Regulatory mechanisms of innate immune signaling in zebrafish embryos

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Cover: Stereo fluorescent image of a one day old zebrafish embryo infected with *Salmonella typhimurium*.

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To honor my parents

With love to my grandmother, brother and sister

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Chapter 1

General Introduction

Regulation of the innate immune response

Protection against harmful agents is a fundamental process in all living beings. These harmful agents include microorganisms, which are the cause of a wide variety of infectious diseases. To combat infections, the body must first detect the presence of microorganisms as non-self materials. The body initially does this by recognizing molecules unique to groups of related microorganisms. These unique microbial molecules are called pathogen-associated molecular patterns or PAMPs (Mogensen, 2009). The recognition of PAMPs activates the immune system, which is traditionally divided into two main branches: innate immunity and adaptive immunity (Janeway and Medzhitov, 2002). Cells of the innate immune system, such as macrophages and neutrophils, contain specific receptors, which are responsible for the recognition of PAMPs (Mogensen, 2009; Beutler, 2009). PAMP recognition will activate various anti-microbial defense mechanisms in these cells. This includes the attraction of other immune cells to the site of infection, which results in a local inflammation that helps to clear the infection. In addition, activated innate immune cells will alert cells of the adaptive immune system, including helper T-cells, cytotoxic T-cells and antibody-producing B-cells. In cooperation with cells of the innate immune system, these adaptive immune cells will mount a highly specific immune response against the infectious agent. Furthermore, once the infection is cleared, the adaptive immune system provides an immunological memory such that the body can respond more effectively should a re-encounter with the same infectious agent occur.

Together, the innate and adaptive immune system form a highly effective barrier against infectious agents. Unfortunately, the immune defenses, especially inflammation, are also harmful to our own body (Beutler, 2009). It is therefore essential that the immune system is tightly regulated to ensure that inflammatory responses are resolved timely. In this thesis the zebrafish is used as an animal model to study mechanisms involved in the regulation of the innate immune response. This introductory chapter gives an overview of the current knowledge of the innate immune system of vertebrates and discusses how various negative control mechanisms are thought to keep the immune response in check.

The innate immune system

Innate immunity is the immunity one is born with and is the first level of defense which subsequently triggers more antigen-specific responses by the adaptive immune system (Janeway and Medzhitove, 2002). Innate immunity comes into action immediately after exposure to microbes. The cell types contributing to the innate immune response include phagocytic cells (neutrophils, monocytes, and macrophages), cells that release inflammatory mediators (basophils, mast cells, and eosinophils), cells that present antigens to the adaptive immune system (dendritic cells), and natural killer cells (NK cells). The innate immune system also has a humoral component, including serum molecules such as complement factors and acute phase proteins. Upon recognition of

microbes, innate immune cells activate several anti-microbial mechanisms and secrete small peptides, called cytokines, as communication signals. While the innate immune system was once believed to be non-specific in nature, the discovery of the Toll-like receptors (TLRs) completely changed the view on innate immunity. The TLRs were recognized as a family of pattern recognition receptors (PRRs) evolved to detect PAMPs (Akira et al., 2001; Beutler, 2009; Mogensen, 2009). The discovery that triggering of TLRs forms an essential bridge between the innate and adaptive immune system led to a greatly renewed interest in the innate branch of the immune system (Poltorak et al., 1998; Beutler, 2009, Steinman, 2006). In 2011 these discoveries were awarded with the Nobel Prize in Medicine.

The innate immune system triggers the activation of the adaptive immune system by production of proinflammatory cytokines and by providing stimulatory signals via major histocompatibility complex (MHC) molecules and costimulatory molecules such as CD40, CD80, and CD86; together, it facilitates a full activation of the immune system for an efficient response to pathogens (Janeway and Medzhitov, 2002; Steinman, 2006).

In addition to TLRs, other main families of PRRs that detect molecules of microbial origin have also been discovered. The function of the 'outward-looking' TLRs that act as cell-surface receptors to recognize bacteria, is complemented by the 'inward looking' nucleotide oligomerization domain (NOD)-like receptors (NLRs) that recognize bacterial molecules in the cytoplasm (Franchi et al., 2012). Similarly, TLRs on endosomal membranes, functioning in viral recognition, work in concert with the cytoplasmic retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs) (Kawai and Akira, 2008; Loo and Gale, 2011). These three families of PRRs will be discussed in detail below. They have critical roles in host control of infection processes, but these receptors and their downstream signalling components have also been associated with inflammatory diseases (Beutler, 2009).

TLRs

PAMPS recognized by TLRs comprise lipids, glycolipids, lipoproteins, proteins and nucleic acids from a large number of microbial taxa including bacteria, viruses, parasites and fungi (Mogensen, 2009). The TLR-dependent recognition of PAMPS occurs in different cellular compartments, among which the plasma membrane, endosomes, lysosomes and endolysosomes (Akira et al., 2006). TLRs were first recognized as pattern recognition receptors when one of them, TLR4, was identified as the long-sought receptor that responds to bacterial lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria that can cause septic shock (Poltorak et al., 1998). Gene targeting and knock-out studies have subsequently demonstrated that each TLR has a distinct function in terms of PAMP recognition and immune responses (Akira et al., 2006; Beutler, 2009; Mogensen, 2009). For example, TLR2, which generally forms heterodimers with TLR1 and TLR6, is involved in the recognition of PAMPs such as bacterial lipoprotein, lipoteichoic acid, peptidoglycan, and

yeast zymosan. As another example, TLR5 recognizes flagellin, the principal component of bacterial flagella. Besides recognizing molecules of microbial origin, there are some TLRs, such as TLR4, which also recognize host molecules that are released by damaged tissue (Osterloh and Breloer, 2007; Miyake, 2007; Piccini and Midwood, 2010)

On the cytoplasmic side, TLRs have a characteristic protein domain called the Toll / interleukin-1 (IL-1) receptor (TIR) domain (Akira et al., 2006). The TIR domain accounts for the major portion of the cytoplasmic domain and serves two purposes. First, it contains an oligomerization site, facilitating dimeric interactions between the TLR subunits. Second, it contains a site that recruits cytoplasmic adaptor proteins that also contain TIR domains. MYD88 was the first discovered member of the TLR adaptor family, after which four other TIR domain–containing adaptors were identified: TIRAP (MAL), TRIF, TRAM, and SARM (O'Neill and Bowie, 2007). Individual TLRs selectively recruit distinct adaptor molecules, thereby resulting in more specific immunological responses to the infecting microbes (Akira et al., 2006). For example, TLR3 and TLR4 generate both type I interferon and inflammatory cytokine responses, whereas cell surface TLR1-TLR2, TLR2-TLR6 and TLR5 induce mainly inflammatory cytokines. MYD88 is used by all TLRs except TLR3, and activates transcription factors such as NF-κB and mitogen-activated protein kinases (MAPKs) to induce inflammatory cytokines. In contrast, TRIF is used by TLR3 and TLR4 and induces alternative pathways that lead to activation of the transcription factors IRF3 and NF-κB and the consequent induction of type I interferon and inflammatory cytokines. TRAM and TIRAP function as sorting adaptors that recruit TRIF to TLR4 and MyD88 to TLR2 and TLR4, respectively. SARM1 is the only adaptor protein for which a negative regulatory role in TLR signaling has been proposed, as discussed further below (Carty et al., 2006). Following the recruitment of adaptors, a signaling complex consisting of IRAK1 and IRAK4 kinases is formed that subsequently activates TRAF6 and downstream MAPK and NF-κB signaling pathways (Gay et al., 2011) (Fig. 1).

NLRs

The importance of NLR family members in immunity was first suggested by the fact that one of them, a trans-activator of MHC gene transcription, class II transactivator (CIITA), was mutated in patients with bare lymphocyte syndrome (Steimle et al., 1993). The human NLR family contains more than 20 members, most of which recognize PAMPs as well as danger signals (Franchi et al., 2012). Among the NLRs, NOD1 and NOD2 recognize the degradation products of bacterial cell wall components, and NLRP3(NALP3) responds to various stimuli by forming the inflammasome complex, which promotes the release of active IL-1β and IL-18 by caspase-1-dependent cleavage of the precursor forms (Franchi et al 2009, 2012; Martinon et al., 2009). Dysregulation of NOD2 signaling is involved in the pathogenesis of a variety of inflammatory disorders, such as Crohn's disease (Billmann-Born, 2011; Rosenstiel and Schreiber, 2009). NOD2 has also been implicated in the development of autoimmune diseases, allergy and asthma (De-Jager et al., 2006; Shaw et al., 2011; Weidinger et al., 2005;

Kabesch et al., 2003; Bogefors, 2010; Daley et al., 2009; Magalhaes et al., 2008; Duan et al., 2010; Tigno-Aranjuez and Abbott, 2012).

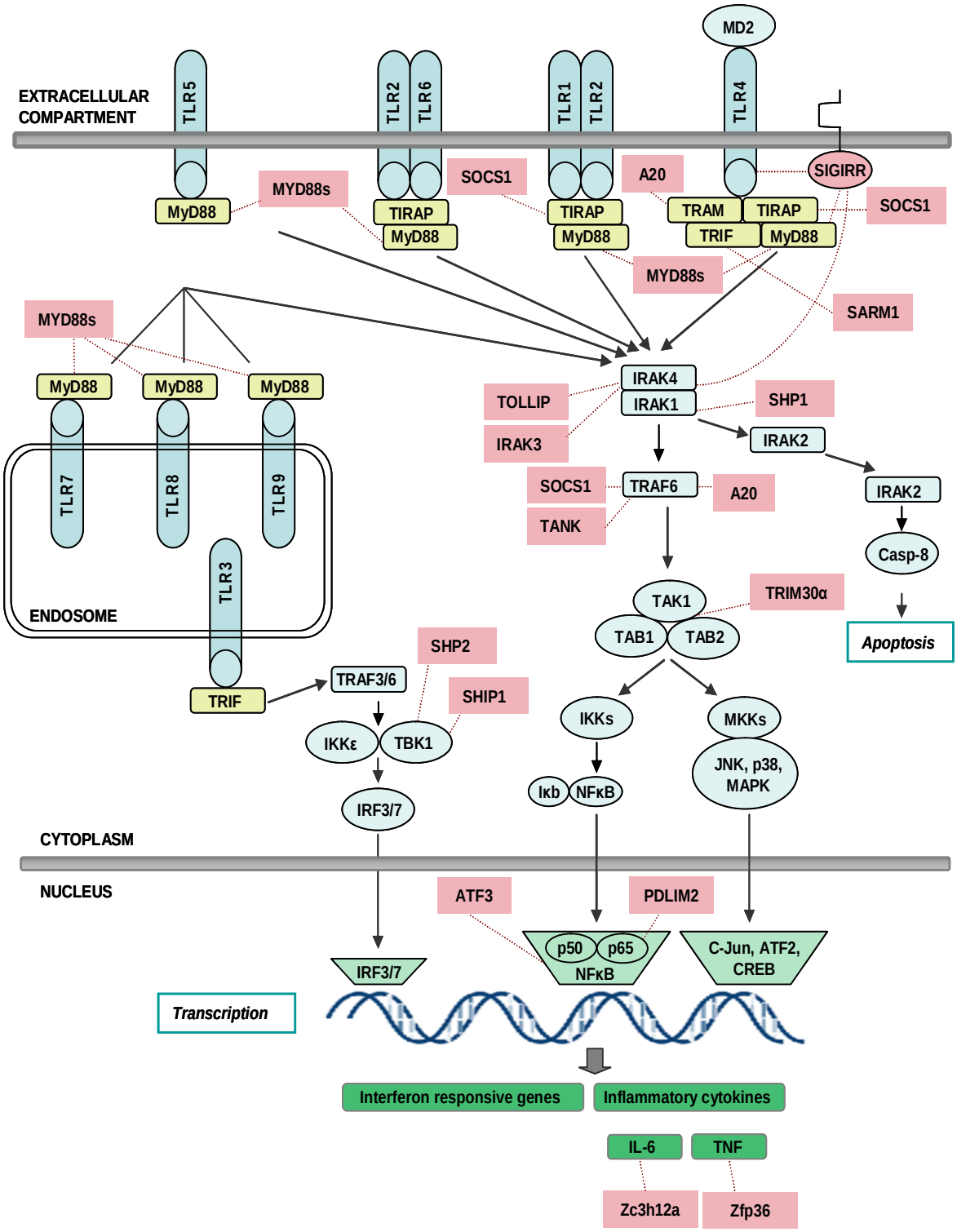

Figure 1. Negative regulation of the TLR pathway. Different negative regulators of the TLR pathway are shown in pink color around their target proteins. See text for details.

RLRs

The RLR family has three members namely, RIG-I, Mda5 and LGP2. They are RNA helicases that detect viral RNA species and signal through the adaptor molecule IPS-1, thus inducing antiviral responses (Yoneyama and Fujita, 2009; Kawai and Akira, 2008). The RLR-mediated signalling cascade culminates in the induction of transcription factors like interferon regulatory factors (IRFs) and NFκB and subsequent expression of interferon-stimulated genes. Like NLRs, RLRs act cooperatively with TLR signalling in the innate response and to modulate the adaptive immune response. Aberrant RLR signaling or dysregulation of RLR expression has also been implicated in the development of autoimmune diseases (Loo and Gale, 2011).

Detection of danger signals by the innate immune system

In addition to PAMP recognition by PRRs, other possible means of pathogen recognition include the modification of host proteins by microbial enzymes, the detection of 'missing self' due to downregulation of major histocompatibility complex (MHC) class I molecules, and the recognition of 'danger' by sensing 'change caused by infection' (Beutler, 2009). By analogy with PAMPs, these danger signals are commonly referred to as danger-associated molecular patterns (DAMPs) (Gallucci and Matzinger, 2001; Bianchi, 2007). Examples of danger signals include extracellular ATP released by damaged cells, crystals, and bacterial toxins. Especially the inflammasome-associated NLRs have central roles in the response to DAMPs, but the importance of DAMP-mediated activation of TLRs is increasingly recognized (Franci et al., 2012; Piccinini and Midwood, 2010). An aberrant response of TLRs, NLRs or other PRRs to such endogenous danger signals is thought to contribute to inflammatory and autoimmune diseases.

Innate immunity and control of autophagy

It has recently become clear that systems for protein degradation are essential for tight control of the inflammatory response. TLR and NLR pathways have been shown to control the autophagy system, which functions to deliver cellular waste materials (e.g. damaged organelles, long-lived proteins, and insoluble protein aggregates) and invading microbes into autolysosomes and is suggested to be involved in the regulation of inflammation (Deretic, 2012). Demonstrating the importance of autophagy for control of the inflammatory response, mutations in the gene encoding the autophagy-related molecule Atg16L1 have been linked to Crohn's disease (Hampe et al., 2007). Macrophages derived from Atg16L1-deficient mice showed more activation of caspase-1 and production of IL-1β and IL-18 in response to LPS (Saitoh et al., 2008). Moreover, intestinal Paneth cells derived from Atg16L1-deficient mice showed higher expression of genes involved in responses to intestinal injury (Cadwell et al., 2008). These results show that Atg16L1 is essential for the suppression of intestinal inflammation.

Negative regulation of the immune system

Sufficient production of inflammatory mediators is essential to clear infections; however, activation of the innate immune system against infections can be a double edged sword for the host. Pro-inflammatory cytokines mediate a positive feedback loop on the innate immune system, and unchecked production of cytokines is hazardous to the host and may cause severe outcomes such as hyperthermia, organ failure, and even death in extreme cases. (Kobayashi and Flavell, 2004). The inflammatory response must therefore be very tightly regulated. The innate immune system is run by a large set of effector molecules, some of which regulate it positively by integration of signals, while others function in the resolution phase to maintain homeostasis. The latter are typically considered as the negative regulators of the system. Negative regulators play critical roles in all pathways of innate and adaptive immunity to keep the systems in balance.

Negative regulation of TLR signaling

Unrestrained TLR signaling can have major deleterious effects on host cells as excessive inflammation may lead to tissue damage and inflammatory disorders. The ability of microbial TLR ligands to trigger various inflammatory diseases has been intensively discussed. Some examples of diseases that may depend on a TLR-mediated microbial trigger include arthritis (Joosten et al 2003; Deng et al., 1999), multiple sclerosis (Waldner et al., 2004; Kerfoot et al., 2004; Ichikawa et al., 2002), myocarditis (Eriksson et al., 2003), diabetes (Lang et al., 2005), atherosclerosis (Yumoto et al., 2005), colitis (Cario, 2010), and Crohn's disease (Man et al., 2011). In the healthy situation, TLR signaling can be suppressed at multiple levels by various negative regulators, which include inhibitory variants of TLR adaptor proteins or downstream kinases, ubiquitin ligases and deubiquitinases, transcriptional regulators, phosphatases, and microRNAs (Kobayashi and Flavell, 2004; Liew et al., 2005; O,Neill 2008; Wang et al., 2009; Contreras and Rao, 2012) Below, we discuss some key negative regulators of TLR signaling whose dysfunctioning have been shown to lead to over-activation of the host immune response (Fig. 1).

SIGIRR (Single Ig IL-1-related receptor): SIGIRR/TIR8 is a member of the TIR domain-containing family of receptors and functions as an inhibitor of interleukin-1 receptor (IL-1R) and Toll-like receptor (TLR) signaling. It has been suggested to function as a blocking receptor by interacting with IL-1R, TLR4, MYD88, IRAK1, and TRAF6, thereby inhibiting IL-1R and TLR-4 signaling (Qin et al., 2005). The ability to dampen signaling from IL-1R family members and TLRs makes SIGGIR a key regulator of inflammation, cancer-related inflammation, and autoimmunity (Xiao et al., 2007; Garlanda et al., 2009).

MYD88s and TAG: MYD88s is a splice variant of MYD88 (Janssens et al., 2002). It lacks the ability to bind IRAK-1. Overexpression of MYD88s reduces the phosphorylation of

IRAK-1 and reduces NF-κB activation upon IL-1 and LPS treatment (Janssens et al., 2002; Burns et al., 2003). A splice variant of TRAM, named TAG (TRAM adaptor with GOLD domain), has also been reported (Palsson-McDermott et al., 2009), TAG can displace the adaptor TRIF from TRAM and thus can act as a specific inhibitor of the MYD88-independent pathway activated by TLR4.

SARM1: In contrast to the other four TLR adaptor molecules MYD88, MAL, TRIF and TRAM, the fifth adaptor, SARM1, was identified as a negative regulator of innate immunity. SARM1 was found to inhibit the function of TRIF, which acts downstream of TLR3 and TLR4 (Carty et al., 2006). SARM1 was also shown to play a role in neuronal morphogenesis, where it interacts with and receives signal from Syndecan2 and controls dendritic arborization of neurons through the MKK4–JNK pathway (Chen et al., 2011).

TOLLIP (Toll interacting protein): TOLLIP represents a ubiquitin-binding protein that interacts with several signaling components in the TLR and IL-1R signaling cascades (Burns et al., 2000; Capeluto, 2011). By modulating signaling and membrane trafficking processes through its interaction with proteins, like IRAK1, or with phosphoinositides, TOLLIP acts as a critical negative regulator of the immune system (Ankem et al., 2011; Capeluto, 2011).

IRAK3: IRAK3 (IRAK-M) acts as a negative regulator of TLR signaling by inhibiting the dissociation of IRAK1 and IRAK4 kinases from the TLR signaling complex. IRAK3-deficient mice showed increased inflammatory responses to bacterial infection and reduced endotoxin tolerance (Kobayashi et al., 2002). Expression of IRAK3 is induced upon TLR stimulation and IRAK3-deficient cells showed increased production of proinflammatory cytokines upon TLR and IL-1R stimulation. Mutations in IRAK3 have been associated with a susceptibility to asthma (Balaci et al., 2007; Pino-Yanes et al., 2012). Furthermore, IRAK3 has been found to suppress TLR7-mediated autoimmunity in a mouse model of systemic lupus erythematosus (Lech et al., 2011) and was found to be of critical importance in down-regulating induction and progression of dextran sodium sulfate (DSS)-induced colitis (Berglund et al., 2010).

TANK: TANK was originally identified as a TRAF-binding protein that has both stimulatory and inhibitory functions (Cheng and Baltimore, 1996). In addition, TANK binding to TBK1 and IKKi has been linked to the activation of both NF-κB and IRF3 (Pomerantz and Baltimore, 1999; Guo and Cheng, 2007; Clark et al., 2011). Recently, it became apparent that TANK is not essential for interferon induction and instead is a potent negative regulator for TLR-mediated induction of proinflammatory cytokines, suppressing constitutive TLR signaling in response to commensal microorganisms (Kawagoe et al., 2009). TANK-deficient mice spontaneously develop autoimmune glomerular nephritis, which is suppressed by treatment with antibiotics or deficiency in MYD88 or IL-6 (Kawagoe et al., 2009). Despite having intact induction of type I

interferons, TANK-deficient macrophages and B cells show more NF-κB activation and IL-6 production in response to TLR ligands. TANK-deficient cells also show enhanced TRAF6 ubiquitination. Therefore, TANK acts as a negative regulator of TRAF6 ubiquitination in both macrophages and B cells.

Zinc finger proteins: A20 (TNFAIP3) is a zinc finger protein produced during TLR stimulation that has two enzymatic activities, acting as an E3 ubiquitin ligase and a deubiquitinase. *In vitro* analysis has shown that A20 restricts NF-κB activation by modulating RIP1 and TRAF6 (Jacque and Ley, 2009; Kawai and Akira, 2010). A20 has also been shown to block the IKK complex upstream of NF-κB (Skaug et al., 2011). A20-deficient mice die prematurely due to severe inflammation and cachexia and are more responsive to LPS and TNF (Lee et al., 2000). Polymorphisms within the A20 genomic locus have been associated with multiple inflammatory and autoimmune disorders, including systemic lupus erythaematosis, rheumatoid arthritis, Crohn's disease and psoriasis (Vereecke et al., 2011). Another zinc finger protein that is induced by TLR agonists is the RING-finger type tripartite-motif protein TRIM30α. This protein was found to interact with the TAB2-TAB3-TAK1 adaptor-kinase complex required for NF-κB activation. TRIM30 α promoted the degradation of TAB2 and TAB3, and inhibited NF-kB activation induced by TLR signaling (Shi et al., 2008). TRIM30α has also been implicated in the negative regulation of inflammasome signaling (Hu et al., 2010). Zc3h12a has a CCCH-type zinc-finger domain and is also induced upon TLR signaling (Matsushita et al., 2009). Zc3h12a targets the 3′ untranslated regions of *IL-6* mRNA and *IL-12p40* mRNA for degradation via its RNase activity. Zc3h12a-deficient macrophages consistently produce remarkably large amounts of IL-6 and IL-12p40 but normal amounts of TNF in response to TLR agonists (Matsushita et al., 2009). Zfp36 (Tristeraprolin) is another zinc-finger protein which prevents the development of autoimmune arthritis (Taylor et al., 1996). Zfp36 has been shown to bind AU-rich elements in the 3′ untranslated region of *TNF* mRNA and remove the poly(A) tail by deadenylation, which leads to degradation (Carrick et al., 2004). Therefore, the Zc3h12a and Zfp36 zinc finger proteins control mRNA stability through different mechanisms.

ATF3: ATF3 is a transcriptional repressor of the ATF/CREB family that is induced during infection and inflammation alongside with the induction of many transcriptional activators (Whitmore et al., 2007; Medzhitov and Horng, 2009; Thompson et al., 2009). Bone marrow derived ATF3-deficient macrophages showed high production of cytokines including IL-12p40, IL-6 and TNF upon LPS stimulation. ATF3 has been proposed to inhibit *IL-6* and *IL-12b* transcription by altering chromatin structure, thereby restricting access to transcription factors (Gilchrist, 2006). Because ATF3 is itself induced by LPS, it seems to regulate TLR4-stimulated inflammatory responses as part of a negative-feedback loop. ATF was shown to protect mice against LPS-induced endotoxic shock (Gilchrist, 2006), and to ameliorate allergen-induced airway inflammation and hyperresponsiveness in a mouse model of human asthma (Gilchrist,

2008). It was also demonstrated that ATF3 protects against atherosclerosis by controlling macrophage lipid metabolic and inflammatory pathways (Gold, 2012).

