b upon *S. typhimurium* infection were reduced under conditions of *traf6* or *myd88* deficiency, but induction was not completely abolished. This showed that infection-induced expression of miR-146 is partially dependent on the Myd88-Traf6 pathway and suggests that parallel signaling routes also contribute to miR-146 induction. The partial dependence on the Myd88-Traf6 pathway suggests that miR-146a and miR-146b may function in feedback control of TLR signaling, like the human and murine counterparts (Taganov et al., 2006, Boldin et al., 2011).

A recent study by Ghani et al., (2011) suggested miR-146a to be required for myeloid cell differentiation in mouse and zebrafish. In this study, in situ hybridization of zebrafish embryos with the pan-leukocytic L-plastin marker suggested an almost complete absence of myeloid cells under conditions of miR-146a morpholino knockdown. This observation is in strong contrast to the phenotype of miR-146a knockout mice, which showed hyperproliferation of myeloid cells leading to autoimmunity (Boldin et al., 2011). Our analysis of miR-146a knockdown in zebrafish embryos also contrasts the data of Ghani et al. We used two morpholinos for miR-146a (one of which was the same as a miR-146a morpholino used by Ghani et al.), and verified the knockdown effect by TaqMan qPCR. With L-plastin immunostaining, which is more sensitive than in situ hybridization, we detected no differences in myeloid cell development between miR-146 morphants and controls over an elaborate time course between 26 and 32 hpf, which comprises the critical embryonic stages when myeloid cells differentiate and enter the circulation. Furthermore, no effect on neutrophil differentiation at 2 dpf was detected. Thus, we found no evidence for an inhibitory effect of miR-146 deficiency on myeloid cell development in zebrafish embryos.

Knockout mice of miR-146a are hyperresponsive to LPS, showing increased up-regulation of pro-inflammatory cytokines, such as TNF and IL-6 (Boldin et al 2011). We used *S. typhimurium* infection of zebrafish embryos, which is accompanied by strong pro-inflammatory gene induction, to analyze the knockdown effect of miR-146a and miR146b by RNA-Seq analysis. We used a combination of morpholinos against miR-146a and miR-146b in the RNA-Seq study to avoid that the two miRNAs might compensate for each other's loss-of-function, as their mature sequences differ only by two nucleotides. The combined morpholino knockdown led to increased induction of *mmp9* during *S. typhimurium* infection; however, this induction of *mmp9* was not accompanied by a general hyperinduction of other pro-inflammatory markers in the RNA-Seq analysis. In addition to *mmp9*, *mmp2* was also up-regulated in miR-146a/b morphants, but independent of *S. typhimurium* infection. One of the matrix metalloproteinase genes, *MMP16*, has previously been described as a target gene for human miR-146b (Xia et al., 2009). *MMP2* and *MMP9* are not predicted target genes of miR-146 in human or zebrafish, but human *MMP9* was found to be down-regulated
upon miR-146a/b overexpression in MDA-MB-231 breast cancer cells and in THP-1 macrophages (Bhaumik et al., 2008; Yang et al., 2011). This down-regulation was suggested to occur via TLR-mediated and NF-κB-dependent pathways rather than by direct targeting of MMP9 (Bhaumik et al., 2008; Yang et al., 2011). Likewise, the induction of zebrafish mmp9 under miR-146a/b knockdown conditions might be an indirect consequence of effects on upstream signaling proteins. In agreement, we have previously shown that mmp9 induction by S. typhimurium infection is mediated by Traf6, which is a known target of miR-146 (Stockhammer et al., 2010; Taganov et al., 2006; Boldin et al., 2011). The observation that an increase of other Traf6-dependent pro-inflammatory markers was not seen in our RNA-Seq analysis of miR-146a/b knockdown may be explained by the fact that mmp9 is the most strongly induced pro-inflammatory marker in S. typhimurium infection (Stockhammer et al., 2009).