PDLIM2: The PDZ-LIM domain protein PDLIM2 was recently shown to play an important role in the termination of NF-κB activation. PDLIM2 functions as a nuclear ubiquitin E3 ligase promoting polyubiquitination of the p65 subunit of NF-κB. Polyubiquitinated p65 was subsequently sequestered by PDLIM2 into discrete intranuclear compartments where proteasome-mediated degradation occurred (Tanaka et al., 2007). PDLIM2 deficiency increased nuclear p65 and proinflammatory cytokine production in response to innate stimuli (Tanaka et al., 2007). PDLIM2 was also found to promote the polyubiquitination and proteasomal degradation of STAT3, thereby disrupting STAT3-mediated gene activation (Tanaka et al., 2011). STAT3 function is critical for the differentiation of T helper 17 (TH17) cells, which are thought to contribute to the pathology of autoimmune and inflammatory diseases, in particular granulomatous inflammation. By targeting of STAT3, PDLIM2 inhibited TH17 cell development and granulomatous responses. Deficiency in PDLIM2 exacerbated granuloma formation. Based on these results PDLIM2 provides a potential therapeutic target for the treatment of autoimmune diseases (Taneka et al., 2011).

SOCS proteins: Suppressor of cytokine signaling (SOCS) proteins function as negative regulators of cytokine signaling (JAK-STAT) pathways but are also induced by TLR stimuli and involved in regulation of TLR signaling (Heeg and Dalpke, 2003). For instance, SOCS1-deficient cells were shown to be hyperresponsive to TLR stimulation (Fujimoto and Naka, 2010). Defects in SOCS protein function contribute to a variety of inflammatory disorders and are also viewed as attractive therapeutic targets in autoimmune diseases (Yoshimura, 2004; Ramgolam and Markovic-Plese, 2011).

SH2 domain containing phosphatases: Like the SOCS proteins, several Src homology (SH2) domain-containing phosphatases also act on multiple signaling pathways involved in the responses to cytokines and microbial triggers. The lipid phosphatase SHIP1 (SH2 domain-containing inositol-5-phosphatase 1) negatively regulates the activation of immune cells primarily via the phosphoinositide 3-kinase (PI-3K) pathway (March and Ravichandran, 2002). However, SHIP1 has also been shown to regulate TLR4-mediated LPS responses by a phosphatase activity and PI-3K-independent mechanism (An et al., 2005). Recently, the zebrafish homolog of SHIP1 was shown to control neutrophil inflammation by limiting the motility of neutrophils and their recruitment to sites of injury (Lam et al, 2012).

Other important SH2 domain-containing regulators of TLR and cytokine responses are the protein tyrosine phosphatases (PTPs), SHP1 and SHP2. These are enzymes that remove phosphate groups from phosphorylated tyrosine residues on proteins. All PTPs carry a highly conserved active site motif C(X)5R (PTP) signature motif, share a common catalytic activity, and possess a similar core structure. Together with tyrosine kinases, PTPs regulate the phosphorylation state of many important

signaling molecules, such as the MAP kinases, and perform important roles in the regulation of cell proliferation and maturation. The human genome carries a total of 107 genes encoding PTPs of which the majority is experimentally verified for the function of the catalytic domain (Alonso et al., 2004). Biochemical and genetic studies demonstrated that PTPs are involved in both positive and negative signaling pathways (Van Vactor et al., 1998). Deregulation of several PTPs leads to a number of pathogenic effects in human diseases (Li and Dixon, 2007). PTPs have been shown to regulate many aspects of TLR and cytokine receptor signaling during innate and adaptive immunity. They are divided into evolutionary distinct classes on the basis of primary structure of the catalytic domain. PTPs can also be classified based on their cellular localization as receptor like PTPs which are transmembrane receptors and non-receptor intracellular PTPs. SHP1 and SHP2 are members of the non-receptor cytosolic PTPs which carry one phosphatase domain at the C-terminus and two tandem SH2 domains at the N-terminus. The SH2 domains act as protein phospho-tyrosine binding domains, and mediate the interaction of PTPs with their substrates. These substrates include cytokine receptors, e.g., erythropoietin or c-kit (Yi et al 1995; Yi and Ihle, 1993), but also intracellular signaling molecules, e.g. tyrosine kinases of the JAK family (Klingmuller et al., 1995).

Both SHP1 and SHP2 have been reported to be involved in the negative regulation of TLR signaling and cytokine receptor signaling (An et al., 2008; An et al., 2006; Croker et al., 2008). More specifically, SHP2 (also known as PTP non-receptor type 11 (PTPN11)) inhibits TRIF-dependent TLR3- and TLR4-mediated signaling, thereby suppressing type 1 IFN and proinflammatory cytokine production. *In vitro* studies suggested that this cytokine suppression by SHP2 is carried out by preventing TANK binding kinase (TBK1) from phosphorylating its substrates upon its interaction with SHP2 after TLR3 activation (An et al 2006). Additionally, SHP2 is known to have several functions as a positive regulator in immunity (Gadina et al., 1998; Salmond et al., 2005). Negative regulatory mechanisms of SHP1 (also known as PTP non-receptor type 6 (PTPN6)) also play important roles in controlling immune signaling pathways. While SHP2 is ubiquitously expressed, SHP1 is predominantly expressed in hematopoietic cells (Yi et al., 1992), and functions as an important regulator of multiple signaling pathways in immune cells. SHP1 has been shown to interact with, and dephosphorylate a wide spectrum of phospho-proteins involved in hematopoietic cell signaling. Loss of function mutations in the SHP1 gene at the motheaten (me/me) locus in mice revealed the role of SHP1 in negative regulatory pathways of cell signaling in immune cells. Motheaten mice suffer from multiple hematopoietic abnormalities and die at an early age (Shultz et al., 1993; Zhang et al., 2000). Lack of SHP1 leads to a decrease in the number of astrocytes and microglia in the brains of motheaten mice (Wishcamper et al., 2001) and was shown to mediate the cytokine activity in astrocytes through negative regulation of the JAK/STAT pathway and by regulating IFN-inducible gene expression (Massa and Wu, 1996; Massa and Wu, 1998). SHP1 deficiency has been linked with many inflammatory and autoimmune diseases in humans. Levels of SHP1 protein and mRNA were found to be significantly lower in the peripheral blood

mononuclear cells (PBMCs) of Multiple Sclerosis patients compared to control individuals (Christophi et al., 2008). Neutrophil- and IL-1-dependent inflammatory disease in SHP1 (Y208N/Y208N) mice was shown to be due to the loss of negative regulation of TLR and IL-1R signaling (Croker et al., 2011). SHP1 also acts as a negative regulator of the signaling pathways involved in the development of chronic inflammatory asthma and lung infections. Defective SHP1 function in asthma contributed to the enhanced inflammatory gene expression during *Mycoplasma pneumoniae* infections (Wang et al., 2012). Selective deletion of SHP1 in B cells was sufficient to cause autoimmune diseases, showing its critical regulation in adaptive immune processes (Pao et al., 2007).

SHP1 was also shown to act as a tumor suppressor gene in many lymphoma, leukemia, and other cancers owing to its antagonistic behaviors to the growth promoting and oncogenic capacities of tyrosine kinases (Wu et al., 2007). In agreement, decreased levels of SHP1 have been observed in many leukemia and lymphoma cell lines. On the other hand, SHP-1 was found to be over-expressed in prostrate, ovarian, and breast cancer cell lines (Wu et al., 2007). These finding indicate that dysregulation of SHP1 function can lead to abnormal cell growth and cause a number of pathologies. While it reduces the TLR-mediated inflammmatory cytokine induction by inhibiting NF-κB and MAP kinases activation, it can promote type 1 IFN production simultaneously (An et al., 2008). The mechanisms underlying both outcomes were linked to interaction between SHP-1 and IRAK1, which results in inhibition of the kinase activity of IRAK1, thereby shifting the balance between proinflammatory cytokine and interferon production (An et al., 2008). A hypomorphic SHP1 mutation in *spin* mutant mice was found to result in inflammatory lesions associated with aberrant macrophage activation in response to TLR stimulation (Croker et al., 2008). MYD88 deficiency was shown to suppress this inflammation, further supporting that SHP1 negatively regulates MYD88-dependent TLR signaling.

MicroRNAs in immune regulation

MicroRNAs (miRNAs) are another recently emerged class of negative regulators in immune pathways, which are now thought to play essential roles in fine-tuning mechanisms of both innate and adaptive responses (Lindsay, 2008; O'Connell et al., 2010; O'Neill et al., 2011) They are evolutionary conserved, short single-stranded RNA molecules, around 22 nucleotides in length, that post-transcriptionally down-regulate the expression of target mRNA sequences by binding to their 3′ untranslated region (UTR). The first miRNA discovered was LIN-4, an important negative regulator of the LIN-14 protein required for development in *Caenorhabditis elegans*, and the subsequent work revealed that miRNAs drive a gene silencing mechanism (Lee et al., 1993; Fire et al., 1998).

The latest version of the miRNA database (miRBase 18, November 2011) lists 1898 mature human miRNAs, thus representing approximately 6-8 % of all human genes. It is thought that each miRNA can target several hundreds of genes and thus it is

estimated that a large portion of the human genome can be regulated by miRNAs (Lewis et al., 2005). With more recent studies it has emerged that the main function of mammalian miRNAs is to decrease target mRNA levels, in contrast to earlier views that miRNAs function only to repress the translation of their target mRNAs (Guo et al., 2010).

Considering their dynamic nature, involvement of miRNAs in regulating the components of TLR signaling and innate immune pathways was no surprise. Based on their highly regulated expression, miRNAs can function as efficient immunomodulators (O'Neill et al., 2011). The first report about the role of microRNAs in the immune system came in 2004 which showed the relative expression of miR-142a, miR-181a, and miR-223 in immune cells (Chen, 2004). MiRNAs were subsequently shown to target mRNAs of proteins responsible for regulation of inflammation, thereby altering the magnitude of the inflammatory response (O'Neill et al., 2011). Many studies have provided evidence that development of cells of the myeloid lineage and differentiation of B and T cells are under control of miRNAs. MiRNAs are now known to be involved in the regulation of a variety of responses including maturation, proliferation, differentiation and activation of immune cells of both the innate and adaptive systems (Lindsay, 2008; Ha, 2011; Rusca and Monticelli, 2011). They have also been suggested to serve as a link between innate and adaptive immune signaling pathways. Furthermore, they have been proposed to have a role in controlling the switch from a strong early pro-inflammatory response to the resolution phase of the inflammatory response and thereby limiting induction of endotoxin tolerance (Quinn and O'Neill 2011; Quinn et al., 2012).

As discussed above, the signaling molecules that comprise innate and adaptive immune signaling pathways are regulated by numerous mechanisms, including physical interactions, conformational changes, phosphorylation, ubiquitylation and proteasome mediated degradation (Carpenter and O'Neill, 2009). It is thought that miRNA-mediated destabilization of these signaling molecules may provide a more energy-efficient way to regulate their activity. In comparison to rapid proteasomal degradation of these signaling molecules, miRNA-mediated control of their expression and function, either by mRNA decay or by translational inhibition, might be more advantageous in infection pathways, as the control of mRNA levels by miRNAs that are themselves induced by infection facilitates the shift from a strong initial immune response to the resolution phase, where the immune response is gradually dampened down (O'Neil et al., 2011).

Regulation of miRNA expression by TLR signaling

The end result of TLR signaling pathways is the activation of pro-inflammatory transcription factors that enhance the transcription of RNA polymerase II sensitive genes such as those encoding cytokines, chemokines, and antimicrobial enzymes. Because miRNAs are also transcribed by RNA polymerase II it is therefore quite logical for miRNA genes to be up-regulated by TLR signalling pathways (Cai et al., 2004; Lee et al., 2004). Several recent studies suggest that miRNAs that are expressed following TLR activation regulate the strength, location, and timing of TLR responses. MiR-155, miR-

21 and miR-146a have been central in much miRNA research due to their enhanced expression levels following TLR activation. Like other TLR-regulated genes, miRNA genes can also be categorized as early or late response genes, as some are induced very early e.g., miR-155; and others at somewhat later time points e.g., miR-21 (O'Neil et al., 2011). Other TLR-responsive miRNAs include miR-132, miR-9, miR-147 and miR-346 (Taganov et al., 2006; Bazzoni et al., 2009; Liu et al., 2009; Alsaleh et al., 2009). These are up-regulated in various cell types after stimulation with TLR ligands. Other miRNAs have been reported to be downregulated after LPS treatment, including let-7i, which is thought to target TLR4 itself, and miR-125b (Chen et al., 2007; Tili et al., 2007). As many of the miRNAs regulated by TLR signaling are also dysregulated in cancer (Calin and Croce, 2006), it is possible that miRNAs form a key link between inflammation and cancer and that the induction of specific miRNAs by TLRs may be a key step in tumor progression (Fig. 2).

miRNA targets in TLR signaling pathways

There is no strong evidence for the direct targeting of TLRs by miRNAs. Only few bioinformatic studies have predicted active miRNA target sites in the 3′ UTRs of TLRs (Chen et al., 2004). However, there are many reports on miRNAs targeting the mRNAs of signaling components downstream of TLRs, of cytokines induced by TLRs, or of TLR signaling regulators. Some examples of these are discussed below and illustrated by Fig. 2.

MiR-146 has been shown to target IRAK1 and TRAF6, both of which are important components of the MYD88-dependent pathway for NF-κB activation downstream of TLR2, TLR4, TLR5, TLR7, TLR8 and TLR9 (Taganov et al., 2006). Mir-146 was also proposed to negatively regulate the MYD88-NF-κB pathway in response to a bacterial infection (Taganov et al., 2006). More recently, IRAK2 has also been reported to be a target of miR-146 (Hou et al., 2009). MiR-145 is reported to target MAL/TIRAP (Starczynowski et al., 2010), and MYD88 is targeted by miR-155, which also targets TAB2, functioning downstream of TRAF6 in MAPK activation ((Tang et al., 2010; Zhou et al., 2010). MiR-348 targets Bruton tyrosine kinase (BTK), recently implicated in NF-κB activation downstream of TLR4, TLR7, TLR8, and TLR9 (Alsaleh et al., 2009). MiRNAs are also shown to target inhibitor of NF-κB kinases (IKKs) which are central to NF-κB activation. For example, miR-223 targets IKKα, miR-199 targets IKKβ, and IKKε is a potential target of miR-155 (Li et al., 2010; Chen et al., 2008; Gottwein et al., 2007; Xiao et al., 2009). Furthermore, the p50 subunit of NF-κB was found to be a direct target of miR-9 (Bazzoni et al., 2009). It is important to note that the above mentioned miRNA targets, including MYD88, MAL/TIRAP, IRAKs, TRAF6, BTK, IKKs, and NF-κB are part of multiple signaling pathways. Thus, the miRNAs targeting these signaling components can not only dampen signaling via multiple TLRs, but also via other PRRs or cytokine receptors, for instance IL-1R and TNFR signaling. (O'Neil et al., 2011). In addition, the 3′ UTRs of many cytokine mRNAs are predicted to contain miRNA target sites (Asirvatham et al., 2008, 2009). However, it remains to be further

established to what extent miRNA targeting of cytokine mRNAs contributes to the regulation of TLR responses.

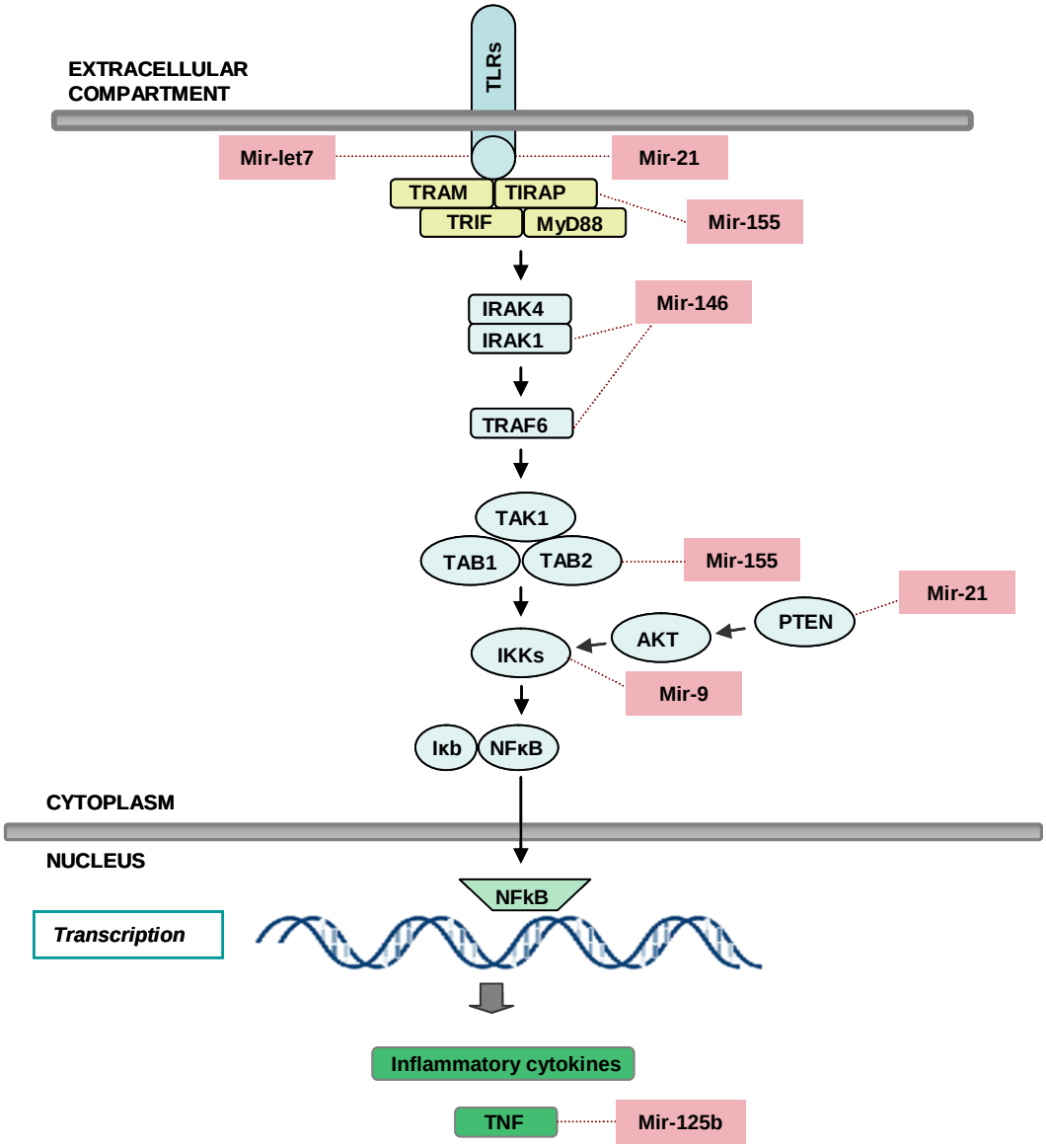

Figure 2. MiRNAs involved in negative regulation of the TLR Pathway. Immune-related miRNAs are shown in pink color around their target proteins. See text for details.

In addition to miRNAs targeting TLR signaling components or downstream effectors like cytokines, there is also evidence of miRNAs targeting TLR signaling regulators. For example, miR-155 seems to have a complex role in pro-inflammatory cytokine responses, as there are reports both of a function as a negative regulator and as a positive regulator. While the negative regulation function of miR-155 relates to the targeting of several TLR signaling components (see above) or to direct targeting of cytokine mRNAs, the positive regulatory function may be explained by the targeting of

SOCS1 and SHIP1, two negative regulators of TLR and cytokine signaling (Wang et al., 2010; O'Connel et al., 2009). Expression of miR-155 was found to be inhibited by the anti-inflammatory cytokine IL-10. This inhibition led to an increase in the expression of the miR-155 target SHIP1, and thereby to a reduction of TLR4 signaling (McCoy et al., 2010). Another link between IL-10 and miRNA regulation is that IL-10 can be induced in response to increased levels of miR-21. The proposed mechanism for this induction is that miR-21 down-regulates the translation initiation factor PDCD4, which inhibits IL-10 translation (Sheedy et al., 2010). Finally, studies of miR-132 recently identified a link between TLR signaling and neuroinflammation. MiR-132 was found to target the gene encoding acetylcholinesterase (ACHE), which hydrolyses acetylcholine, an important neurotransmitter in the cholinergic anti-inflammatory pathway. Acetylcholine can attenuate TLR signaling by inhibiting nuclear translocation of NF-κB in macrophages. An increased miR-132 expression downstream of TLR signaling results in repression of ACHE and thus increases acetylcholine-mediated negative regulation of TLR induced signals (Shaked et al., 2009).

miRNA in immune-related diseases

Emerging evidence has demonstrated that miRNAs are differentially expressed in autoimmune diseases and that miRNA regulation may impact the development or prevention of autoimmunity (Pauley et al., 2009; Iborra et al., 2012). Autoimmune diseases that have been associated with miRNA dysregulation include rheumatoid arthritis, systemic lupus erythematosus, primary biliary cirrhosis, ulcerative colitis, psoriasis, idiopathic thrombocytopenic purpura, primary Sj¨ogren's syndrome, and MS (Padgett et al., 2009; Dai et al., 2007; Alevizos and Illei, 2010; Ha, 2011). As is the case for other regulators of the immune response, many studies suggest that a proper balance of miRNAs production and withdrawal is critical to avoid inflammatory outbreaks in these diseases. Many of the miRNAs that are dysregulated in autoimmune diseases overlap with those showing aberrant expression in cancer, where inappropriate control of inflammation is also an important risk factor.

Multiple miRNA profiling reports have shown increased expression of TLR-induced miRNAs, like miR-21, miR-132, miR-146a, and miR-155 in patients with rheumatoid- and osteoarthriits, psoriasis, and atopic eczema (Jones et al., 2009; Stanczyk et al., 2008; Nakasa et al., 2008; Murata et al., 2010; Stanczyk et al., 2010; Ilopoulos et al., 2008; Sonkoly et al., 2007). However, a lack of miR-146a was detected in patients with systemic lupus erythematosus (Dai et al., 2007). As another example, loss of miR-145 and miR-146a has been shown to result in 5q syndrome, which is a haematopoietic abnormality, eventually leading to acute myeloid leukaemia (Starczynowski et al., 2010). Reduced expression levels of miR-146a and other anti-inflammatory miRNAs, like miR-21 and miR-132, in patients with autoimmune diseases are easier to interpret than the up-regulated levels that are also often observed. In these cases, currently unknown target genes of these miRNAs might be involved in

pathogenesis. Alternatively, the anti-inflammatory effects of these miRNAs cause disease by interfering with appropriate immune responses (O'Neill et al., 2011).

As proposed by O'Neill et al. (2011), a persistent low-grade inflammation in inflammatory diseases may results in dysregulated miRNA expression, which leads to disease progression (O'Neil et al., 2011). Another factor which may play an important role in miRNA-related pathogenesis is the occurrence of mutations or deletions in the 3′ UTRs of target mRNAs.For example, a polymorphism in the 3′ UTR of the IRAK1 mRNA (a target gene of miR-146a) is associated with susceptibility to rheumatoid arthritis (Chatzikyriakidou et al., 2010).

In addition to the numerous studies about the link between miRNAs and autoimmune disorders, the role of miRNAs in allergic inflammation is also recently emerging. Demonstrating the potential for therapeutic modulation of miRNA in treatment of inflammatory diseases, a study in mice showed a reduced asthmic response to the house dust mite antigen when miR-126 was selectively blocked (Mattes et al., 2009). The more we understand the complex relationships between inflammatory pathogenesis and miRNAs, the bigger the possibilities for additional therapeutic strategies to control these diseases.

Zebrafish as an infection and inflammation model

Zebrafish has proven to be a versatile tool to study host-pathogen interactions and an excellent model for the study of human diseases (Renshaw and Trede, 2012). Various infection models for bacterial, viral, and fungal pathogens using zebrafish as a host have recently been developed (Meijer and Spaink, 2011). Zebrafish is also recognized as a good model for studying inflammatory diseases, such as inflammatory bowel disease (Love et al., 2007; Oehlers et al., 2011). Despite the absence of lungs, zebrafish models can even help understand the molecular basis of respiratory diseases (Renshaw et al., 2007; Martin et al., 2009). Zebrafish has filled the gap between mouse and invertebrate models because it has a similar immune system as higher vertebrates and additionally provides a transparent system to visualize infection and inflammation processes in real time. Effective gene knockdown technology using antisense morpholino oligonucleotides has boosted reverse genetic screens in zebrafish (Bedell et al., 2011). Furthermore, zebrafish is an ideal model for forward genetic mutagenesis screens (Streisinger et al., 1981; Patton and Zon, 2001). As an example, the application of this screening technology recently led to the discovery of a new tuberculosis susceptibility locus (LTA4H) that is conserved between zebrafish and humans (Tobin et al., 2010; Tobin et al., 2012)

There is striking similarity in the immune system between humans and zebrafish with comparable immune cell types (Tobin et al., 2012). Like humans, adult zebrafish strongly rely on a functional adaptive immune system to control infections. For example, zebrafish that are impaired in adaptive immune responses (*rag1* mutants) are hypersusceptible to tuberculosis. Moreover, wild type zebrafish, like humans, are able to restrict the growth of tuberculosis bacteria by the formation of immune cell

aggregates known as granulomas (Swaim et al., 2006). However, during the first weeks of embryonic and larval development zebrafish rely solely on their innate immune system, because adaptive immunity is not yet matured (Lam et al., 2004). Therefore, these embryonic and larval stages provide a useful model to investigate the function of the innate immune system in the absence of adaptive responses (Meijer and Spaink, 2011; Cui et al., 2011). TLR genes have been shown to be broadly expressed during all stages of embryonic development (van der Sar et al., 2006). Furthermore, several studies have demonstrated the function of TLR signaling in response to exposure of zebrafish cells or embryos to microbes or PAMPs (Phelan et al., 2005; van der Sar 2006; Stockhammer, 2009, 2010). Likewise, the anti-microbial function of NLRs, NOD1 and NOD2 has also been found to be essential to combat *Salmonella* infection in zebrafish larvae. (Oehlers et al., 2011).

The innate immune system of zebrafish embryos responds very differently to acute pathogens, such as *Salmonella typhimurium*, and a pathogen that causes chronic disease, like *Mycobacterium marinum*. First of all, *S. typhimurium* and *M. marinum* cause a different pathology (Fig. 3). *S. typhimurium* proliferates rapidly and causes a lethal infection within approximately one day after intravenously injecting one-day old embryos. Injection of a similar dose of *M. marinum* bacteria leads to the formation of tissue aggregates of infected and uninfected macrophages and neutrophils that can be considered as early stages of granuloma formation, a hallmark of tuberculosis infection. Secondly, the expression profiles induced by *S. typhimurium* and *M. marinum* are specific and follow a different time course (van der Vaart et al., 2012). Within hours, *S. typhimurium* infection induces the expression of many pro-inflammatory genes and transcriptional activators of the immune response (Stockhammer 2009, van der Vaart et al., 2012). During *M. marinum* infection such genes are not significantly induced until a clear granulomatous infection has developed (van der Sar, 2009; van der Vaart et al., 2012). Concomitant with the induction of pro-inflammatory genes and transcriptional activators, several negative regulators of the TLR pathway (for example *atf3*, *socs1*, *socs3*, and *irak3*) were found to be up-regulated during *S. typhimurium* infection in zebrafish embryos (Stockhammer et al., 2009). Additionally, the zebrafish homolog of the anti-inflammatory cytokine IL-10 is also rapidly induced after infection. These gene induction profiles suggest a tight feedback control of the inflammatory response. The inflammatory response may also be controlled at the protein level by additional factors, such as the phosphatase Ptpn6, the well conserved zebrafish homolog of the human negative regulator SHP1. Finally, miRNAs, which are highly conserved among all vertebrates, are induced upon bacterial infection in zebrafish and may play a role in dampening inflammation (Ordas, 2010).

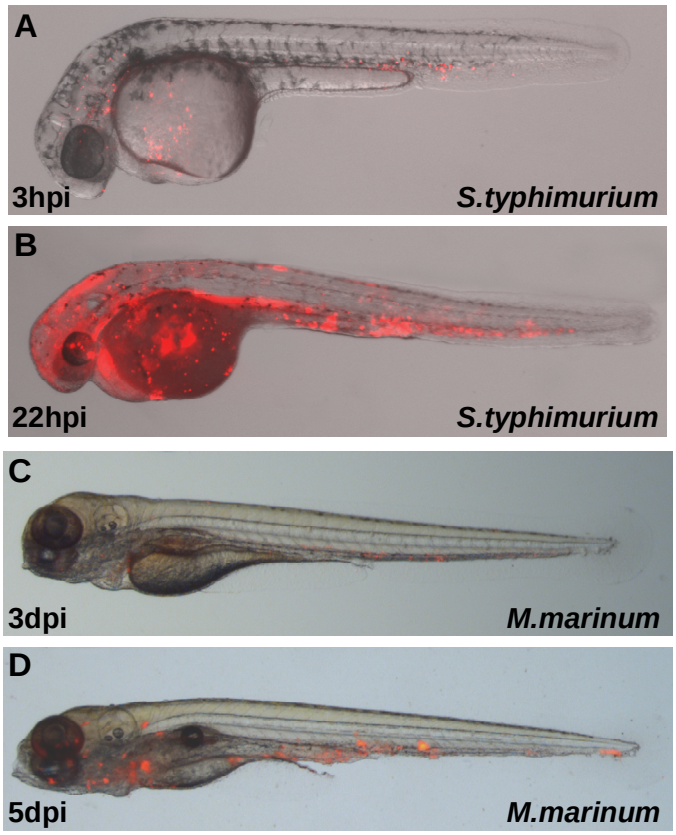

Figure 3. Disease progression in zebrafish larvae infected with *Salmonella typhimurium* and *Mycobacterium marinum.* Embryos were infected at 28 hpf with *S. typhimurium* (A, B) or *M. marinum* (C, D) by injection into the caudal vein. *S. typhimurium* rapidly proliferates and causes lethal infection within a day after injection. In contrast, *M. marinum*-infected cells migrate deeper into tissues and attract other non-infected cells to form tissue aggregates in which the bacteria can persist chronically. These tissue aggregates of *M.marinum*-infected cells are considered as the early stages of tuberculous granulomas.

Outline of the thesis

The studies described in this thesis made use of the zebrafish as a model to gain insight into the molecular mechanisms that regulate the vertebrate innate immune response. As discussed in this introductory chapter, dysfunctioning of these regulatory mechanisms may underlie many inflammatory disorders in humans.

Chapter 2 reports on a knockdown analysis of *ptpn6*, the homolog of the human gene encoding the SHP1 non-receptor protein tyrosine phosphatase. Like the human gene, zebrafish *ptpn6* shows specific expression in immune cells. Knockdown analysis in zebrafish resulted in a late larval phenotype that is associated with increased expression levels of inflammatory genes. Furthermore, infection of embryos with *S. typhimurium* or *M. marinum* resulted in a hyperactivation of the innate immune response under conditions of *ptpn6* knockdown These results support the function of

ptpn6 as a negative regulator of the innate immune response. Hyperinduction of the innate immune response in *ptpn6* knockdown embryos was found to impair their ability to control *S. typhimurium* and *M. marinum* infections, suggesting that loss of the *ptpn6*-mediated negative control mechanisms causes a pathological inflammatory response that is incompatible with a functional immune response to these infections.

In **Chapter 3**, recently developed fluorescent reporter lines were used to separate specific immune cell populations from zebrafish larvae by fluorescence activated cell sorting (FACS). In addition, *M. marinum* infected cells were FACS-separated based on the bacterial fluorescence marker. These cell populations were subjected to RNA-Sequencing analysis (RNA-Seq) using a cDNA amplification kit that allowed the analysis of RNA samples down to concentrations below the nanogram range. Using this novel technology, the specific transcriptome profiles of macrophages, neutrophils, immature T-cells and *M. marinum*-infected cells could be compared and many specific markers for the different cell types and the infection condition could be determined. In addition, these cell-specific transcriptome profiles were analyzed under conditions of knockdown of the negative regulator *ptpn6*, which provided insight in the pathways activated under pathological conditions of inflammation.

Chapter 4 focuses on the zebrafish homologs of miR-146a and miR-146b, which have putative target sites in the mRNAs of the TLR-pathways genes IRAK1 and TRAF6 in both human and zebrafish. The results show that induction of miR-146a and miR-146b expression during bacterial infection in zebrafish embryos is controlled by the Myd88-Traf6 pathway. Furthermore, this chapter reports on a morpholino knockdown analysis of miR-146a and miR-14bb and the effects of their down-regulation on the transcriptome and bacterial burden.

As summarized and discussed in **Chapter 5**, the studies in this thesis have contributed to a better understanding of regulatory mechanisms that control the innate immune response in zebrafish embryos and support the use of zebrafish embryo and larval models for inflammatory and infectious diseases.

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Chapter 2

Deficiency in hematopoietic phosphatase Ptpn6/Shp1 hyperactivates the innate immune system and impairs control of bacterial infections in zebrafish embryos

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Abstract

Deficiency in the protein-tyrosine phosphatase SHP1/PTPN6 is linked with chronic inflammatory diseases and hematological malignancies in humans. Here we exploited the embryonic and larval stages of zebrafish (*Danio rerio*) as an animal model to study *ptpn6* function in the sole context of innate immunity. We show that *ptpn6* knockdown induces a spontaneous inflammation-associated phenotype at the late larval stage. Surprisingly, glucocorticoid treatment did not suppress inflammation under *ptpn6* knockdown conditions but further enhanced leukocyte infiltration and pro-inflammatory gene expression. Experiments in a germ-free environment showed that the late larval phenotype was microbe independent. When *ptpn6* knockdown embryos were challenged with *Salmonella typhimurium* or *Mycobacterium marinum* at earlier stages of development, the innate immune system was hyperactivated to a contra-productive level that impaired the control of these pathogenic bacteria. Transcriptome analysis demonstrated that KEGG pathways related to pathogen recognition and cytokine signaling were significantly enriched under these conditions, indicating that *ptpn6* functions as a negative regulator that imposes a tight control over the level of innate immune response activation during infection. In contrast to the hyperinduction of pro-inflammatory cytokine genes under *ptpn6* knockdown conditions, anti-inflammatory *il10* expression was not hyperinduced. These results demonstrate that *ptpn6* has a crucial regulatory function in preventing host-detrimental effects of inflammation and is essential for a successful defense mechanism against invading microbes.

Introduction

The innate immune system has been conserved in evolution from invertebrate to vertebrate organisms and plays an indispensable role in host protection against infections. The vertebrate innate immune system has been demonstrated to not only function as the first line of defense against microorganisms, but also to be required for activating the secondary adaptive defenses. However, if the innate immune system goes unchecked, the production of inflammatory mediators can cause considerable tissue damage. Recent studies indicate that defects in the initial sensing of microorganisms and allergens by the innate immune system can contribute to autoimmune and autoinflammatory diseases, which were classically viewed as specific disorders of the adaptive immune system (Beutler, 2009; Drexler and Foxwell, 2010; Theofilopoulos et al., 2011). In healthy individuals the innate immune response is tightly controlled by complex regulatory mechanisms that prevent excessive and chronic inflammation (O'Neill, 2008). The SHP1 phosphatase, encoded by the *PTPN6* gene, has been recognized as a critical factor in this process of negative regulation.

SHP1 belongs to the family of protein-tyrosine phosphatases (PTPs), which dephosphorylate phosphotyrosyl residues in proteins that are phosphorylated by protein-tyrosine kinases (PTKs). PTPs and PTKs function in a variety of cellular

processes, from cell survival to proliferation, differentiation, migration and immune responses. SHP1 (PTPN6) and SHP2 (PTPN11) are closely related non-receptor type PTPs, each having two Src homology 2 (SH2) domains amino-terminal to the phosphatase catalytic domain (Zhang et al., 2000; Tsui et al., 2006; Pao et al, 2007). While SHP2 is expressed ubiquitously, SHP1 is predominantly expressed in hematopoietic cell lineages, and it has been implicated in the regulation of a diverse range of cytokine receptors, growth factor receptors, and immunoreceptors. SHP1 has been shown to associate with immunoreceptor tyrosine-based inhibition motifs (ITIMs) in these receptors (Zhang et al., 2000; Tsui et al., 2006), and has been proposed to bind to ITIM-like motifs in various kinases, including IRAK1, ERK1/2, p38, JNK, JAK2, JAK3, TAK1, IKKα, and LYN (Abu-Dayyeh et al., 2008; Abu-Dayyeh et al., 2010).

SHP1 has been extensively studied after the discovery of two naturally occurring mutant mouse strains: motheaten (*me*), considered to carry a null allele of the *Ptpn6* gene, and motheaten viable (*mev*), which encodes a phosphatase with approximately 20% of wild type catalytic activity (Shultz et al., 1993; Tsui et al., 1993; Kozlowski et al., 1993). These mice suffer from severe immune disorders, with spontaneous inflammatory activity affecting multiple organs, including the lungs, kidney, joints, and skin, the latter resulting in their typical 'motheaten' appearance. The mutations result in lethal pneumonitis by three (*me*) or nine (*mev*) weeks of age. Backcrossing of *mev* mice to *rag1* null mutants (that do not contain mature T and B cells) did not alleviate the *motheaten* inflammatory disease, indicating that the function of myeloid cells rather than the function of the adaptive immune system is required for major aspects of the *shp1* mutant phenotype (Yu et al., 1996). Furthermore, pulmonary inflammation in *mev* mutants was found to depend strongly on the function of mast cells (Zhang et al., 2010). A viable hypomorphic allele of *Ptpn6, spin* (spontaneous inflammation), carrying a point mutation in one of the SH2 domains, was later described that elicits chronic inflammatory and autoimmune disease (Croker et al., 2008). Inflammation in *spin* mutants was triggered by the presence of microbes and found dependent on production of IL-1, subsequent IL-1 signaling, and the presence of neutrophils (Croker et al., 2008; Croker et al., 2011). Consistent with these findings, SHP1 was shown to negatively regulate Toll-like receptor (TLR)-mediated production of pro-inflammatory cytokines by suppressing the activation of MAPKs and the transcription factor NF-κB (An et al., 2008).

SHP1 has been associated with several human inflammatory diseases. In patients with psoriatic inflammatory skin disease, deficient SHP1 expression in T-cells has been observed (Eriksen et al., 2010). Furthermore, macrophages of multiple sclerosis (MS) patients display SHP1 deficiency concomitant with enhanced expression of genes mediating inflammatory demyelination in MS pathogenesis (Christophi et al., 2009). Finally, it has been suggested that altered expression of SHP1 may also be associated with human allergies and asthmatic disease, based on recent studies in mice that indicate a role of SHP1 in mast cells and allergic inflammatory responses (Zhu et al., 2010). In addition, SHP1 is considered a putative tumor suppressor. Decreased expression of SHP1 has been observed in many types of human lymphomas and

leukemias. The reduced levels of SHP1 in these malignancies have been attributed to mutations, epigenetic regulation, and post-transcriptional mechanisms (Wu et al., 2003; Witkiewicz et al., 2007). SHP1 has also been implicated as a negative regulator of insulin signaling and clearance of insulin in the liver, and has been linked to progression of diabetic retinopathy (Dubois et al., 2006; Geraldes et al., 2009).

As many recent studies have shown, the zebrafish embryo model has specific advantages not only for developmental biology but also for studying immunity, inflammation and infections (Lieschke and Trede, 2009; Martin and Renshaw, 2009; Meijer and Spaink, 2011). The embryo model is particularly useful for studying responses of the innate immune system, as macrophages and neutrophils develop during the first two days of embryogenesis, when the adaptive immune system is not yet in place (Lam et al., 2004). The zebrafish genome encodes orthologs of the majority of human PTPs, including *shp1/ptpn6* and *shp2/ptpn11a* (van Eekelen et al., 2010). We have previously shown that hematopoietic expression of *shp1/ptpn6* is conserved in zebrafish embryos and controlled by the transcription factor Pu.1 (Spi1), like its human counterpart (Zakrzewska et al., 2010; Wlodarski et al., 2007). The zebrafish embryo model was also exploited to study the role of *shp2* in early development and to investigate the cell biological effects of activating and inactivating mutations in Shp2 protein that underlie the Noonan and LEOPARD syndromes in humans. Defective Shp2 signaling induced cell movement defects as early as gastrulation and zebrafish embryos expressing Noonan or LEOPARD Shp2 displayed craniofacial and cardiac defects, reminiscent of human symptoms (Jopling et al., 2007).

Here we used morpholino knockdown to study the effect of *ptpn6* deficiency in zebrafish embryos. No visible phenotypic effects of *ptpn6* knockdown were observed during early development, but morphant larvae developed a late phenotype at 5 to 6 days post fertilization (dpf). Skin lesions in these morphants were reminiscent of phenotypes of the murine *ptpn6* mutants, *motheaten* and *spin*, which suffer from severe inflammation leading to patches of hair loss and foot lesions, as discussed above (Tsui et al., 1993; Croker et al., 2008). Based on leukocyte infiltration and pro-inflammatory gene expression we concluded that also the zebrafish *ptpn6* morphant phenotype is associated with an inflammatory response. We describe infection experiments of *ptpn6* morphants with bacterial pathogens at 1 dpf, which is several days prior to the manifestation of the late inflammation-associated phenotype. We observed that *ptpn6* morphants responded to bacterial challenge with increased induction of pro-inflammatory genes compared to wild type embryos, yet their ability to control these infections was severely impaired, indicating that this is not a functional response. In conclusion, our data demonstrate the role of *ptpn6* as a negative regulator of the innate immune system, which is important for a functional innate immune response during bacterial infections.

Materials and methods

Zebrafish husbandry

Zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). Zebrafish lines used in this study included AB/TL, *Tg(mpx:gfp)i114* (Renshaw et al., 2006), *Tg(fli1:EGFP)* (Lawson and Weinstein, 2002) and *Tg(-1.0pomca:GFP)zf44;Tg(prl:RFP)zf113* (Liu et al., 2006). Embryos were grown at 28.5–30°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 whole mount in situ hybridization and immunostaining were kept in egg water containing 0.003% 1-phenyl-2-thiourea (Sigma-Aldrich) to prevent melanization.

Morpholino knockdown

Morpholino oligonucleotides (Gene Tools) were diluted to the desired concentration in 1× Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 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 *ptpn6* two morpholinos were used, one targeting the exon 11/intron 11-12 splice junction (MO1: 5'ACTCATTCCTTACCCGATGCGGAGC3'; 0.0625 mM), and one targeting the translation start site (MO2: 5'CTGTGAAACCACCGAACCATCTTCC3'; 0.20 mM). As a control we used the standard control (SC) morpholino from GeneTools at the same concentrations as the *ptpn6* MOs.

Whole-mount in situ hybridization, immunodetection, TUNEL assay, and myeloperoxidase activity assay

For all assays embryos were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Whole-mount in situ hybridization (WISH) using alkaline phosphatase detection with BM Purple substrate (Roche Diagnostics) was carried out as previously described (Stockhammer et al., 2009). Digoxigenin-labeled *mfap4* and *mpx* probes were generated as described in (Zakrzewska et al., 2010). Immunofluorescence stainings were performed with 1:500 dilutions of polyclonal rabbit Ab against phospho-Histone H3 (Santa Cruz) and L-plastin (Mathias et al., 2007). For detection, Alexa Fluor 568/488 goat anti-rabbit IgG secondary Ab (Molecular Probes) as described in (Cui et al., 2011) were used. DNA fragmentation during apoptotic cell death was examined by TUNEL using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore) according to the manufacturer's instructions. Embryos were fixed, permeabilized, treated with Proteinase K and refixed in 4% PFA as for WISH. Embryos were then fixed in ethanol:acetic acid 2:1 for 15 min at -20ºC followed by PBS washes. After 15 min incubation in the equilibration buffer embryos were transferred to working strength TdT enzyme solution supplemented with 0.3% TritonX-100, and incubated for 1 h on ice followed by 1 h at 37ºC. The reaction was stopped by a 5 min wash in stop solution at room temperature, followed by 45 min

incubation at 37ºC and PBS washes. Subsequent immunodetection with alkaline phosphatase-conjugated anti-DIG Fab fragments and BM purple staining was performed as for WISH. Histochemical staining for Mpx activity was performed with the Peroxidase Leukocyte Kit (Sigma-Aldrich) as described in (Cui et al., 2011).

RNA isolation, quantitative RT-PCR, and RT-MLPA

RNA isolation and quantitative RT-PCR (qPCR) analysis was performed as described in (Stockhammer et al., 2009). Primer sequences for *ppial*, *il1b*, *mmp9*, *lgals*, *mpeg1*, *cxcr3.2*, *mfap4*, and *marco* are described in (Zakrzewska et al., 2010; Stockhammer et al., 2009). Additional primer sequences used were:
lyz: FW5'TGTCCTCGTGTGAAAGCAAGAC3', REV5'AGAATCCCTCAAATCCATCAAGCC3';
mpx: FW5'AAGACAATGCACGAGAGC3', REV5'GCAATGAAGCAAGGAACC3';
csf1r: FW 5'CTGCTGGTCGTAGAGGAG3', REV 5'TGTGAAGTCAGAGGAGG3';
ncf1: FW5'CACAGGATGGCTGAAACATACG3, REV5'TAGTGCTGGCTGGGAAAGAATC3'.
Reverse transcription – multiplex ligation-dependent probe amplification (RT-MLPA) was performed as described in (Rotman et al., 2011).

Germ-free experiments

For generating germ-free embryos we used a natural breeding method described in (Pham et al., 2008). Eggs were washed 3× in antibiotic gnotobiotic zebrafish medium (GZM) prior to performing morpholino injections in a down-flow cabinet using sterilized needles and equipment. Following injections, eggs were immediately washed with antibiotic GZM and were successively treated with PVP-I and bleach solutions. Embryos were grown in 6 well plates at 28°C wrapped in aluminum foil and sterile antibiotic GZM was refreshed daily. At the end of the experiment sterility was tested by plating media from the germ-free and conventionally raised embryos on tryptic soy agar plates.

Chemical treatments

Betamethasone 17-valerate (1 μM) , beclomethasone (25 μM), and prednisolone (25 μM) were dissolved in 0.1% DMSO and added directly to egg water at 1 dpf or 3 dpf, solutions were refreshed daily. Controls were treated with DMSO only. $CuSO_4$-induced inflammation was performed as in (d'Alencon et al., 2010).

Infection experiments

Salmonella typhimurium infections were performed using the *S. typhimurium* 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. After injections embryos were transferred to fresh egg water and incubated at 28°C. For plating assays, infected embryos were homogenized using a Retsch mixer mill with a metal bead for 1 min at maximum speed.

Microarray analysis

For microarray analysis of *S. typhimurium* infection in *ptpn6* morphants, three independent infection experiments were performed. In each experiment, RNA was isolated from pools of 15-20 embryos per treatment group. Knockdown of *ptpn6* was performed using MO1 and control embryos were injected with Danieau buffer/phenol red. The *ptpn6* morphants and control embryos were infected at 28 hpf with *S. typhimurium* SL1027 bacteria, or mock-injected with PBS as a control. RNA extraction was performed at 8 hpi. Microarray analysis was performed using a previously described custom-designed 44K Agilent chip (Agilent Technologies) (Stockhammer et al., 2009). All RNA samples were labeled with Cy5 and hybridized against a Cy3-labeled common reference, which consisted of a mixture of all samples from the infection study. Labeling, hybridization and data analysis using Rosetta Resolver 7.0 was performed as previously described (Stockhammer et al., 2009). The raw data were submitted to the Gene Expression Omnibus database under accession no. GSE34930. KEGG pathway analysis was performed using DAVID v6.7 (http://david.abcc.ncifcrf.gov/home.jsp) (Huang da et al., 2009).