While the overall knockdown effect observed in our RNA-Seq analysis was relatively minor and no general hyperinduction of inflammation markers was observed, apolipoprotein-mediated lipid transport emerged as an infection-inducible pathway under miR-146a/b knockdown conditions. Numerous studies have linked apolipoproteins to immunoregulation and host defense (Khovidhunkit et al., 2004; Li et al., 2008) MiR-146a has been suggested to be involved in negative regulation of oxidized low-density lipoprotein- (LDL) accumulation in macrophages (Yang et al., 2011). Lipid accumulation in macrophages is associated with the inflammatory processes that lead to atherosclerosis. The expression of miR-146a was found to be down-regulated upon oxidized LDL stimulation of THP-1 macrophages. Furthermore, miR-146 overexpression reduced intracellular LDL cholesterol content and secretion of IL6, IL8, and MMP9 via TLR4-mediated signaling. A similar effect on LDL accumulation was observed by silencing miR-155, another important miRNA regulator of immune processes (Huang et al., 2010). Our results support the inhibitory function of miR-146 in lipid-mediated inflammatory responses and its proposed application as a potential therapeutic for atherosclerosis treatment (Yang et al., 2011).

Several genes in the p53 pathway, including tp53 itself, were up-regulated in miR-146 morphants as compared to controls under infected as well as non-infected conditions. This might be attributed to the well known off-target effects of morpholino oligonucleotides (Robu et al., 2007). However, as miR-146 has been frequently linked with cancer, a direct effect on the p53 pathway cannot be excluded (Labbaye and Testa, 2012; Sassen et al., 2008; Visone and Croce, 2009). In fact, one of the genes in the p53 pathway up-regulated by miR-146 knockdown, cdkn1a (p21), is an experimentally validated target of miR-146a in human (Borgdorff et al., 2010). In total we found 73 genes which were significantly up-regulated in miR-146 infection as compared to control infection. Besides cdkn1a, only one other gene, fibrinogen beta chain (fjb), showed an overlap with the predicted targets of zebrafish miR-146a and miR-146b in miRBase. Fibrinogen has roles in cell adhesion, hematopoiesis, and in coagulation and complement cascades associated with primary defense against bacterial infections (Rivera et al., 2007). Expression levels of other known targets of miR-146 involved in
innate immunity, such as irak1, traf6, irf5 and stat1, were not affected, but miR-146-dependent modulation of these genes may occur post-transcriptionally.

By targeting components of TLR signaling miR-146 has been shown to function as a negative regulator of the innate immune response (Taganov et al., 2006; Boldin et al., 2011). However, in our study of S. typhimurium infection in zebrafish embryos, miR-146 knockdown did not make a strong impact on the induction of proinflammatory genes. Notably, the effect of miR-146 knockdown was minor in comparison with knockdown analysis of ptpn6, which encodes a SH2-domain phosphatase that functions as a negative regulator of innate immunity (An et al., 2008; Croker et al., 2008). In the same experimental set-up, S. typhimurium infection of zebrafish embryos under knockdown of ptpn6 resulted in hyperinduction of mmp9 and a wide range of cytokines, other immune effectors genes, and transcriptional regulators of the immune response (Chapter 2), while in case of miR-146a/b knockdown only mmp9 was hyperinduced. Furthermore, hyperinflammation in ptpn6 morphants impaired control of S. typhimurium infection, while miR-146 knockdown had no such effect. These results support that Ptpn6 functions as a much stronger negative feedback regulator than miR-146a/b in the early response of zebrafish embryos to S. typhimurium infection. This would be consistent with the idea that miRNAs function in more subtle fine-tuning of the immune response (O’Neill, 2011)

Compared to acute lethality of S. typhimurium infection, M. marinum infection shows a chronic progression in zebrafish embryos and larvae. An increased bacterial burden of M. marinum was observed after combined knockdown of miR-146a and b. MiR-146b knockdown alone was sufficient for a significant increase of bacterial burden compared with the infection level of the control group, suggesting that miR-146b rather than miR-146a regulates responses to this type of infection. Similarly, knockdown of ptpn6 also resulted in an increased bacterial burden of M. marinum infection (Chapter 2). Together, these results suggest that both ptpn6 and miR-146b-mediated control mechanisms are protective and are required for a functional innate immune response to M. marinum infection.