Microscopy and image analysis

Bright field images of embryos were obtained with a Leica M165C stereomicroscope equipped with a DFC420C digital color camera (Fig. 1; Fig. 2 A-D; Fig. 3 E-H; Supplementary Fig. S1 C, D) Composite images of different focal planes were created with Adobe Photoshop. Fluorescence images were taken with a Leica MZ16FA stereo fluorescence microscope equipped with a DFC420C digital color camera (Fig. 2 E-L ; Fig. 3 A, B, I-L; Fig. 5 C-F, H-M; Supplementary Fig. S1 E-H, K, L;). Overlay images of bright field and fluorescence stereomicroscopy were made in Adobe Photoshop. Confocal microscopy was performed with a Leica TCS SPE confocal microscope (Fig. 3 C, D: HC PLAN APO objective 20x/0.70 NA; Fig. 4 I-T: HCX APO objective 40x/0.80 NA; Fig. 5 N-Q) or Zeiss LSM5 Exciter system (Fig. 4 A-F: APO objective 10x/0.3 N/A). Maximum intensity projections of z-stacks of different focal planes were obtained using image J. Pixel counts on stereo fluorescence images were performed as described in (Stoop et al., 2011).

Results

Knockdown of *ptpn6* causes a late phenotype in zebrafish larvae that is characterized by enhanced proliferation, apoptosis, and inflammation

In order to study the function of the *ptpn6* gene in zebrafish, a knockdown study of this gene was performed using a splice blocking morpholino that causes deletion of the phosphatase catalytic domain (MO1) and a translation blocking morpholino (MO2) (Supplementary Fig. S1 A, B). Both morpholinos did not have strong effects on embryo morphology at 1-3 dpf, except that the heads and eyes of *ptpn6* morphants were slightly smaller compared with the controls (Fig. 1 A, B, H, I; Fig. 2). Furthermore, L-plastin immunostaining, *in situ* hybridization with *mfap4* and *mpx* markers, and Mpx activity assays demonstrated that macrophage and neutrophil numbers and the migratory responses of these cells towards injury were normal (Fig. 2).

However, at later stages of development, pleiotropic effects on larval morphology were observed that became progressively severe. At 3 dpf, some embryos showed a minor oedema of the heart cavity (Fig. 1 B, I), which became more prominent at 4 dpf (Fig. 1 C, J). At 5-6 dpf, morphant larvae additionally developed lesions on the eyes and skin, and in severe cases they also developed oedema between the trunk and the yolk sac and yolk extension (Fig. 1 D-G, K-N). After injection with MO1 between 80-90% of the larvae at 5-6 dpf showed these phenotypic abnormalities, while the effect of MO2 injection was lower, with only 5-10% of larvae displaying severe oedema and skin lesions (Supplementary Fig. S1 C, D). The heart beat frequency was unaffected in *ptpn6* morphants until 4 dpf, but was approximately 40% reduced in larvae of 6 dpf, as the likely result of the increased cardiac oedema at this stage (data not shown). Morpholino injection of *Tg(fli1:EGFP)* embryos did not reveal defects in vascular development (Supplementary Fig. S1 E-H).

Since mammalian *ptpn6*/*shp1* has been implicated in negative regulation of growth factor, MAPK, and NF-κB signaling pathways, we investigated proliferation, apoptosis, and inflammation in *ptpn6* morphants. Phosphohistone H3 immunolabeling revealed increased numbers of mitotic cells in *ptpn6* morphants in a specific region on the dorsal side of the brain and in the retina at 5 dpf (Fig. 3 A-D). Furthermore, increased numbers of apoptotic cells were detected with a TUNEL assay, particularly in the brain and in the region of cardiac oedema at this stage (Fig. 3 E-H). To determine if the phenotype of the zebrafish *ptpn6* morphants was associated with inflammation, we performed immunofluorescence staining using anti-L-plastin Ab, which stains all leukocytes (Mathias et al., 2007). The results showed that leukocytes in 5 dpf *ptpn6* morphants accumulated around the oedemic area of the heart cavity and at skin lesions, while they had largely disappeared from their normal location in the caudal hematopoietic tissue (Fig. 3 I-L). In addition, we checked the expression of the pro-inflammatory genes *il1b* and *mmp9*. Quantitative RT-PCR (qPCR) showed that, concomitant with the appearance of the strong phenotypic defects at 5-6 dpf, the

expression levels of *il1b* and *mmp9* were enhanced in *ptpn6* MO1 and MO2 morphant embryos compared to the controls at 5dpf and 6dpf, but not at 4dpf (Fig. 3 M-P). We conclude, based on leukocyte infiltration and enhanced *il1b* and *mmp9* expression, that the late pleiotropic phenotype of *ptpn6* morphant larvae is associated with inflammation.

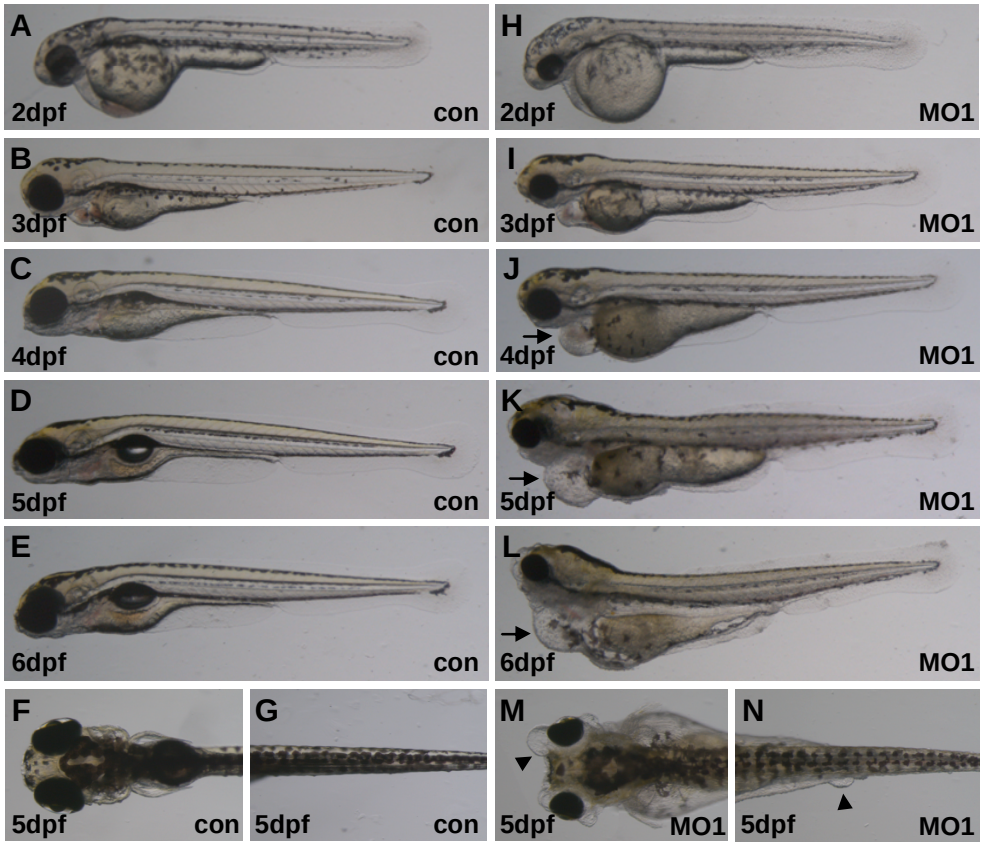

Figure 1. Late pleiotropic phenotype in *ptpn6* morphant zebrafish larvae. (A-G) Embryos injected with standard control morpholino (con). (H-N) embryos injected with *ptpn6* morpholino (MO1). Phenotypes are shown at 2-6 dpf in lateral (A-E, H-L) or dorsal view (F, G, M, N) with the anterior side to the left. Dorsal views show head and trunk (F, M) or the tail region (G, N). Skin lesions in *ptpn6* morphants are indicated with arrow heads and cardiac oedema is indicated with arrows.

The *ptpn6* morphant phenotype is independent of the presence of microbes

Since inflammation might be triggered by the presence of microbes, we investigated whether the zebrafish *ptpn6* morphant phenotype also developed in a germ-free environment. To this end, eggs were bleached, treated with iodine, and cultured in the presence of antibiotic and antifungal compounds following established protocols (Pham et al., 2008). At the end of the experiment sterility was confirmed by plating culture medium on tryptic soy agar plates, showing that bacterial colonies developed from

conventionally reared larvae but not from germ-free cultured larvae**.** When reared in the germ-free environment, 80-90% of the *ptpn6* morphant larvae developed oedema and skin lesions, similar to the conventionally raised morphant larvae. Therefore, the inflammation-associated phenotype of *ptpn6* morphants is apparently not driven by the presence of culturable microbes.

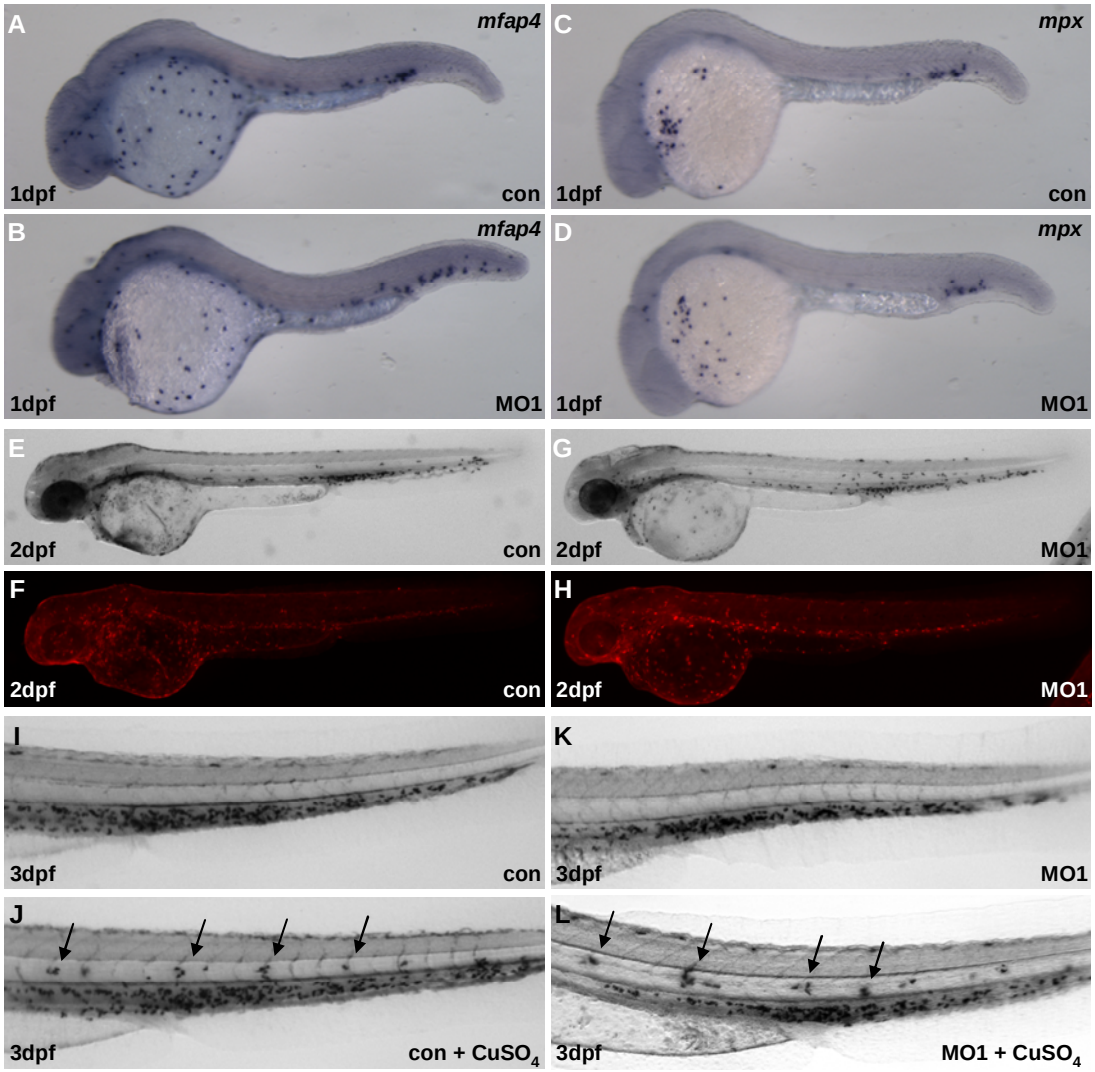

Figure 2. Unaltered leukocyte development and migratory response in *ptpn6* morphants. (A, B) In situ hybridization with macrophage marker *mfap4* (Zakrzewska et al., 2010) at 1 dpf. (C, D) In situ hybridization with neutrophil marker *mpx* at 1 dpf. (E, G) Histochemical staining for Mpx enzyme activity at 2-3 dpf. (F, H) immunolabeling of the same embryos as in E and G with Ab against the general leukocyte marker L-plastin. (I-L) Histochemical staining for Mpx enzyme activity in larvae incubated for 2 h with (J, L) or without (I, K) 10 µM CuSO4 at 3 dpf. Chemically induced inflammation by CuSO4 treatment (d'Alençon *et al* 2010) is due to damage of hair cells of the lateral line neuromasts, which attracts neutrophils (arrows in J, L). Embryos were injected with standard control (con) or *ptpn6* morpholino (MO1) and are shown in lateral view with the anterior to the left. The images are representative examples of ≥ 20 larvae in each group.

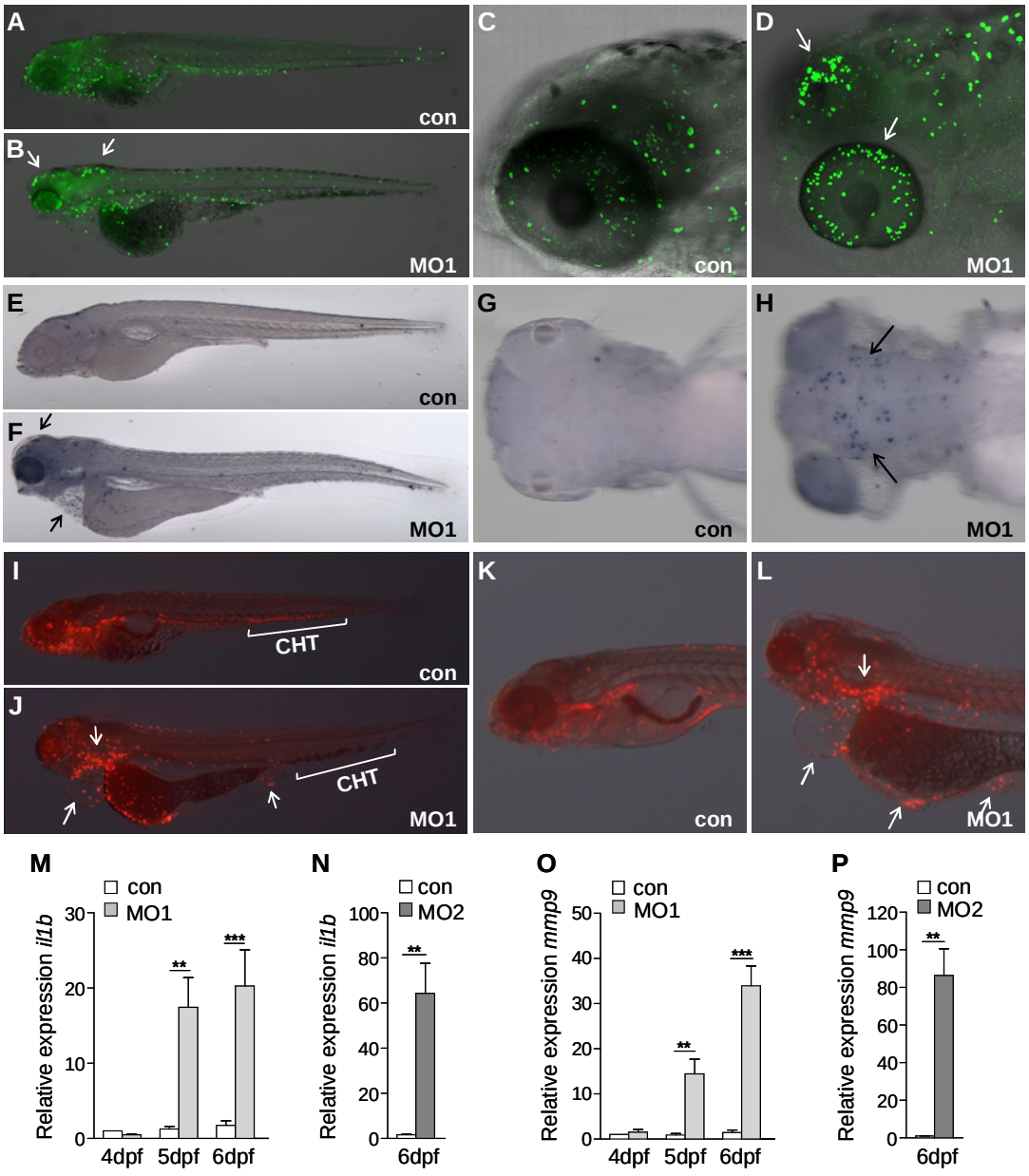

Figure 3. Enhanced proliferation, apoptosis, and inflammation in *ptpn6* morphants. (A-D) Phosphohistone H3 immunolabeling (E-H) TUNEL assay. (I-L) Immunolabeling with Ab against the general leukocyte marker L-plastin. Embryos were injected with standard control (con) or *ptpn6* morpholino (MO1) and all assays were performed at 5 dpf. Larvae or head details are shown in lateral (A- D, E, F, I-L) or dorsal (G, H) view with the anterior to the left. Stereo microscope images (A, B, E-L) and confocal Z-stack projections (C, D, transmitted light and fluorescence overlay) are representative examples of ≥ 20 larvae in each group. Arrows indicate regions with increased numbers of proliferating cells (B, D), increased numbers of apoptotic cells (F, H), and accumulation of leukocytes around sites of cardiac oedema and skin lesions (J, L) in *ptpn6* morphants. Also note the absence of immune cells in the caudal haematopoietic tissue (CHT) of *ptpn6* morphants (J) compared with the control (I). (M-P) Increased expression of proinflammatory genes in *ptpn6* morphants. Embryos were injected with splice blocking (MO1) or translation blocking (MO2) morpholinos targeting *ptpn6* or with standard control

morpholino (con). RNA was isolated at 4-6 dpf from pools of 10-20 larvae, which were picked randomly in the case of con and MO1 morpholino injections. In the case of MO2, which has a lower penetrance, 10-20 larvae showing the oedema and skin lesion phenotype were selected. Gene expression levels of *il1b* (M, N) and *mmp9* (O,P) were determined by qPCR and relative expression levels are shown with the lowest expression level set at 1. Data are the mean ± SEM of three independent experiments. Asterisks indicate significant differences (**, $P < 0.01$; ***, $P < 0.001$) tested with an unpaired t-test.

Glucocorticoid treatment enhances the *ptpn6* morphant phenotype

The immunosuppressive action of glucocorticoids is known to be conserved between zebrafish and mammals (Schaaf et al., 2009). Since the *ptpn6* morphant phenotype was associated with enhanced pro-inflammatory gene expression and leukocyte infiltration of affected tissues, we tested whether these effects could be suppressed by glucocorticoid treatment. Surprisingly, treatment with the synthetic glucocorticoid betamethasone 17-valerate, which was previously demonstrated to act as a potent glucocorticoid in zebrafish (Schoonheim et al., 2010), enhanced rather than suppressed the development of oedema and skin lesions in *ptpn6* morphants. Treatment with two other glucocorticoids, beclomethasone and prednisolone, also enhanced the development of oedema and skin lesions (data not shown). The affected tissues in morphants treated with betamethasone 17-valerate were accompanied by a more abundant leukocyte infiltration, as demonstrated using a transgenic marker line for neutrophils (*Tg(mpx:gfp)i114,* (Renshaw et al., 2006) and by L-plastin-immunostaining of neutrophils and macrophages (Fig. 4 A-F). In addition, approximately 10% of *ptpn6* morphants that were treated with betamethasone 17-valerate from 1 dpf died at 5 dpf, while all untreated morphants were viable until 7-8 dpf. In line with the enhanced phenotype, the induction levels of *il1b* and *mmp9* were also further increased in morphants treated with betamethasone 17-valerate as compared to the control group (Fig. 4 G, H). The increase of *il1b* and *mmp9* at 5 dpf was more pronounced when betamethasone 17-valerate treatment was performed for 4 days starting at 1 dpf compared to a 2-day treatment starting at 3 dpf (Fig. 4 G, H). However, no general insensitivity to glucocorticoids was observed. Expression of *fkbp5*, a well-known glucocorticoid receptor target gene (Schaaf et al., 2009), was inducible by betamethasone 17-valerate in both *ptpn6* morphants and control embryos (data not shown). In addition, betamethasone 17-valerate could still repress the expression of the *pomc* gene in the anterior lobe of the pituitary gland of *ptpn6* morphants (Fig. 4 I-T), a phenotype often used to score for glucocorticoid responsiveness (Schoonheim et al., 2010). Overall, these results show that glucocorticoids are not able to suppress the inflammatory response observed in *ptpn6* morphants, and that instead they unexpectedly enhance this response.

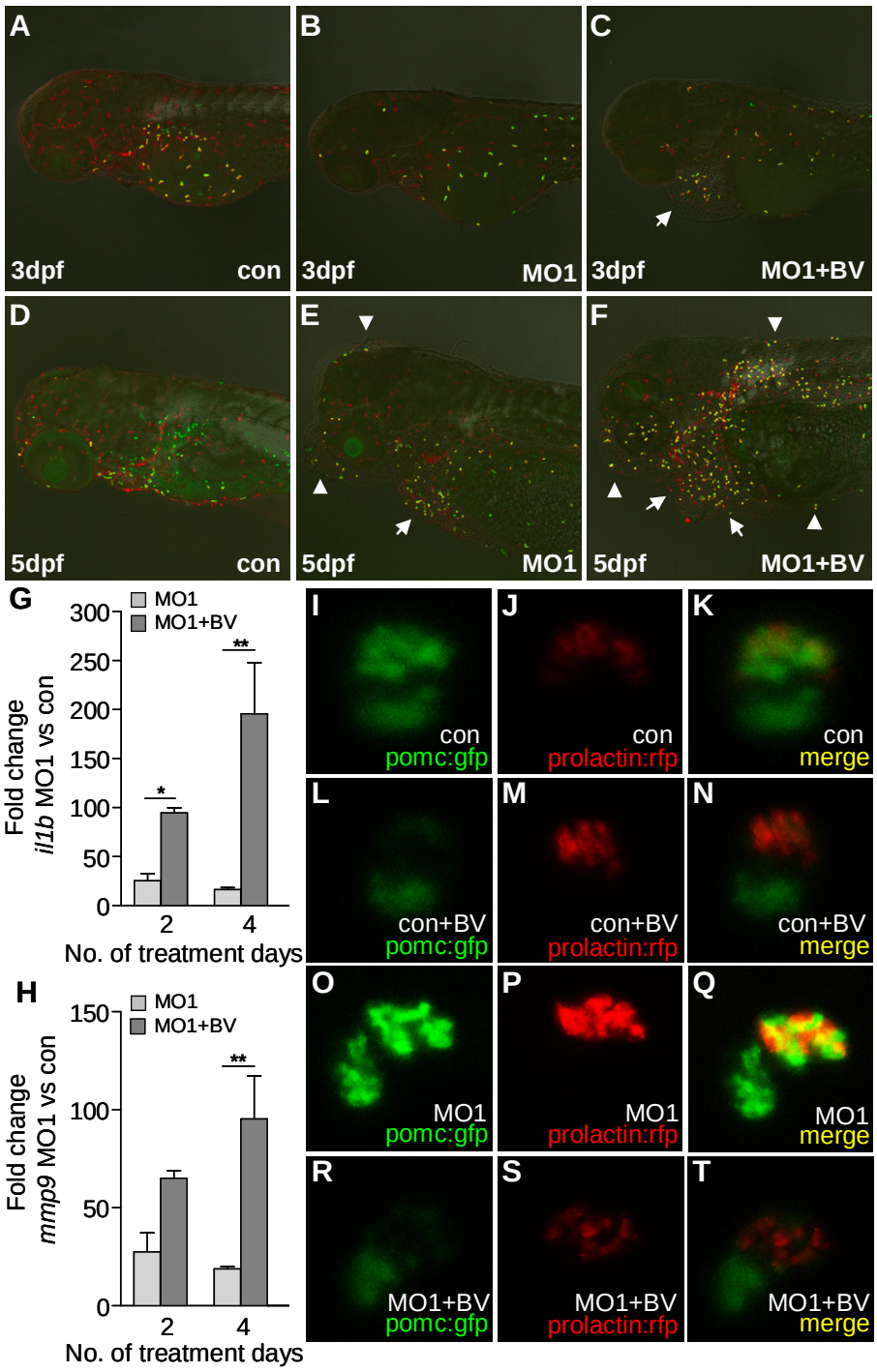

Figure 4. Enhanced leukocyte infiltration and inflammatory gene expression by glucocorticoid treatment of *ptpn6* morphants. (A-F) Embryos of the *Tg(mpx:gfp)i114* neutrophil marker line were injected with standard control (con) or *ptpn6* morpholino (MO1) and treated at 1 dpf with 1 µM betamethasone 17-valerate (BV) in 0.1% DMSO or with 0.1% DMSO alone as a control. Immunolabelling with Ab against the general leukocyte marker L-plastin was performed at 3 and 5 dpf. Confocal Z-stacks (fluorescence and transmission overlay) of the larval heads (lateral view, anterior to the left) are representative examples of ≥

20 larvae in each group. Accumulation of leukocytes around sites of cardiac oedema and skin lesions in *ptpn6* morphants is indicated with arrows and arrowheads, respectively. (G,H) Embryos were injected with standard control or *ptpn6* (MO1) morpholino and treated with betamethasone 17-valerate (BV) for 2 days starting at 3 dpf or for 4 days starting at 1 dpf. RNA was isolated at 5 dpf from pools of 10-20 larvae. Gene expression levels of *il1b* (G) and *mmp9* (H) were determined by qPCR and are represented as the fold increase of expression level of the MO1 group relative to that of the control morpholino group. Data are the mean ± SEM of three independent experiments. Asterisks indicate significant differences (* , $P < 0.05$; **, $P < 0.01$) tested by two-way ANOVA analysis with Bonferroni method as post-hoc test. (I-T) Glucocorticoid repression of *pomc* expression in the anterior lobe of the pituitary gland in *ptpn6* morphants and control embryos. Double transgenic zebrafish embryos expressing prolactin:rfp in the anterior lobe of the pituitary gland and pomc:gfp in the anterior and posterior lobes (*Tg(-1.0pomca:GFP)zf44;Tg(prl:RFP)zf113*, Liu et al., 2006) were injected with standard control (con, I-N) or *ptpn6* (MO1, O-T) morpholinos and treated from 1 dpf with 1 μM betamethasone 17-valerate (BV) in 0.1% DMSO (L-N, R-T) or with 0.1% DMSO as a control (I-K, O-Q). The pituitary gland was imaged at 5 dpf and confocal Z-stacks are representative of 15-20 embryos per group. In *ptpn6* morphants the pituitary gland is twisted to the right compared to that in control embryos, but pomc:gfp expression in the anterior lobe is down-regulated by glucocorticoid treatment similar as it is in the control embryos.

Knockdown of *ptpn6* impairs the ability of embryos to combat *S. typhimurium* and *M. marinum* infections

To investigate the function of *ptpn6* in the innate immune response to infection, we challenged *ptpn6* morphants and control embryos by intravenous injection of *S. typhimurium* bacteria. Importantly, bacterial injections were performed at 28 hpf, i.e. several days before the appearance of inflammation and other phenotypic effects in *ptpn6* morphants. In both *ptpn6* MO1 morphants and controls, *S. typhimurium* infection was lethal around 24-30 hours post infection (hpi). However, CFU counts at 2, 8 and 20 hpi showed that *S. typhimurium* proliferated faster in the *ptpn6* morphants (Fig. 5 A). Subsequently, we performed infections with the non-pathogenic *S. typhimurium* LPS O-Ag mutant strain Ra (van der Sar et al., 2003). While this Ra strain hardly proliferated in control embryos, clear proliferation of the DsRed-labeled Ra bacteria was observed in approximately 55% of *ptpn6* MO1 morphants at 1 day post infection (Fig. 5 B-D). At 5 days post infection (dpi), control embryos had cleared the infection or showed very low DsRed fluorescence signal, while the majority of *ptpn6* morphants were heavily infected or had died at 5 dpi (Fig. 5 B, E, F). Increased proliferation of wild type and Ra *S. typhimurium* bacteria was also observed in embryos injected with *ptpn6* MO2 (Supplementary Fig. S1 I-L).

Next we examined the response of *ptpn6* morphants to infection with *M. marinum*, a pathogen known to cause a chronic infection in zebrafish embryos, whereby infected and uninfected macrophages cluster into aggregates that resemble tuberculous granulomas (Davis et al., 2002; van der Sar et al., 2009). At 3 dpi *ptpn6* morphants showed increased fluorescence signal of mCherry labeled *M. marinum* bacteria and increased numbers of granuloma-like aggregates compared to control embryos (Fig. 5 G-I). At 4 and 5 dpi, granulomas in *ptpn6* morphants further increased in size compared

to those in control embryos (Fig. 5 G, J-M). Furthermore, immunohistochemical analysis using an L-plastin Ab of granulomas at 4 dpi showed that the bacteria were mostly extracellular in *ptpn6* morphants, while in control embryos bacteria were mostly contained within L-plastin-labeled leukocytes (Fig. 5 N-Q).

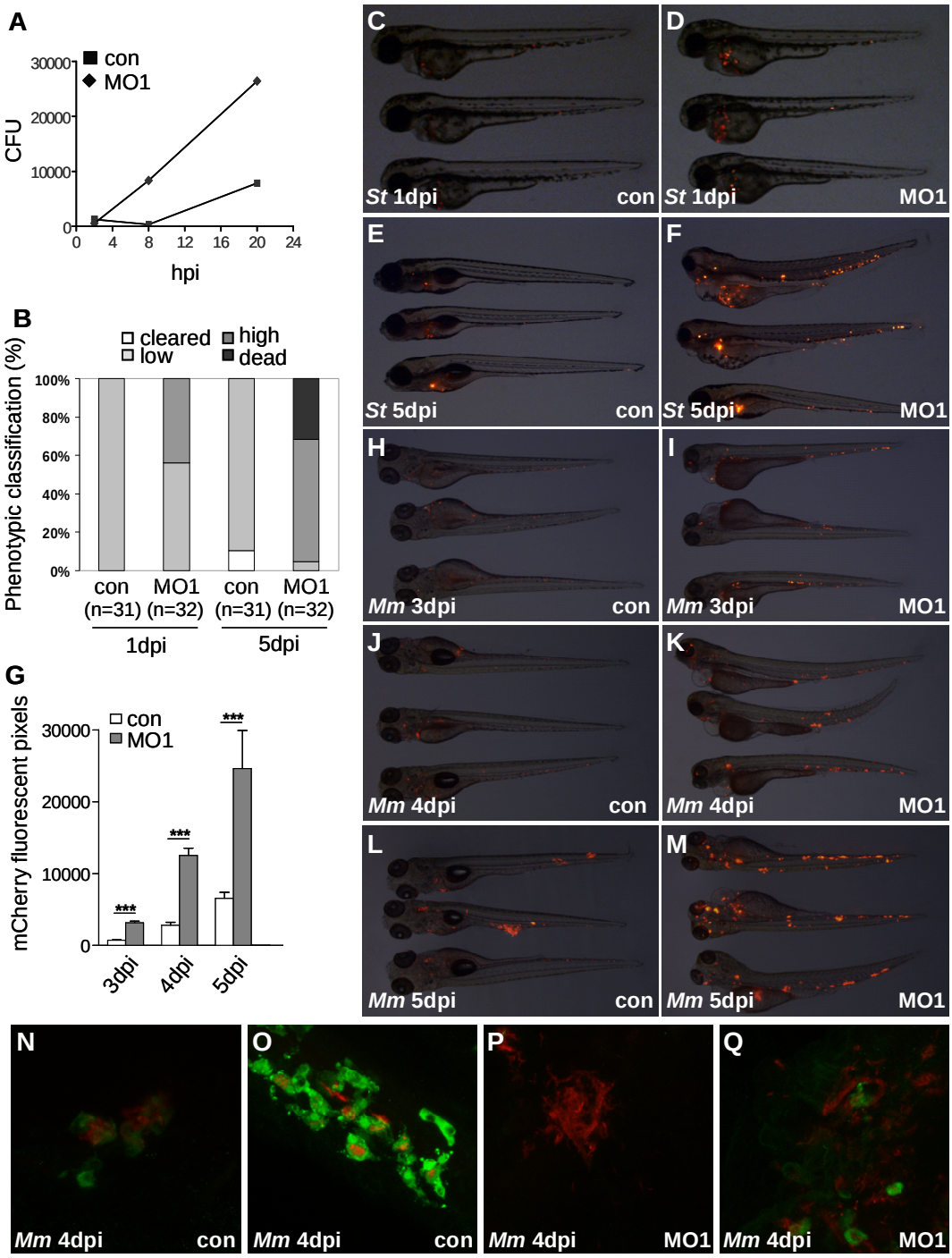

Figure 5. Impaired control of *S. typhimurium* and *M. marinum* infection in *ptpn6* morphants. (A) Infection with *S. typhimurium* wild type strain. Embryos were injected with

standard control morpholino (con) or *ptpn6* MO1 and infected with *S. typhimurium* at 28 hpf. Groups of 5 embryos were crushed in PBS at 2, 8, and 20 hpi, and dilutions were plated for CFU counting on LB medium with carbenicillin selection of the DsRED marker plasmid in *S. typhimurium*. A representative example of three independent experiments is shown. (B-F) Infection with *S. typhimurium* LPS O-Ag mutant Ra strain. Embryos were injected with standard control morpholino (con) or *ptpn6* MO1 and infected with *S. typhimurium* Ra at 28 hpf. The bacterial burden was analyzed at 1 and 5dpi based on fluorescence of the DsRED marker plasmid. A quantification of phenotypes (B) and stereo fluorescence images (lateral view, anterior to the left,) of 3 embryos per group (C-F) are shown for a representative example of three independent experiments. The bacterial burden in embryos at 1 dpi was scored as low (representative image in C) or high (representative image in D). At 5 dpi embryos had either cleared the infection, or had died, or showed low (representative image in E) or high (representative image in F) bacterial burden. (G-Q) Infection with *M. marinum* Mma20 strain. Embryos were infected with approximately 100 CFU at 28 hpf, and formation of *M. marinum* granulomas was analyzed at 3, 4 and 5 dpi based on fluorescence of the mCherry marker plasmid. Fluorescence images of more than 60 embryos per treatment group accumulated from two independent experiments were analyzed with pixel quantification software (Stoop et al., 2011) including an uninfected group as the blank. Pixel quantification data ± SEM (G) and representative fluorescence images (lateral view, anterior to the left) of 3 embryos per group (H-M) are shown. (N-Q) Confocal Z-stacks of granulomas in *ptpn6* MO1 morphants and control embryos.

In conclusion, when challenged with bacteria during early development, prior to the manifestation of the late inflammation-associated phenotype, *ptpn6* morphants were severely impaired in their ability to control the progression of infection. This impaired control was observed with *S. typhimurium* and *M. marinum* strains that induce very different pathologies, indicating a general inability of *ptpn6* morphants to mount a functional immune response.

Bacterial challenge of *ptpn6* morphants leads to hyperinduction of *il1b* and *mmp9* gene expression

To further investigate the function of *ptpn6* in the innate immune response to infection, we performed qPCR analysis for two genes, *il1b* and *mmp9*, which were previously shown to represent robust pro-inflammatory markers associated with bacterial infection (Stockhammer et al., 2009) and with the late phenotype observed after *ptpn6* knockdown (see above). We chose to analyze the response to *S. typhimurium* infection at 8 hpi (36 hpf) based on a previous time-course analysis (Stockhammer et al., 2009). As expected, control embryos showed a strong induction of *il1b* and *mmp9* expression levels. Upon knockdown of *ptpn6* with MO1 or MO2, the induction levels of *il1b* and *mmp9* were significantly higher than in the control embryos (Fig. 6 A, B). Therefore, we conclude that *ptpn6* functions as a negative regulator of *il1b* and *mmp9* induction during *S. typhimurium* infection. Unlike the strong pro-inflammatory gene expression that is induced by *S. typhimurium* infection at 8 hpi (Fig. 6 A, B), control embryos or *ptpn6* morphants infected with *M. marinum* did not show significant *il1b* and *mmp9* induction at this time point (Fig. 6 C, D). However, at 3 dpi *mmp9* (but not *il1b*) expression was increased in *M. marinum*-infected *ptpn6* morphants compared to

uninfected morphants and infected controls (Fig. 6 C, D). In summary, a hyperinduction of pro-inflammatory genes was observed upon bacterial infections of *ptpn6* morphants, yet their ability to control these infections was impaired.

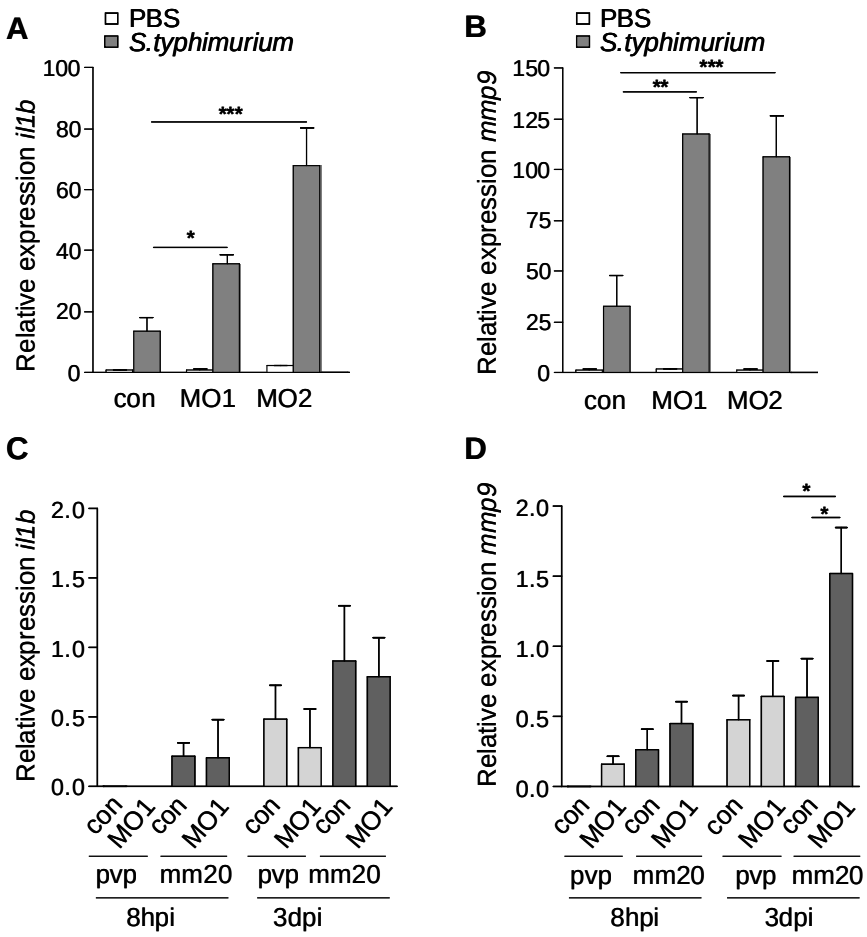

Figure 6. Increased induction of *il1b* and *mmp9* expression in *ptpn6* morphants challenged with bacterial pathogens. (A, B) qPCR analysis of *il1b* (A) and *mmp9* (B) expression in RNA samples of PBS-injected or *S. typhimurium*-infected control embryos (con) and *ptpn6* morphants (MO1 and MO2). Embryos were injected at the 1-2 cell stage with standard control (con) or *ptpn6* (MO1 or MO2) morpholinos, injected with PBS or with 250 CFU of *S. typhimurium* at 28 hpf, and RNA was isolated at 8 hpi for qPCR analysis. Relative expression levels are shown with the lowest expression level set at 1. Values are the means ± SEM of three biological replicates. (C, D) Gene expression levels of *il1b* (C) and *mmp9* (D) in response to *M. marinum* infection. Embryos were injected with standard control morpholino (con) or *ptpn6* MO1, approximately 100 CFU of *M. marinum* bacteria were injected at 28 hpf, and RNA was isolated at 8 hpi or 3 dpi. Relative expression levels are shown with the lowest expression level set at 1. Data are plotted on a logarithmic scale and are the mean ± SEM of three replicate experiments. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) tested by two-way ANOVA analysis with Bonferroni method as post-hoc test.

Microarray analysis demonstrates an overall enhancement of the innate immune response to *S. typhimurium* infection upon *ptpn6* knockdown

The *S. typhimurium* infection model was chosen for further investigation of the specific effects of *ptpn6* knockdown on the innate immune response by microarray analysis. The advantage of this model for functional analysis of *ptpn6* was that the response to *S. typhimurium* infection can be analyzed at 36 hpf (8 hpi) (Stockhammer et al., 2009; Ordas et al., 2011), thus avoiding that the microarray analysis is affected by secondary effects of the late inflammatory phenotype (observed at 4-6 dpf). RNA samples from infected and mock-injected *ptpn6* MO1 morphants or control embryos from three replicate experiments were analyzed using a common reference approach (Fig. 7 A). First we analyzed the basal expression differences between mock-injected *ptpn6* morphants and control embryos. KEGG pathway analysis showed that p53 signaling and cell cycle were the most significantly affected pathways, and minor effects were observed on cytosolic DNA-sensing, sucrose metabolism and pyrimidine metabolism (Fig. 7 B). There was little overlap with the genes that were induced by infection in *ptpn6* morphants: only 7% of the microarray probes that showed basal expression differences between controls and *ptpn6* morphants were responsive to infection in *ptpn6* morphants (Fig. 7 B). The infection-responsive gene set showed significant alteration of many KEGG pathways related to the immune response, such as TLR, NLR, RIG-I, p53, MAPK, and JAK-STAT signaling (Fig. 7 B). For further analysis of *ptpn6* function we concentrated on the differences between the responses to infection in *ptpn6* morphants and controls.

The total number of probes showing significant responsiveness to infection was approximately 2-fold larger in *ptpn6* morphants than in controls (Fig. 7 C). Furthermore, the absolute fold changes of many infection-responsive probes were larger in *ptpn6* morphants (see Supplementary Table 1 for a complete overview of the microarray data). In fact, a total of 598 probes (representing 258 different genes) showed significantly higher up-regulation in *ptpn6* morphants and 78 probes (representing 51 different genes) showed significantly stronger down-regulation (Fig. 8). Analysis of the gene group with higher up-regulation in *ptpn6* morphants showed significant overrepresentation of TLR, NLR, RIG-I, p53, MAPK, JAK-STAT and other immune-related KEGG pathways (Fig. 7 C). More specifically, genes showing higher up-regulation in *ptpn6* morphants included cytokine/chemokine/interferon genes such as *il1b*, *il8*, *tnfa*, *tnfb*, and *ifnphi1*, matrix metalloproteinases such as *mmp9* and *mmp13*, and many transcriptional regulators of the ATF, CEBP, AP1, NFκB, and STAT families (Fig. 8). The higher up-regulation of *il1b* and *mmp9* was consistent with the qPCR experiments described above (Fig. 6 A, B) and with qPCR validation of the samples used for the microarray study (Supplementary Fig. S2 A, B). As observed in previous *S. typhimurium* infection studies, several negative regulators of immunity signaling are induced concomitantly with the induction of pro-inflammatory genes (Stockhammer et al., 2009; Ordas et al., 2011). Similar to the increased induction of pro-inflammatory

genes, infected *ptpn6* morphants also showed increased induction levels of several of these negative regulators, such as *irak3*, *socs3a* and *socs3b,* and NFκB inhibitor genes (*nfkbiaa*, *nfkbiab*, *nfkbib*, *nfkbiz*). In contrast, the anti-inflammatory cytokine gene *il10* (represented by 4 probes on the array) did not show increased induction in *ptpn6* morphants.

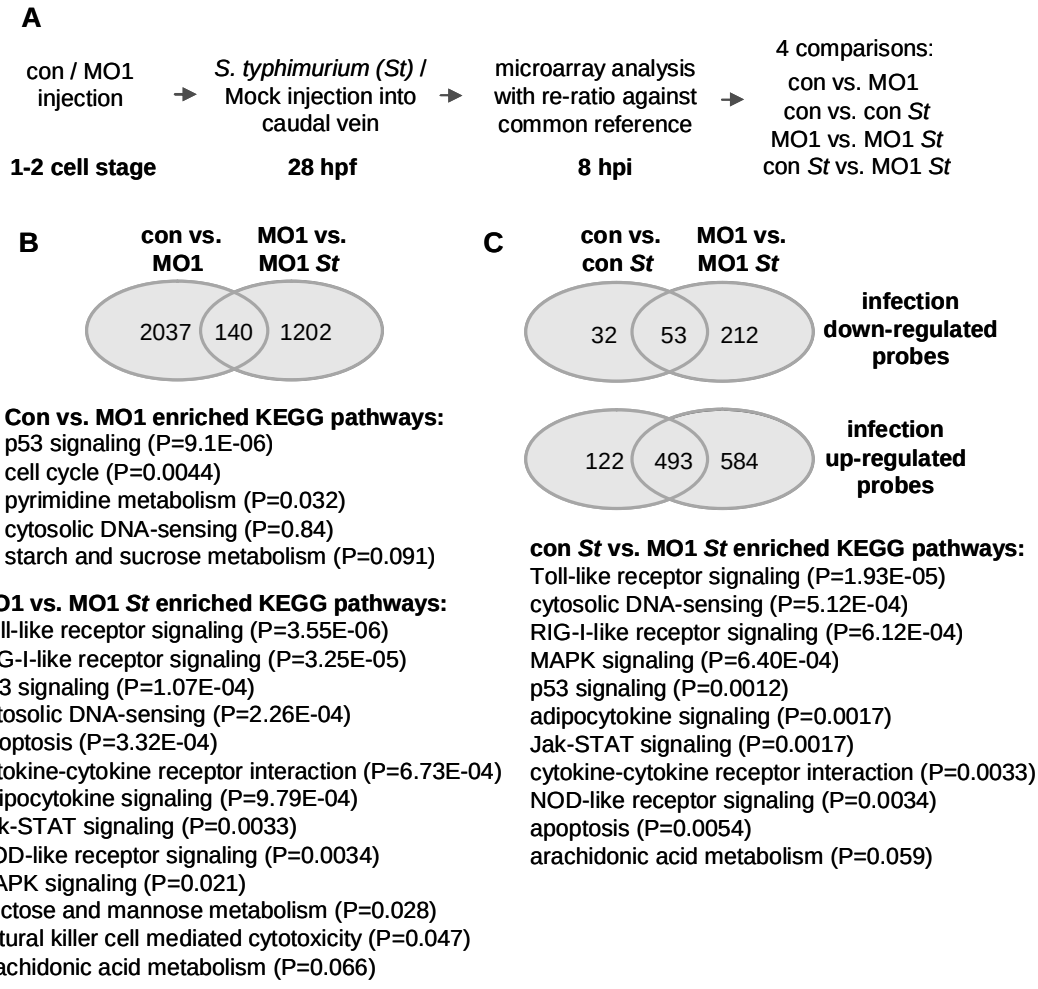

Figure 7. Enhanced innate immune response to *S. typhimurium* in *ptpn6* morphants. (A) Experimental set-up of the microarray analysis. Embryos were injected with *ptpn6* MO1 or Danieau's buffer at the 1-2 cell stage, and approximately 250 CFU of *S. typhimurium* bacteria were injected into the caudal vein at 28 hpf after the onset of the blood circulation, or PBS was injected as a control. Microarray analysis was performed on RNA samples extracted from pools of 15-20 embryos at 8 hpi. RNA samples from the four treatment groups (control/PBS, control/*S. typhimurium*, *ptpn6* MO1/PBS, *ptpn6* MO1/*S. typhimurium*) were obtained from three independent experiments and hybridized against a common reference consisting of RNA from all treatment groups. Next, four expression ratios were derived by Rosetta Resolver re-ratio analysis of the sample data against the common reference. The significance cut-offs were set an absolute fold change ≥1.5 and $P \leq 0.0001$. (B) Venn diagram showing the overlap between the effect of *ptpn6* knockdown on basal gene expression levels (con vs. MO1) and the effect of *S. typhimurium* on gene expression in *ptpn6* morphants (MO1 vs MO1 *St*). The numbers of probes

with significantly changed expression are shown in the Venn diagram and significantly enriched KEGG pathways for each comparison are indicated below. (C) Venn diagrams showing comparisons of the numbers of probes that were up-regulated or down-regulated by *S. typhimurium* infection in control embryos (con vs. con *St*) or in *ptpn6* morphants (MO1 vs. MO1 *St*). KEGG pathways that were significantly enriched in the dataset of infected *ptpn6* morphants compared with the dataset of infected controls (con *St* vs. MO1 *St*) are indicated below. A complete overview of the microarray data is given in Supplementary Table 1.