Acknowledgements

We thank Hans Jansen (ZF-screens B.V., Leiden, The Netherlands) for RNA-Seq services and Julien Rougeot for help with statistical analysis of RNA-Seq data. We also thank Ulrike Nehrdich and Davy de Witt for fish care, and members of the molecular cell biology group for helpful discussions. This work was supported by the Smart Mix Program of The Netherlands Ministry of Economic Affairs and the Ministry of Education, Culture and Science, by the European Commission 6th Framework Project ZF-TOOLS (LSHG-CT-2006-037220), and by a fellowship of the Higher Education Commission of Pakistan to ZK.
Supplementary data

A. Stem-loop dre-miR-146a:

\[
\begin{align*}
\text{GAGUUUGU} & \quad \text{UCU} & \quad \text{Cu} & \quad \text{Gu} & \quad \text{aggiacug}a & \quad \text{uccauauag}u & \quad \text{U} \\
\text{AAUUAACA} & \quad \text{AAUUUGU} & \quad \text{AA} & \quad \text{GG} & \quad \text{UUUUGAGCA} & \quad \text{UGGUACUCU} & \quad \text{G} \\
\text{UCGUCUGU} & \quad \text{CU} & \quad \text{C} & \quad \text{UC} & \quad \text{C} & \quad \text{C} & \quad \text{UGAAAA}
\end{align*}
\]

B. Stem-loop dre-miR-146b:

\[
\begin{align*}
\text{GC} & \quad \text{UCU} & \quad \text{GCU} & \quad \text{aaggiacug}a & \quad \text{auuccg}u & \quad \text{U} \\
\text{CG} & \quad \text{AGG} & \quad \text{UGAA} & \quad \text{UUCUCGACUC} & \quad \text{UGAA} & \quad \text{CCCG} & \quad \text{UG}
\end{align*}
\]

Supplementary Figure 1. Target sites of miR-146a and miR-146b morpholinos on their respective miRNAs. The stem-loop sequences of the zebrafish miR-146a and miR-146b homologs, dre-miR-146a (A) and dre-miR-146b (B) are shown with the miRNA guide strand in lower case. Regions targeted by the morpholinos are indicated in blue (146aMO1 and 146bMO1) or yellow (146aMO2 and 146bMO2), and overlap between two morpholino regions is shown in green.

Supplementary Figure 2. Increased mmp9 expression in S. typhimurium-infected miR-146a/b morphants. Expression of mmp9 was analyzed by qPCR in the two independent RNA sample series that were used for RNA-Seq analysis. Embryos were injected with standard control morpholino (scMO) or with a combination of morpholinos against miR-146a and miR-146b (146a/bMOs), and infected with S. typhimurium (inf) or injected with PBS (mock). The mmp9 induction level upon S. typhimurium infection was significantly higher in miR-146a/b morphants than in control embryos, consistent with the results of RNA-Seq analysis (Supplementary Table 1). Relative expression levels are shown with mock control set at 1. Data are the mean ± SEM of two independent experiments. Asterisks indicate significant differences (*, P < 0.05; **, P <0.01) tested by one-way ANOVA analysis with Tukey’s method as post-hoc test.

Supplementary Table 1. Genes showing significantly up- or down-regulated expression in S. typhimurium-infected miR-146a/b morphants compared with S. typhimurium-infected control embryos. Supplementary table can be found online at:
https://www.dropbox.com/s/pxf1672d40ab9b9/Chapter4suppl.table1.xlsx
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