The smaller gene group that showed stronger down-regulation upon infection in *ptpn6* morphants than in the controls included two CCL chemokine genes (*ccl1* and *ccl-c11a*) and several leukocyte markers such as *coro1a*, *cpa5*, *cxcr3.2*, *lgals9l1*, *lcp1*, *lyz*, and *mpx* (Fig. 8). However, there was no generally enhanced down-regulation of leukocyte markers in infected *ptpn6* morphants, as *mpeg1* was less repressed in infected *ptpn6* morphants than in infected controls, and *csf1r* and *mfap4* showed unaltered expression under all conditions. qPCR analysis of leukocyte markers showed the same trend as the microarray data, particularly increased down-regulation of *lgals9l1*, *lyz*, and *mpx* during infection, less pronounced down-regulation of *mpeg1*, and unaltered expression of other markers, including *csf1r* and *mfap4* (Supplementary Fig. S2 C-F). In conclusion, microarray analysis indicated that under *ptpn6* knockdown conditions, embryos responded to *S. typhimurium* infection by an enhanced gene induction profile of the innate immune response.

Increased pro-inflammatory gene induction in *S. typhimurium*-infected *ptpn6* morphants is confirmed by RT-MLPA analysis

In addition to the microarray analysis, we used a recently described RT-MLPA assay that allows the simultaneous semi-quantitative PCR analysis of 34 innate immune genes (Rotman et al., 2011). Further confirming the microarray results, RT-MLPA analysis showed over 2-fold increased *S. typhimurium*-induced up-regulation in *ptpn6* morphants compared to controls for several cytokine/chemokine/interferon genes (*ccl-c5a*, *cxcl-c1c*, *ifnphi1*, *il1b*, *il8*, and *tnfa*), immune-related transcription factor genes (*fos*, *jun*, *junb*, *nfkb2*, and *rel*), the matrix metalloproteinase gene, *mmp9,* and the acidic chitinase gene, *chia.6* (Fig. 9). Additionally, five other genes that did not meet the significance thresholds in the microarray analysis (*ccl20*, *ccl-c24i*, *cxc46*, *fkbp5*, *tlr5a*) also showed over 2-fold higher up-regulation in *ptpn6* morphants based on RT-MLPA (Fig. 9). Of note, in both microarray and RT-MLPA analysis, the anti-inflammatory *il10* gene showed equal induction levels during *S. typhimurium* infection of *ptpn6* morphants and controls (Fig. 9, Supplementary Table 1). Based on the increased pro-inflammatory gene induction profile of *S. typhimurium*-infected *ptpn6* morphants, observed in microarray analysis and RT-MLPA, we conclude that *ptpn6* functions as a negative regulator of the innate immune response upon infection.

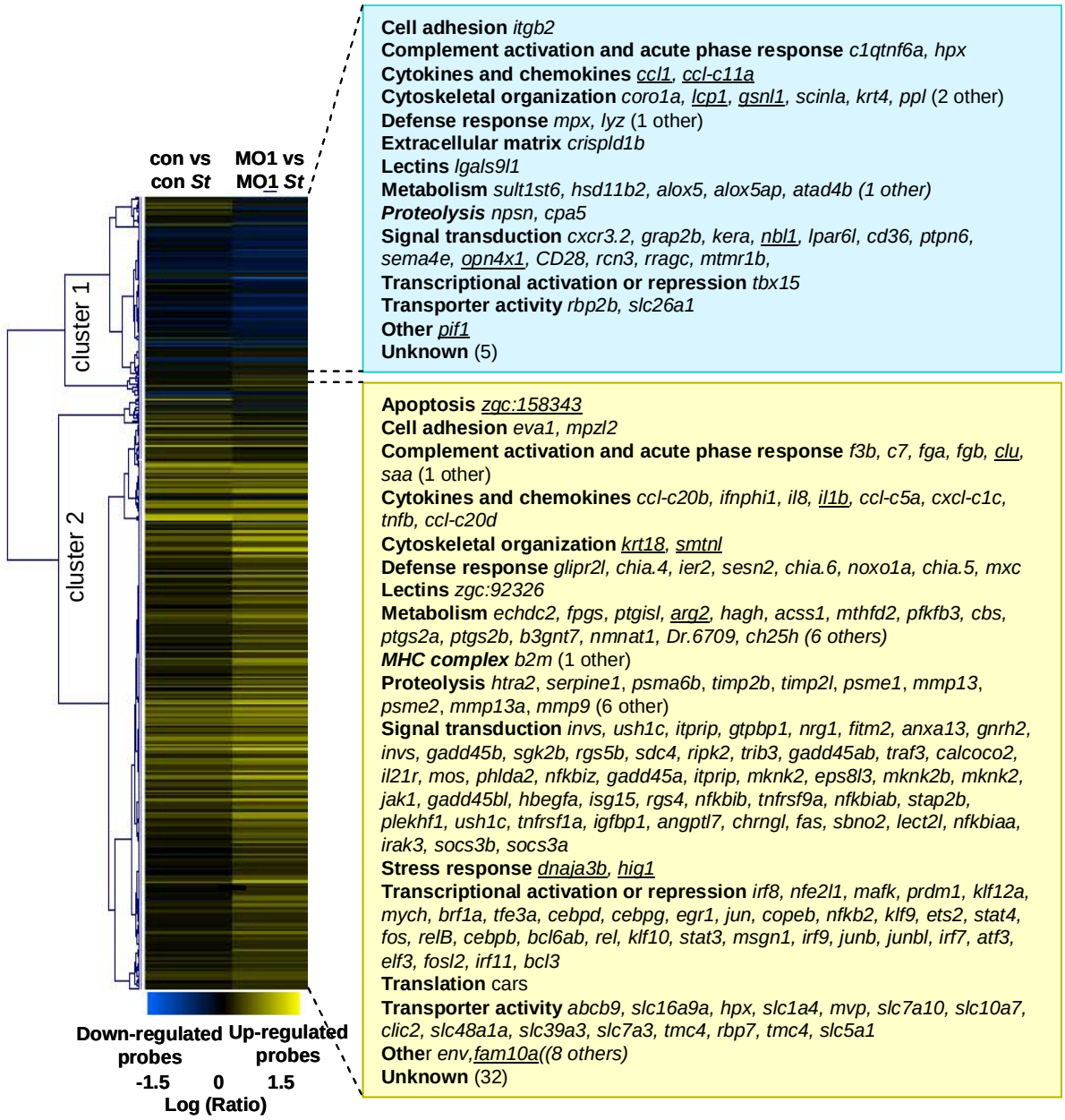

Figure 8. Gene groups showing higher up-regulation or stronger down-regulation upon *S. typhimurium* challenge of *ptpn6* morphants compared with *S. typhimurium* challenge of control embryos. A two-dimensional hierarchical clustering (average link, cosine correlation) was performed of the probes that were up-regulated or down-regulated by *S. typhimurium* infection in control embryos or in *ptpn6* morphants (fold change ≥1.5 and $P \leq 0.0001$). Up-regulated probes are indicated by increasingly brighter shades of yellow, and down-regulated probes are indicated by increasingly brighter shades of blue. Cluster 1 contains 78 probes (representing 51 different genes) that showed a stronger down-regulation in *S. typhimurium*-infected *ptpn6* morphants than *S. typhimurium*-infected control embryos, and cluster 2 contains 598 probes (representing 258 different genes) with stronger up-regulation in the *ptpn6* morphants. The gene symbols corresponding to the probes with stronger up- or down

regulation are indicated next to the two clusters, categorized in functional annotation groups. Only gene symbols of genes with assigned gene names are indicated in the figure, the number of other genes without a gene name in each annotation group is indicated between brackets; the full list is given in Supplementary Table 1. Symbols of genes that also showed differential expression in mock-injected *ptpn6* morphants compared to mock-injected control embryos are underlined.

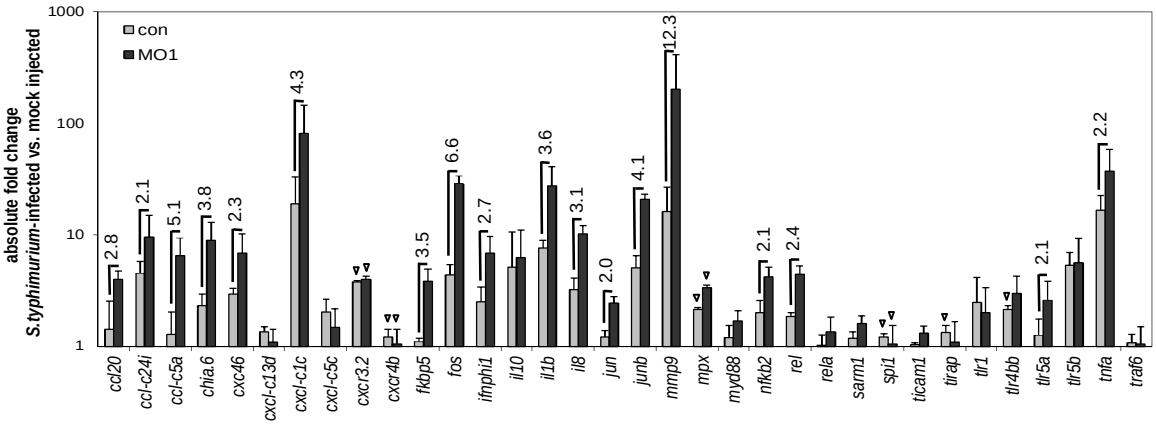

Figure 9. Hyperinduction of proinflammatory genes in *S. typhimurium*-infected ptpn6 morphants shown by RT-MLPA analysis. Relative expression levels of 34 innate immune response genes were determined as described in Rotman et al. 2011. The figure shows the absolute fold difference in expression of *S. typhimurium*-infected control embryos (con) and *ptpn6* morphants (MO1) versus the corresponding mock-injected groups. Data are plotted on a logarithmic scale. Values are the means ± standard deviation of three independent sample sets, which were the same as previously used for microarray analysis. Down-regulated expression is indicated with triangles. Numbers indicate ≥ 2-fold-change differences in up-regulated expression between *S. typhimurium*-infected *ptpn6* morphants and *S. typhimurium*-infected control embryos.

Discussion

The association of SHP1/PTPN6 deficiency with several types of chronic inflammatory disorders and with lymphoid and myeloid malignancies has raised substantial interest in this hematopoietic phosphatase as a drug target (Kundu et al., 2010). Here we exploited the zebrafish embryo as a novel animal model for studies on the function of the *ptpn6* gene. The late onset of adaptive immunity during zebrafish development permits studying *ptpn6* function in the sole context of innate immunity during embryo and larval development. Our results show that *ptpn6* deficiency in this model leads to pleiotropic defects at the late larval stage, accompanied by leukocyte infiltration and up-regulated inflammatory gene expression. Moreover, when embryos were challenged with bacteria during earlier stages of development, *ptpn6* knockdown enhanced the innate immune response to this infection. Despite this increased inflammatory response, *ptpn6*-deficient embryos were severely impaired in their ability to control infections, indicating the crucial importance of negative regulation by *ptpn6* for a balanced and functional innate immune response.

Inflammation-associated effects of *ptpn6* deficiency

Under normal culture conditions, without challenge by infection, *ptpn6* morphant larvae developed severe oedema and skin lesions by 5 to 6 days of age. The affected tissues in zebrafish *ptpn6* morphants were strongly infiltrated by leukocytes and increased numbers of TUNEL-positive apoptotic cells were also detected in these areas. Simultaneously, the overall expression levels of pro-inflammatory markers *il1b* and *mmp9* were over 10-fold increased. The observed inflammation in zebrafish *ptpn6* morphants is consistent with the function of mammalian SHP1 as a negative regulator of cytokine signaling, and is most likely caused by increased cytokine excretion by leukocytes which have been shown to exclusively express *ptpn6* (Zakrzewska et al., 2010). It remains unknown whether inflammation is the primary cause or the consequence of oedema and skin lesions in *ptpn6* morphants.

The observed phenotype is highly reminiscent of the severe skin inflammation observed in murine *Ptpn6* mutants, *me*, *mev,* and *spin* (Shultz et al., 1993; Tsui et al., 1993; Croker et al., 2008). In mice, *Ptpn6* deficiency, besides causing inflammation, was also associated with hyperproliferation of immune cells (Van Zant and Shultz, 1989), but we did not detect enhanced proliferation of myeloid cells during zebrafish embryonic and larval development. Among the three mutant alleles of *Ptpn6* in mice, *spin* causes the least severe functional knockdown (Croker et al., 2008). Inflammatory foot lesions of *spin* mutants did not develop when homozygotes were raised in a germ-free environment, showing the requirement of the normal microbiota to trigger this phenotype (Croker et al., 2008). However, *me* homozygotes born into a specific pathogen-free colony survived no better than under conventional conditions (Lutzner and Hansen, 1976). Similarly, we found that the late inflammation-associated phenotype of *ptpn6* morphant zebrafish larvae was equally severe when cultured using an established protocol to generate germ-free conditions (Pham et al., 2008). Complete germ-free conditions cannot be guaranteed during morpholino injections, but at least a major reduction of microbes was achieved in our experiments, since no bacterial colonies were observed when the culture medium of morpholino-injected larvae was plated on rich culture medium. Therefore, like in the *me* mutant, most likely another mechanism than the response to microbes is responsible for the development of spontaneous inflammation in *ptpn6* morphants.

In addition to the general inflammation-associated defects in *ptpn6* morphants, the dorsal area of the brain contained increased numbers of apoptotic cells as well as increased numbers of cells positive for the proliferation marker phosphohistone H3. Deregulation of pathways such as MAPK and NF-κB signaling, known to be affected by mammalian SHP1, may explain enhanced proliferation and apoptotic cell numbers in *ptpn6* morphants. The expression of *ptpn6* in wild type embryos and larvae by whole mount *in situ* hybridization was only detectable in myeloid cells and in the larval thymus (Zakrzewska et al., 2010). However, it cannot be excluded that *ptpn6* is also expressed in other tissues at lower levels, which may explain the specific hyperproliferative area in the brain of *ptpn6* morphants. In rodents, *Ptpn6* expression is

also predominantly hematopoietic, but has been detected in other tissues as well, including the CNS (Massa et al., 2000; Horvat et al., 2001).

Treatment with glucocorticoids enhanced the inflammatory phenotype, which is surprising since glucocorticoids are well known for their anti-inflammatory effects. They are widely used clinically to treat a variety of human immune-related diseases (Barnes, 2006) and their immune-suppressive effects appear to be well conserved between mammals and fish (Schaaf et al., 2009). Most anti-inflammatory effects of glucocorticoids are a result of the inhibitory interaction between the glucocorticoid receptor (GR) and transcription factors like AP-1 and NF-κB which are important for the up-regulation of many pro-inflammatory genes (De Bosscher and Haegeman, 2009). However, interaction with other transcription factors can enhance the activity of these proteins, and this may even lead to specific pro-inflammatory effects of glucocorticoids. In particular, interactions between GR and members of the STAT family, like STAT3, -5, and -6 have been demonstrated to be synergistic in nature (Zhang et al., 1997; Biola et al., 2000; Hermoso et al., 2004; Stoecklin et al., 1997; Rogatsky and Ivashkiv, 2006; Engblom et al., 2007). Interestingly, the JAK/STAT signaling pathway has been shown to be negatively regulated by SHP1 in many studies (Valentino and Pierre, 2006). Thus, the enhancement of the inflammatory phenotype upon *ptpn6* knockdown in zebrafish by glucocorticoids might be explained by a synergistic interaction between the GR and transcription factors, like the members of the STAT family, that have become activated due to the Ptpn6 deficiency (Fig. 10). Since in humans SHP1/PTPN6 deficiency has been shown to be possibly involved in the pathogenesis of several immune-related diseases, care should be taken when patients suffering from these diseases are treated with glucocorticoids. The SHP1 deficiency could be the cause of resistance to glucocorticoid treatment that is observed in a significant subpopulation of patients (Barnes and Adcock, 2009) and could theoretically even underlie a worsening of the disease state in response to this therapy.

Function of *ptpn6* in the response to bacterial infections

Knockdown of *ptpn6* impaired the ability of zebrafish embryos to control the proliferation of two bacterial pathogens that induce very different disease pathologies in the zebrafish embryo model: *S. typhimurium*, which causes acute disease, and *M. marinum*, which causes a chronic disease where bacteria persist in granulomatous aggregates. Even the growth of a normally non-pathogenic strain (*S. typhimurium* LPS O-Ag Ra mutant) could not be efficiently controlled and caused lethality. In contrast, murine *spin* mutants displayed increased resistance to *Listeria monocytogenes* (Croker et al., 2008). As discussed above, the *spin* phenotype is ascribed to a hypomorphic allele of *Ptpn6*, which may explain why this mutant is immunocompetent while zebrafish *ptpn6* morphants are not. The immunodeficiency of *ptpn6* morphants was apparent at early developmental stages well before the spontaneous increase in basal levels of pro-inflammatory genes and developmental defects that occur later during larval development. We found that the expression levels of immune-related transcription factor genes and many effector genes of the innate immune response were

hyperinduced upon infection in *ptpn6* morphants. In agreement, the whole set of hyperinduced genes showed significant enrichment of KEGG pathways related to pathogen recognition and cytokine signaling. These results support the function of *ptpn6* as a negative regulator of the innate immune response to bacterial infection. Since *ptpn6* morphants displayed decreased resistance, the hyperactivation of their innate immune response is apparently contra-productive for the organism's defense against bacterial pathogens.

The hyperinduction of innate immune response genes upon infection of *ptpn6* morphants was specific, since for example the expression levels of several myeloid markers were unchanged or reduced. In addition to many pro-inflammatory cytokines and transcription activators of the immune response, we also observed hyperinduction of other negative regulators than *ptpn6* itself. Apparently, in the absence of *ptpn6*, increased induction of other negative regulators was insufficient to prevent excessive inflammation and a contra-productive defense response against bacterial pathogens. This may be explained by the presumed inhibitory effect of Ptpn6 on many cytokine and immuno-receptors as well as central kinases in innate immunity signaling pathways (Abu-Dayyeh et al., 2008; Abu-Dayyeh et al., 2010), (Fig. 10). Furthermore, the anti-inflammatory cytokine gene *il10* was not induced to higher levels in infected *ptpn6* morphants than in controls. The fact that increased production of pro-inflammatory cytokines is not counteracted in *ptpn6* morphants by increased anti-inflammatory IL10 production is a possible explanation for their non-functional immune response against bacterial pathogens.

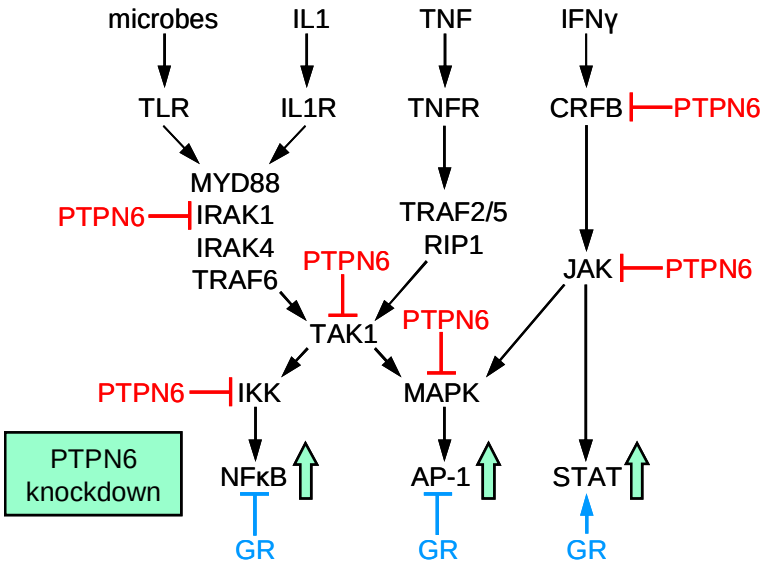

Figure 10. Model of PTPN6/SHP1 function in innate immunity signaling and interaction with glucocorticoid signaling. PTPN6/SHP1 has been proposed to inhibit cytokine receptors (like the CRFB family) as well as several kinases in TLR and cytokine signaling pathways (Abu-Dayyeh et al., 2008, Abu-Dayyeh et al., 2010). The putative inhibition motifs in these proteins are evolutionary conserved (Supplementary Table 2). Under conditions of PTPN6 deficiency the activation of transcriptional regulations like NFκB, AP-1 and STATs is enhanced (green arrows). Upon stimulation with glucocorticoids, the GR inhibits NF κB and AP-1 activity, but may have a synergistic interaction with STATs.

Interestingly, virulence factors of *Leishmania* parasites have been proposed to block macrophage functions by activation of SHP1 (Abu-Dayyeh et al., 2008; Gomez et al., 2009). Similarly, SHP1 activation by lipoarabinomannan (ManLAM) has been suggested as a host evasion strategy of *Mycobacterium tuberculosis* (Knutson et al., 1998; Rajos et al., 2002). Our results show the important regulatory function of this phosphatase in the host innate immune response to bacterial pathogens, which therefore could indeed be an attractive target for bacteria to manipulate. In our study, deficiency of *ptpn6* favored growth of *S. typhimurium* and *M. marinum* despite an enhanced innate immune response to these pathogens. These results are in line with studies of zebrafish embryos defective in TNF signaling or eicosanoid biosynthesis, which indicated that the outcome of *M. marinum* infection is worsened either when the fish produce high levels of anti-inflammatory lipoxins inhibiting TNF production, or when the fish produce pro-inflammatory leukotrienes and excessive levels of TNF (Clay et al., 2008; Tobin et al., 2010; Tobin et al., 2012). We have shown that the role of *ptpn6* is crucial for tightly regulating the induction levels of many key players in the innate immune response, identified by our microarray analysis, and conclude that the loss of *ptpn6* function results in a non-functional immune response to *S. typhimurium* and *M. marinum* infections.

Acknowledgements

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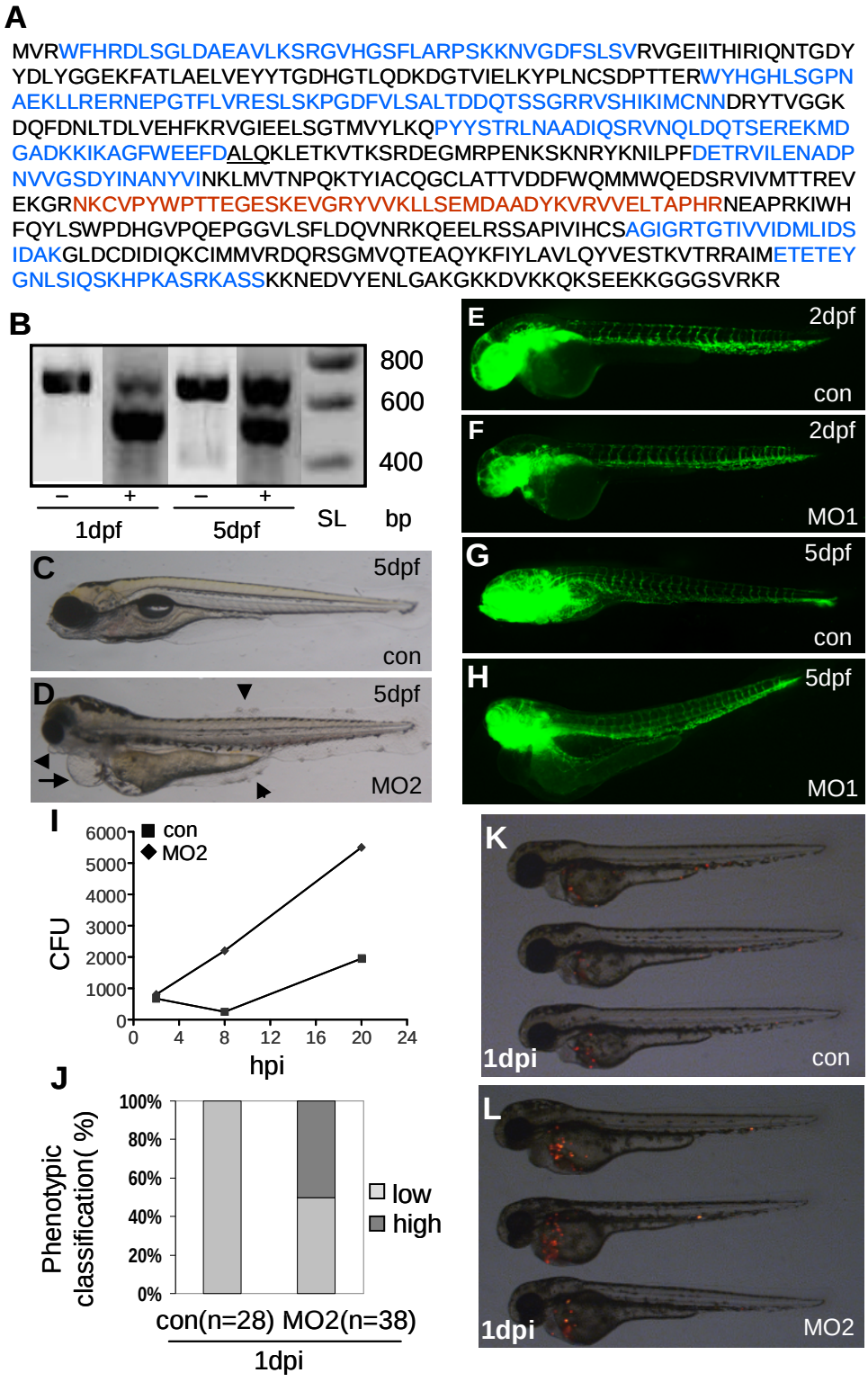
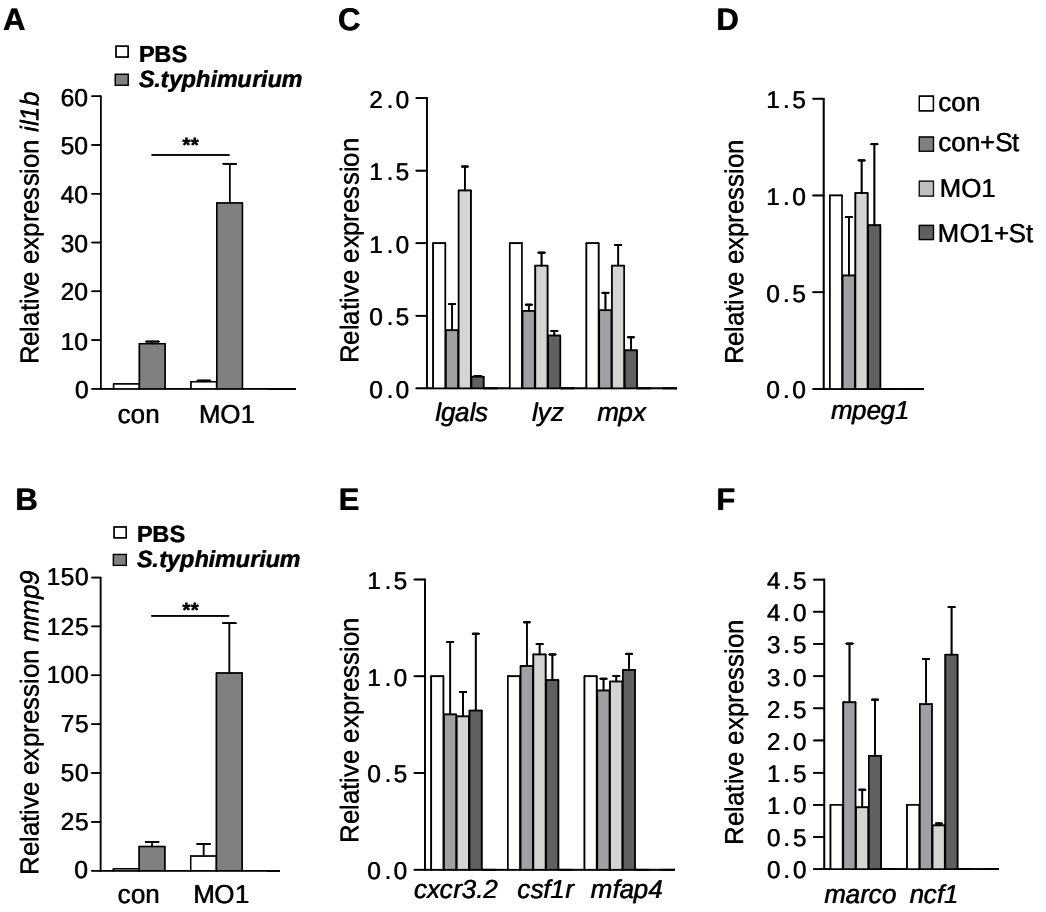
Supplementary data



Supplementary Figure S1. Splice blocking morpholino effect and phenocopy with translation blocking morpholino. (A) The Ptpn6 protein sequence (Reference sequence NP_956254) is encoded by 16 exons. Amino acid sequences derived from different exons are indicated with alternating black and blue colours. The amino acid sequence deleted by injection of the splice blocking morpholino (MO1) is indicated in red. This sequence comprises the phosphatase catalytic domain. We note that the *ptpn6* genomic sequence on chromosome 16 in Ensembl Zv9 is erroneously split over two genes, ENSDARG00000089043, comprising coding exons 1-7, and ENSDARG00000013916, comprising coding exons 8-16. (B) Efficiency of the splice blocking morpholino. Embryos were injected at the 1-2 cell stage with Danieau's buffer (-) or with 1 nl of 0.06 mM *ptpn6* MO1 in Danieau's buffer (+) and RNA was isolated at 1 and 5 dpf. RT-PCR with forward primer in coding exon 8 (ATATTCAGAGCAGAGTAAATCAG) and reverse primer in exon 13 (TTCGTCACCTTCGTTTCC) results in a 698 bp product for the intact mRNA and a 563 bp product when exon 12 is skipped due to MO1 knockdown. MO1 knockdown of the intact mRNA is near complete at 1 dpf and partial at 5 dpf. SL, size ladder. (C) Phenotype of 5 dpf larva injected with standard control morpholino (con). (D). Phenotype of 5 dpf larva injected with translation blocking morpholino (MO2). In different experiments 5-10% of MO2-injected larvae showed cardiac oedema (arrow) and skin lesions (arrowheads) similar as observed with MO1 (Fig.1). (E-H) Fli1:EGFP embryos were injected with standard control mo (con, E,G) or *ptpn6* MO1 (F,H), Representative fluorescence images (lateral view, anterior to the left) were taken at 2 dpf (E, F) and 6 dpf (G, H). (I-L) Phenocopy of the *S. typhimurium* infection phenotype with a translation blocking morpholino (MO2). (I). Infection with *S. typhimurium* wild type strain. Embryos were injected with standard control morpholino (con) or *ptpn6* MO2 and infected with *S. typhimurium* at 28 hpf. Groups of 5 embryos were crushed in PBS at 2, 8, and 20 hpi, and dilutions were plated for CFU counting on LB medium with carbenicillin selection of the DsRED marker plasmid in *S. typhimurium*. A representative example of three independent experiments is shown. (J-L). Infection with *S. typhimurium* LPS mutant Ra strain. Embryos were injected with standard control morpholino (con) or *ptpn6* MO2 and infected with *S. typhimurium* Ra at 28 hpf. The bacterial burden was analyzed at 1 dpi based on fluorescence of the DsRED marker plasmid. A quantification of phenotypes (J) and stereo fluorescence images (lateral view, anterior to the left) of 3 embryos per group (K, L) are shown for a representative example of three independent experiments. The bacterial burden in embryos at 1 dpi was scored as low (representative image in K) or high (representative image in L).



Supplementary Figure S2. qPCR analysis of *il1b*, *mmp9*, and leukocyte-specific genes in *ptpn6* morphants and control embryos infected with *S. typhimurium*. (A, B) Increased *ilb* (A) and *mmp9* (B) induction in infected *ptpn6* morphants compared to infected controls. (C-F) qPCR analysis of leukocyte-specific genes. qPCR analysis was performed on RNA samples from mock-injected (PBS) or *S. typhimurium*-infected (St) control embryos (con) and ptpn6 morphants (MO1). Relative expression levels are shown with the lowest expression level set at 1. Values are the means ± SEM of three independent sample sets, which were the same as previously used for microarray analysis. Asterisks in A and B indicate significant differences (**, P <0.01; **) tested by two-way ANOVA analysis with Bonferroni method as post-hoc test. Data for the leukocyte-specific genes were not significant by two-way ANOVA analysis and Bonferroni post-hoc test, but showed the same trend as the microarray data, i.e. stronger *S. typhimurium*-mediated down-regulation of *lgals9l1*, *lyz* and *mpx* show in *ptpn6* morphants than in controls (C), less pronounced *S. typhimurium*-mediated down-regulation of *mpeg1* in *ptpn6* morphants than in controls (D), unchanged expression of *cxcr3.2*, *csfr1*, and *mfap4* during *S. typhimurium* infection of *ptpn6* morphants and controls (E), and *S. typhimurium*-inducible expression of MARCO receptor homolog *LOC571584* and *ncf1* in both *ptpn6* morphants and controls (F).

Supplementary Table 1. microarray data of the *ptpn6* knockdown effect on the immune response to *Salmonella typhimurium* infection**.** Supplementary table can be found online at: https://www.dropbox.com/s/t3gyu0euewldyyv/Chapter2.suppl.table1.xlsx

Supplementary Table 2. Putative SHP1 binding sites in immune-related kinases**.** Supplementary table can be found online at: https://www.dropbox.com/s/5hgn84e9apiymgp/Chapter2.suppl.table2.xlsx

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Chapter 3

RNA-Seq profiling of leukocyte populations and mycobacterium-infected cells from wild type and *ptpn6*-deficient zebrafish larvae

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Abstract

The zebrafish is an excellent model for studying inflammatory and infectious diseases due to the optical transparency of its embryonic and larval stages and the availability of transgenic reporter lines that express fluorescent proteins in different leukocyte subtypes. However, the gene expression signatures of myeloid and lymphoid cell types in zebrafish and homologies with the human system are still largely unknown. Here we performed RNA-Seq profiling of eGFP-positive macrophages, neutrophils, and early T-cells from zebrafish larvae, which were obtained by fluorescence activated cell sorting (FACS) of transgenic *mpeg1:egfp*, *mpx:egfp*, and *lck:egfp* lines. Our RNA-Seq analysis resulted in comprehensive gene signatures for the different cell types and gave insight in genes that are more abundant or more specific than the currently used markers and thus highly useful for developing new reporter lines and lineage-specific antibodies. *Mycobacterium marinum*, which infects and persists in macrophages, markedly altered the gene expression signature of this cell type. *M. marinum*-infected cells displayed a general down-regulation of macrophage marker genes, along with up-regulation of ribosomal protein genes and genes involved in oxidative phosphorylation, proteolysis, ion transport, chromatin assembly, lipid metabolism, carbohydrate binding, and immunosuppression. The macrophage gene signature was also specifically altered under knockdown conditions of *ptpn6*, a protein tyrosine phosphatase gene known as a negative regulator of immune responses. In particular, deficiency of *ptpn6* resulted in a strong up-regulation of matrix metalloproteinase genes *mmp9* and *mmp13a* in uninfected macrophages as well as in *M. marinum*-infected cells. The up-regulation of these genes may be a major cause of the inflammatory phenotype and increased *M. marinum* susceptibility of *ptpn6*-deficient zebrafish larvae.

Introduction

The processes that occur during inflammation and immune responses to combat infection are orchestrated by complex changes in the gene expression profiles of different immune cell populations (Chaussabel et al., 2010). The analysis of cellular transcriptomes has been greatly advanced by recent developments in deep sequencing technology (Wang et al., 2009; Martin and Wang, 2011). RNA sequencing (RNA-Seq) is a powerful method to characterize transcriptional landscapes and discover novel transcripts or alternative splice forms (Cloonan et al., 2008; Mortazavi et al., 2008; Nagalaskhmi et al 2008; Sultan et al., 2008; Trapnell et al., 2010). RNA-Seq has also proved to be an accurate method for quantitative analysis of differential gene expression (Mortazavi et al., 2008). In this respect, RNA-Seq analysis has several advantages over microarray technology, including a higher sensitivity, a lower background, and independency of prior knowledge of the transcriptome (Marioni et al., 2008; Malone and Oliver, 2011). In RNA-Seq analysis, cDNA read lengths of over a hundred nucleotides can now routinely be obtained with the use of paired-end technology to link the ends of short cDNA fragments (Fullwood et al., 2009). These

reads are subsequently aligned to a reference database of exons or transcripts, and read count data is compared between samples using different statistically algorithms in R/Bioconductor packages such as DESeq, DEGseq, bayseq, and EdgeR (Anders and Huber, 2010; Hardcastle and Kelly, 2010; Robinson et al., 2010; Wang et al., 2010). The application of RNA-Seq technology has great potential to advance the understanding of the immune system during health and disease.

The immune system of vertebrates consists of two cooperative components, the innate and adaptive immune system (Janeway and Medzhitov, 2002). The innate immune response, which forms the first line of defence against infections, is initiated by phagocytic cells such as macrophages and neutrophils. In zebrafish embryos, these cells are the first to develop and consequently, the fish are solely dependent on their innate immune system during the early life stages (Herbomel et al., 1999; Le Guyader et al., 2008; Ellet and Lieschke, 2010). The first cells of the adaptive immune system, expressing early T-cell markers, can be detected by 4 days post fertilization (dpf), after the larval thymus is invaded by hematopoietic precursor cells (Kissa et al., 2008). However, the adaptive immune system is not considered to be functional until the fish reach several weeks of age (Lam et al., 2004). Therefore, zebrafish embryo and larval models are well suited for studies that specifically address the function of the innate immune defenses. Owing to the transparency of zebrafish at these early life stages, high-resolution imaging of the behavior of immune cells and interaction with pathogens is possible using fluorescent transgenic reporter lines (Hall et al., 2009). Furthermore, the combination of efficient forward and reverse genetics makes the zebrafish a very powerful model system (Lawson and Wolfe, 2011). Exploiting these unique advantages, many embryo/larval-based zebrafish models for human inflammatory and infectious diseases have recently been developed and are contributing to a better understanding of human disease mechanisms (Renshaw et al., 2007; Meeker and Trede, 2008; Meijer and Spaink, 2011; Oehlers et al., 2011; Renshaw and Trede, 2012).

In our previous work, transcriptome responses of zebrafish embryos during infection were analyzed at the whole organism level (Stockhammer et al., 2009; Stockhammer et al., 2010; van der Sar et al., 2009; Ordas et al., 2011; van Soest et al., 2011; Van der Vaart et al., 2012). In addition, we determined an expression profile of early myeloid precursor cells obtained from one-day-old embryos by fluorescence activated cell sorting (FACS) of a *spi1:egfp* transgenic line (Zakrzewska et al., 2010). Other recently developed fluorescent reporters can now be used for cell-specific analysis of different immune cell populations in zebrafish larvae. In this study, we used *mpeg1:egfp* (Ellett et al., 2011), *mpx:egfp* (Renshaw et al., 2006), and *lck:egfp* (Langenau et al., 2004) transgenic lines to determine the expression profiles of macrophages, neutrophils, and early T-cells, respectively. In addition, we used the mCherry marker present in a *Mycobacterium marinum* strain to isolate and profile infected cells from zebrafish larvae. Using an ultra-low input cDNA amplification method we could obtain libraries for Illumina RNA-Seq analysis from RNA quantities down to 100-200 pg. To detect transcripts that are induced under pathological inflammatory conditions, we also analyzed the profiles of macrophages, neutrophils, immature T-cells, and *M. marinum*-

infected cells under knockdown conditions of the *ptpn6* gene. This gene encodes a protein tyrosine phosphatase (Shp1) that is conserved between human and zebrafish and that functions as a negative regulator of the innate immune response (An et al., 2008; Croker et al., 2008; Chapter 2). The knockdown of *ptpn6* in zebrafish was previously shown to enhance the expression of pro-inflammatory genes and to result in a hyperactivation of the innate immune response during infection (Chapter 2). Our current data show a major effect of *ptpn6* knockdown on matrix metalloproteinase gene expression in uninfected macrophages as well as *M. marinum*-infected cells.

Materials and Methods

Zebrafish husbandry

Zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). Zebrafish lines used in this study included AB/TL, *Tg(mpx:egfp)i114* (Renshaw et al., 2006), *Tg*(*mpeg1:egfp)gl22* (Ellett et al., 2011) and Tg(*lck:egfp)cz2* (Langenau et al., 2004). Embryos were grown at 28.5–30°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).

Morpholino knockdown

Morpholino oligonucleotides (Gene Tools) were diluted to the desired concentration in 1× Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 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 *ptpn6* a splice morpholino targeting the exon 11/intron 11-12 splice junction (*ptpn6* MO1: 5'ACTCATTCCTTACCCGATGCGGAGC3'; 0.0625 mM) was used (Chapter 2).

Infection experiments

For infection experiments, a Mma20 strain of *Mycobacterium marinum* expressing mCherry in a pSMT3 vector was used (van der Sar et al., 2004). Glycerol stocks of the bacteria were prepared as described by Benard et al., (2012) and microinjected into the caudal vein of embryos at 28 hours post fertilization (hpf), using a dose of 100-150 CFU per embryo. After injections, embryos were transferred to fresh egg water and incubated at 28°C.

Fluorescent Activated Cell Sorting (FACS)

Macrophages, neutrophils and early lymphocytes from 5-6 dpf larvae were isolated by FACS as described in Cui et al. (2011). Briefly, live embryos were rinsed in calcium free Ringer for 15 min. Digestion was performed with 0.25% trypsin for 60-90 min at 28°C. Digestion was stopped with 1 mM $CaCl_2$ and 10% fetal calf serum. The resultant single cell suspension was centrifuged and washed with PBS and resuspended in Leibovitz

medium L15 supplemented with 1% fetal calf serum, 0.8 mM CaCl2, 50 units/ml penicillin and 0.05 mg/ml streptomycin. FACS was performed at 4°C using FACSAriaIII (BD Biosciences) with the BD FACSDiva software (version 6.1.3). For collecting mCherry-positive cells a Coherent Sapphire solid-state 561 nm yellow green laser with 36 mW power was used. Laser settings applied were 600LP, 615/20 BP. For sorting EGFP positive cells a Coherent Sapphire solid-state 488 nm laser with 15.4 mW power was used. Laser settings applied were 505 LP, 530/30 BP. The percentage of mCherry-positive cells in the life gate of cell suspensions from Mma20-infected embryos (from ±200 embryos) was between 0.1 to 1% over a time of ±25 min. The percentages of eGFP-positive cells in the life gate were: 0.09-0.2% over a time of ±20 min for lck:egfp cell suspensions (from ±150 embryos), 0.02-0.04% over a time of ±30 min for mpx:egfp cell suspensions (from ±250 embryos), and 0.04-0.1% over a time of ±25 min for mpeg1:egfp cell suspensions (from ±250 embryos). For negative controls in all cases a maximum of 500,000 non-fluorescent cells were obtained from the whole cell suspension. Fluorescent and non-fluorescent cell fractions were separately collected in supplemented L15 medium with addition of 10% zebrafish embryo extract (ZFIN) and RNA isolation was performed directly after sample collection.

RNA isolation and Illumina sequencing

RNA extraction of the cell fractions was done using the RNAqueous-Micro Kit (Ambion). RNA quality was checked by lab-on-a-chip analysis with an Agilent Bioanalyzer 2100 using the RNA 6000 Pico kit (Agilent, Santa Clara). RNA samples from non-fluorescent cell fractions had RIN values above 8. RNA integrity of samples from fluorescent cells was judged by the presence of ribosomal peaks in lab-on-a-chip analysis, but the quantity was generally too low for an accurate estimation of the RIN value and concentration. A total of 50 pg to 10 ng of RNA was used to make RNA-Seq libraries, using the Clontech SMARTer Ultra Low RNA Kit for Illumina Sequencing according to the manufacturer's instructions (Clontech Laboratories, Inc. Mountain View, CA, USA). After shearing the cDNA the Illumina TruSeq DNA Sample Preparation Kit v2 (Illumina Inc., San Diego, USA) was used to make the libraries. In the manufacturer's instructions three modifications were made. In the adapter ligation step the adapters were diluted 20-fold. 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 library amplification step was done with 15 cycles. 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).

Results and Discussion

RNA-Seq analysis of FACS-sorted macrophages from *mpeg1:egfp* transgenic zebrafish

To determine a gene expression signature for macrophages, we took advantage of a recently described zebrafish line that expresses enhanced GFP (eGFP) under control of the *mpeg1* gene promoter (Ellett et al., 2011). For isolation of eGFP-labeled macrophages we dissociated 5 or 6 day old larvae by trypsinization and subjected the resulting single cell suspensions to FACS sorting. The eGFP-labeled cells constituted between 0.04 to 0.1% of the total cell population and by sorting for 25 min we could obtain 4000-10,000 eGFP-labeled cells from pools of approximately 250 dissociated larvae. After testing different RNA isolation methods, we concluded that the RNAqueous-Micro Kit (Ambion) yielded good quality RNA from such small quantities of cells. The SMARTer Ultra Low RNA Kit for Illumina Sequencing was used to make RNA-Seq libraries. We succeeded in making RNA-Seq libraries even from samples with an estimated RNA input below 100 pg, where ribosomal peaks were just above the detection limit of lab-on-a-chip analysis with the RNA 6000 Pico kit. Approximately 10 million paired-end reads were obtained per library. To determine the reproducibility of the method we compared the results of RNA-Seq analysis of 4 biological replicates. For each replicate DESeq analysis identified several hundreds of genes with enriched expression in the eGFP-positive cell fraction versus the eGFP-negative fraction. Pairwise comparison of the replicates showed an overlap of around 50% of significant genes between each pair, and 29% of the significant genes were common to all 4 replicates (Supplementary Table 1). This demonstrates that consistent results can be obtained from RNA-Seq analysis of FACS-sorted eGFP-labeled macrophages, despite that there are many steps in the procedure where variation can be introduced (different pools of embryos, dissociation and FACS sorting, cDNA amplification from ultra low RNA start material). By combining the 4 replicates, which increases the statistical power of DESeq analysis (Anders and Huber, 2010), we identified a total of 925 genes with significantly enriched expression in macrophages (Supplementary Table 2). As expected, gene ontology analysis using DAVID (Huang et al., 2009) showed specific enrichment of KEGG pathways and GO-terms related to the immune system (Table 1).

Table 1. KEGG pathways and Gene Ontology terms enriched in macrophage and neutrophil cell fractions*

Category	Term	Fold Enrichment	
		mpeg1+	mpx+
KEGG pathways	cytokine-cytokine receptor interaction	4,4	4,6
	lysosome	4,1	
	Toll-like receptor signaling pathway	4,6	
	natural killer cell mediated cytotoxicity	4,6	
	intestinal immune network for IgA production	8,9	
	NOD-like receptor signaling pathway	5,4	7,8
	Jak-STAT signaling pathway	3,2	
	apoptosis	3,3	
	VEGF signaling pathway	3,3	
GO:Biological Process	immune response	5,4	
	defense response	7,2	8,0
	innate immune response	9,8	
	response to bacterium	7,4	16,6
	ntigen processing and presentation	5,2	
	chemotaxis	16,8	
GO:Molecular Function	cytokine binding	8,6	
	cysteine-type endopeptidase activity	5,2	
	peptidase activity, acting on L-amino acid peptides	2,2	2,9
	nucleotide receptor activity, G-protein coupled	9,5	
	purinergic nucleotide receptor activity, G-protein coupled	9,5	
	peptidase activity	2,1	2,8
	cysteine-type peptidase activity	3,6	
	fucosyltransferase activity	12,8	
	endopeptidase activity	2,4	3,9
	nucleotide receptor activity	8,7	
	purinergic nucleotide receptor activity	8,7	
	cytokine receptor activity	8,0	
	lipid binding	2,7	
	voltage-gated chloride channel activity	12,1	
	metalloendopeptidase activity		9,2
GO:Cellular Component	MHC class II protein complex	7,5	
	vacuole	6,2	

*Ensembl ID codes of the significantly enriched genes in eGFP-positive macrophages from *mpeg1:egfp* transgenic zebrafish (mpeg1+) or in eGFP-positive neutrophils from *mpx:egfp* transgenic zebrafish (mpx+) were analyzed using DAVID. The table shows the fold enrichment of KEGG pathways and GO terms for Biological Process, Molecular Function, and Cellular Component in the eGFP-positive cell fractions with a false discovery rate of less than 5%.

Comparison of macrophage, neutrophil, and early T-cell gene signatures

In addition to the *mpeg1:egfp* line for macrophages, we used *mpx:egfp* and *lck:egfp* transgenic lines for FACS sorting and RNA-Seq analysis of neutrophil and early T-cell populations. First, we looked at the expression levels of several marker genes that are commonly used to identify different leukocyte and lymphoid subpopulations in

zebrafish (Table 2). As expected, *mpeg1*, *mpx*, and *lck* RPKM values (read count per kilobase per million mapped reads) were highest in the eGFP-positive cell fractions of macrophages, neutrophils, and T-cells, respectively. Other macrophage markers, including *csf1r*, *cxcr3.2*, *irf8*, *marco*, *mfap4*, and *mhc2dab* were also enriched in *mpeg1:egfp*-positive cells. Among all macrophage markers, *mfap4* showed the highest expression level, and *mhc2dab* showed the highest specificity, with an over 60-fold higher RPKM value than in *mpx:egfp*-positive neutophils. In addition to *mpx*, neutrophils showed higher expression of *lyz*, *mmp9* and *mmp13a* than macrophages. The *lck:egfp*-positive cell fraction showed highly specific expression of T-cell markers, which apart from *lck* included *cd2*, *cd28*, *cd4*, *ikzf1* (*ikaros*), *rag1*, and *rag2*. However, expression of *cd8* was not detectable, suggesting that cytotoxic T-cell activity is not yet present at 5 dpf. Finally, the hematopoietic transcription factor gene *spi1* was specific for the myeloid cell fractions, while *coro1a*, *lcp1* (*L-plastin*), *ptprc* (*cd45*), and *ptpn6* were commonly expressed in myeloid and lymphoid cells.

More than 70% of all 283 genes that were significantly enriched in *mpx:egfp*-positive neutrophils compared to the eGFP-negative background were also significantly enriched in *mpeg1:egfp*-positive macrophages (Fig. 1). In contrast, only 11% of all 2578 genes enriched in *lck:egfp*-positive T-cells were also enriched in either one or both of the myeloid cell fractions (Fig. 1). Next, we searched for genes in the *mpeg1:egfp* gene set with higher expression level or better macrophage specificity than *mpeg1*, which had an RPKM of 126 in *mpeg:egfp*-positive cells and 12 in *mpx:egfp*-positive cells. Genes with higher abundance than *mpeg1* belonged to the families of chemokines, immunoglobulins, olfactomedins, granulins, cathepsins, fibrinogens (*mfap4* and 2 paralogues), lectins, transmebrane receptors, complement factors (C1q), MHC II class proteins, and also included some predicted non-coding RNAs (Table 3A). Among these, the most specific marker for macrophages was *granulin 2* (*grn2*), which was expressed at over 10-fold higher level than *mpeg1* and showed an approximately 300-fold higher RPKM in macrophages than in the neutrophil or T-cell fractions. Other highly specific macrophage markers were the immunoglobulin gene *dicp1*.1, the transmembrane receptor gene *ocstamp*, and MHC class II genes, like *cd74* and *mhc2dab*. In neutrophils, the lysozyme (*lyz*) and nephrosin (*npsn*) genes were expressed at approximately 28- and 4-fold higher level, respectively, than *mpx* (Table 3B). However, the specificity of *mpx* (17-fold higher RPKM than in macrophages) was better than that of *lyz* (12-fold higher) and npsn (8-fold higher). In addition, interleukin 34 (*il34* (1 of 2)) also showed high neutrophil specificity (21-fold higher RPKM than in macrophages), but its expression level was 4-fold lower than that of *mpx*. Markers for T-cells expressed at higher or similar level as *lck* were all highly specific for lymphoid cells and barely or not detectable in myeloid cells (Table 4). These markers included for example *rag1*, cytokine receptor genes (*ccr9b*, *il17r*), various other receptor genes (T-cell-specific immunoglobulins, a member of the signaling lymphocyte activation (SLAM)-family, *p2rx1*, *rorc*), and the transcription factor gene *foxp3a*. In conclusion, our RNA-Seq analysis identified several abundant and specific markers for macrophages, neutrophils,

and T-cells, that are good candidates for developing new transgenic lines and antibodies for distinguishing leukocyte lineages in the zebrafish model.

Table 2. Expression levels of commonly used lineage markers for different leukocyte cell types*

Lineage specificity	Gene symbol	Ensembl ID	mpeg1+	mpx+	lck+	GFP-
macrophage-enriched	*csf1ra*	ENSDARG00000007889	9	3	0	1
	cxcr3.2	ENSDARG00000041041	62	26	0	3
	irf8	ENSDARG00000056407	25	9	0	3
	marco	ENSDARG00000059294	4	0	0	0
	mfap4	ENSDARG00000090783	980	130	3	14
	mhc2dab	ENSDARG00000079105	192	3	0	1
	mpeg1	ENSDARG00000055290	126	12	0	5
neutrophil-enriched	*lyz*	ENSDARG00000057789	1461	18294	0	64
	mpx	ENSDARG00000019521	37	662	0	2
	mmp9	ENSDARG00000042816	357	1341	15	34
	mmp13a	ENSDARG00000012395	320	638	5	13
common myeloid	*spi1*	ENSDARG00000000767	340	253	1	7
lymphoid	*cd2*	ENSDARG00000091109	0	0	31	1
	cd28	ENSDARG00000095070	3	8	14	2
	cd4	ENSDARG00000070668	0	0	27	1
	ikzf1	ENSDARG00000013539	5	5	24	2
	lck	ENSDARG00000059282	1	0	237	3
	rag1	ENSDARG00000052122	0	0	409	11
	rag2	ENSDARG00000052121	0	0	48	2
common leukocyte	*coro1a*	ENSDARG00000054610	164	170	197	19
	lcp1	ENSDARG00000023188	63	92	14	10
	ptprc	ENSDARG00000071437	49	54	23	3
	ptpn6	ENSDARG00000089043/ ENSDARG00000013916	82	97	25	8

*RNA-Seq libraries were prepared from eGFP-positive (+) and negative (-) cell fractions obtained by dissociating and FACS-sorting 5-6 dpf larvae of transgenic lines expressing enhanced GFP under control of the mpeg1, mpx, or lck promoters. RPKM values (read counts per kilobase per million mapped reads) are averaged from 4 biological replicates of eGFP-positive cells from mpeg1:egfp larvae (mpeg1+, 2x 5dpf, 2x 6 dpf), 3 biological replicates from mpx:egfp larvae (mpx+, 5 dpf), and 2 biological replicates from lck:egfp larvae (lck+, 5 dpf). The RPKM value for eGFP-negative cells is the average of all libraries. Enriched expression in macrophages, neutrophils, or T-lymphoblasts is indicated in orange, green, and blue, respectively. RPKM values in the range of 10-50, 50-100, 100-200, 200-500, and over 500 are shown with increasingly darker shades of color. Genes classified as macrophage-enriched have at least 2-fold higher expression than in neutrophils, and genes classified as neutrophil-enriched have at least 2-fold higher expression than in macrophages.

Table 3. Myeloid-specific markers*

Gene symbol	Annotation group	Ensembl ID	mpeg1+	mpx+	lck+	GFP-
A. Macrophage markers						
si:ch211-149o7.4	chemokines (interleukin8-like)	ENSDARG00000079736	5852	245	316	91
si:ch211-149p10.2	immunoglobulin v-set family	ENSDARG00000079553	2633	422	95	86
si:dkey-25o1.6	chemokines (interleukin8-like)	ENSDARG00000093608	2002	60	7	22
CR384059.2	olfactomedin glycoprotein family	ENSDARG00000086947	1646	183	1	16
CR384059.1	olfactomedin glycoprotein family	ENSDARG00000074322	1552	166	0	15
grn2	growth factors	ENSDARG00000088641	1294	5	4	3
si:ch211-122l24.4	chemokines (interleukin8-like)	ENSDARG00000090873	1265	101	0	11
ctsl.1	cathepsin protease family	ENSDARG00000003902	1119	91	1	14
zgc:173915	fibrinogen family (paralogue of mfap4)	ENSDARG00000088745	1108	82	2	17
DKEY-119G10.5	long intervening non-coding RNA	ENSDARG00000087623	996	42	18	7
mfap4	fibrinogen family	ENSDARG00000090783	980	130	3	14
vmp1	endomembrane system	ENSDARG00000012450	642	52	18	18
havcr1	immunoglobulin v-set family receptor	ENSDARG00000040178	571	46	3	8
lgals2a	lectins	ENSDARG00000054942	535	82	3	14
lygl1	glycoside hydrolases	ENSDARG00000056874	484	82	7	9
DKEY-119G10.4	long intervening non-coding RNA	ENSDARG00000095801	438	70	0	3
si:dkey-5n18.1	C1q complement family	ENSDARG00000043436	435	34	3	4
DKEY-6N3.3	long intervening non-coding RNA	ENSDARG00000095820	427	24	0	2
si:ch1073-403i13.1	MHC class II	ENSDARG00000001832	422	29	4	6
cd74	MHC class II	ENSDARG00000036628	346	6	20	5
cd74a	MHC class II	ENSDARG00000009087	277	12	8	3
ocstamp	transmembrane receptor	ENSDARG00000022139	237	3	0	2
mhc2dab	MHC class II	ENSDARG00000079105	192	3	0	1
zgc:194314	lipid metabolism	ENSDARG00000078859	189	24	3	5
si:dkey-15g19.2	non-coding RNA	ENSDARG00000095693	169	4	2	6
si:zfos-2330d3.3	fibrinogen family (paralogue of mfap4)	ENSDARG00000089667	161	7	4	2
rnaset2l	ribonuclease T2 family	ENSDARG00000058413	145	6	0	2
BX649485.1	immunoglobulin v-set family	ENSDARG00000089473	145	26	6	1
CABZ01074899.1	gamma-glutamyl hydrolase	ENSDARG00000025237	142	25	0	3

grn1	growth factors	ENSDARG00000089362	140	16	3	2
mpeg1	membrane attack complex	ENSDARG00000055290	126	12	0	5
sftpb (2 of 2)	surfactant proteins	ENSDARG00000067566	118	14	6	5
dicp1.1	immunoglobulin family	ENSDARG00000091993	109	1	3	4
ctssb.2	cathepsin protease family	ENSDARG00000013771	109	14	0	2
si:dkeyp-2h4.2	MHC class II	ENSDARG00000031745	103	3	2	3
B. Neutrophil markers						
lyz	glycoside hydrolases	ENSDARG00000057789	1461	18294	0	64
npsn	metalloendopeptidases	ENSDARG00000010423	266	2347	0	7
mpx	peroxidases	ENSDARG00000019521	37	662	0	2
si:dkey-238m4.4	unknown	ENSDARG00000093248	66	454	0	1
sult2st1	sulfate transferases	ENSDARG00000086446	31	375	4	7
alox5ap	leukotriene biosynthesis	ENSDARG00000054755	49	294	0	3
CU682604.2	lamin B receptor family	ENSDARG00000075664	15	224	0	3
il6r	cytokine receptors	ENSDARG00000070398	21	188	0	3
il34 (1 of 2)	cytokines	ENSDARG00000091003	8	171	1	3
il34 (2 of 2)	cytokines	ENSDARG00000069128	21	152	10	7
sult2st1	sulfate transferases	ENSDARG00000033170	15	118	2	5

*Macrophage-specific genes (A) were selected based on significantly enriched expression in mpeg1:egfp-positive cells (mpeg1+, log2 fold change > 2, adjusted P-value <0.1), a minimum RPKM value of 100, and >5-fold higher RPKM than in mpx:egfp-positive cells (mpx+), >10-fold higher RPKM than in lck:egfp-positive cells (lck+), and >20-fold higher RPKM than in eGFP-negative cells. Neutrophil-specific genes (B) were selected based on significantly enriched expression in mpx:egfp-positive cells (log2 fold change > 2, adjusted P-value <0.1), a minimum RPKM value of 100, and >5-fold higher RPKM than in mpeg1:egfp-positive cells, >10-fold higher RPKM than in lck:egfp-positive cells, and >20-fold higher RPKM than in gfp-negative cells. RNA-Seq library preparation, averaging of RPKM values from biological replicates, and the use of increasingly darker shades of different colors to indicate higher expression levels were as in Table 2. The full lists of significantly enriched genes in mpeg1:egfp-positive and mpx:egfp-positive cells with fold change and P-value are given in Supplementary Table 2.

Table 4. Lymphoid-specific markers*

Gene symbol	Annotation group	Ensembl ID	mpeg1+	mpx+	lck+	GFP-
CT867973.1	T-cell receptor immunoglobulin family	ENSDARG00000075807	1	0	1718	19
ccr9b	cytokine receptors	ENSDARG00000068310	0	0	664	5
CT583625.4	immunoglobulin V-set domain	ENSDARG00000094770	3	2	650	4
rag1	recombination	ENSDARG00000052122	0	0	409	11
zgc:171686	signaling lymphocyte activation molecule (SLAM)-family receptors	ENSDARG00000076721	1	2	406	6
p2rx1	ion-gated receptor family	ENSDARG00000016695	3	1	276	8
lck	tyrosine kinase family	ENSDARG00000059282	1	0	237	3
ms4a17c.1	membrane proteins	ENSDARG00000094643	1	0	196	3
BX323450.2	sphingolipid activator protein (saposin) family	ENSDARG00000058673	1	1	192	3
rorc	nuclear receptor superfamily	ENSDARG00000087195	0	0	155	4
plp2 (2 of 2)	endomembrane system	ENSDARG00000042972	1	0	140	5
foxp3a	transcription factors	ENSDARG00000055750	0	0	138	1
si:ch211-132g1.1	non-coding RNA	ENSDARG00000040250	6	0	132	2
sh2d1a (1 of 2)	SH2-domain family	ENSDARG00000074854	2	2	123	2
rhoh	ras family	ENSDARG00000070121	0	2	116	1
il7r	cytokine receptors	ENSDARG00000078970	5	3	103	3

*Genes specific for early T-lymphocytes in zebrafish larvae were selected based on significantly enriched expression in lck:egfp-positive cells (lck+, log2 fold change > 2, adjusted P-value <0.1), a minimum RPKM value of 100, and >10-fold higher RPKM than in mpeg1:egfp-positive cells (mpeg1+), >10-fold higher RPKM than in mpx:egfp-positive cells (mpx+), and >20-fold higher expression than in gfp-negative cells. RNA-Seq library preparation, averaging of RPKM values from biological replicates, and the use of increasingly darker shades of different colors to indicate higher expression levels were as in Table 2. The full list of significantly enriched genes in lck:egfp-positive cells with fold change and P-value is given in Supplementary Table 2.

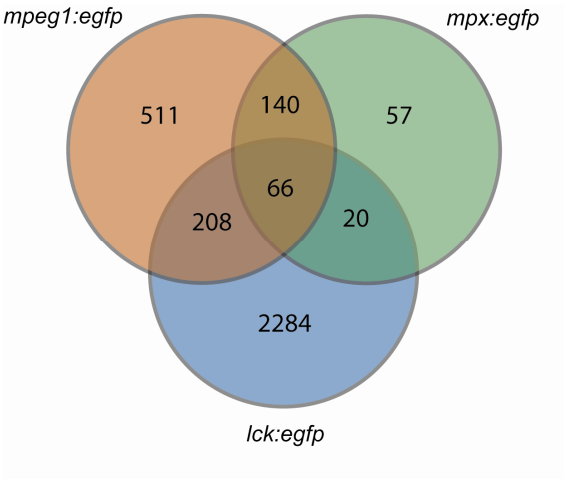


Figure 1. Overlap between genes enriched in GFP-positive cell fractions from mpeg1:egfp, mpx:egfp, and lck:egfp larvae. RNA-Seq libraries were prepared from GFP-positive (+) and negative (-) cell fractions obtained by dissociating and FACS-sorting 5-6 dpf larvae of transgenic lines expressing enhanced GFP under control of the mpeg1, mpx, or lck promoters. DESeq analysis was used to compare the read count data of the GFP-positive versus the GFP-negative cell fractions with 4 biological replicates of mpeg1:egfp larvae (2x 5dpf, 2x 6 dpf), 3 biological replicates of mpx:egfp larvae (5 dpf), and 2 biological replicates of lck:egfp larvae (5 dpf). The Venn-diagram shows the overlap between genes enriched in the GFP-postive cell fractions of the different transgenic lines (log2 fold change >2 and adjusted P-value <0.1).

Expression profile of *M. marinum*-infected cells

The mycobacterium genus comprises several intracellular pathogens that reside in macrophages, including the human pathogen *Mycobacterium tuberculosis* and the fish pathogen *M. marinum*. The hallmark of infectious diseases caused by *M. tuberculosis* or *M. marinum* is that infected macrophages are manipulated to attract uninfected macrophages and other immune cells to form tissue aggregates, known as tuberculous granulomas. These granulomas form a niche for the chronic persistence of mycobacteria inside their host. Zebrafish embryos are a well-characterized model to study the early stages of *M. marinum*-macrophage interaction and granuloma formation (Davis et al., 2002, Ramakrishnan, 2012). To investigate the effect of *M. marinum* on gene expression in macrophages, we infected 1-day-old embryos with mCherry-labeled *M. marinum* Mma20 bacteria and isolated the infected cells by FACS sorting at 4 days post infection (dpi) when early stage granulomas are formed. We compared the RNA-Seq profile of the Mma20-infected cells to the eGFP-negative background from uninfected *mpeg1:egfp* larvae, and investigated the overlap between Mma20-positive cells and eGFP-positive macrophages from uninfected *mpeg1:egfp* larvae (Fig. 2). This comparison showed that out of 188 genes enriched in Mma20-infected cells (Supplementary Table 3), only 40 were overlapping with the gene set of uninfected macrophages. The overlapping genes included some of the most specific macrophage

markers that we described above, such as *grn2* and the MHC class II gene *cd74*. Expression of *mfap4* and two *mfap4* paralogues was also detected, however, *mpeg1* was notably absent. Furthermore, the majority of the 40 macrophage-specific genes were expressed at over 10-fold lower levels in Mma20-infected cells compared with uninfected macrophages. One possible explanation could be that only a small portion of the Mma20-infected cell population consists of macrophages. However, this is very unlikely, because it has been well described that *M. marinum* is rapidly phagocytosed by macrophages after injection of bacteria into the blood circulation, and that the infection spreads to newly attracted macrophages, which phagocytose infected macrophages undergoing apoptosis (Davis and Ramakrishnan., 2009). Furthermore, a preliminary RNA-Seq analysis of red/green double fluorescent *mpeg1:egfp*-cells infected with mCherry-labelled Mma20 also shows strong down-regulation of macrophage markers (data not shown). Therefore, the down-regulation of macrophage markers is more likely due to *M. marinum*-induced transformation of infected macrophages . Indeed, it is well known that infected macrophages in tuberculous granulomas are manipulated to adopt epithelial and multinucleate morphologies (Russell, 2011) and such morphological changes have also been observed in the granulomas of zebrafish larvae (Davis et al., 2002).

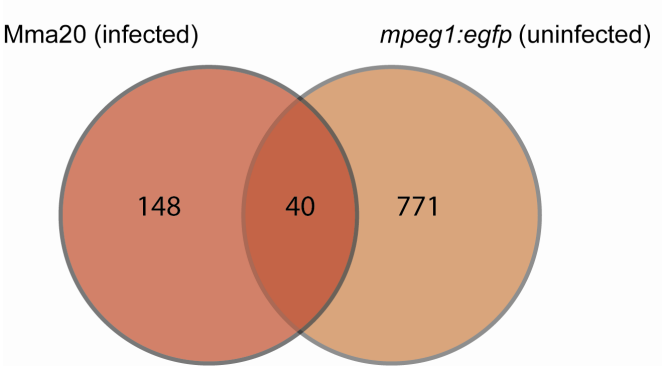


Figure 2. Overlap between genes enriched in M. marinum-infected cells and genes enriched in macrophages. Embryos were infected by intravenous injection of mCherry-labeled *M. marinum* Mma20 bacteria at 1 dpf, and at 4 dpi (5dpf) RNA-Seq libraries were prepared from mCherry-positive and negative cell fractions obtained by FACS-sorting. Read counts of the mCherry-positive fraction from two biological replicates of infected embryos were compared by DESeq analysis against the GFP-negative cell fractions of two biological replicates of uninfected *mpeg1:egfp* larvae (5 dpf). The Venn-diagram shows the overlap of genes enriched in Mma20-infected cells with genes enriched in *mpeg1:efgp*-positive macrophages from two biological replicates of uninfected larvae (log2 fold change >2 and adjusted Pvalue <0.1.

The genes enriched in Mma20-infected cells that did not overlap with macrophage markers (148 out of 188 genes) were analyzed using DAVID, which identified five enriched clusters of GO-terms: ribosome, oxidative phosphorylation, proteolysis, ion transport, and chromatin assembly (Supplementary Table 4). This enrichment may reflect activation of protein translation and defense mechanisms in the

infected cells. For example, the ion transport cluster contained the antimicrobial hepcidin gene (*hamp1*), and it has recently emerged that ribosomal protein genes, such as *rps3* which was up-regulated, have extra-ribosomal regulatory functions in the innate immune system (Gao and Hardwidge, 2011). It is an interesting possibility, warranting further investigation, that the increased expression of genes involved in chromatin assembly might be associated with the mycobacterium-induced morphological changes in macrophages or suppression of the macrophage immune defenses. By manual inspection of the gene list we defined three additional annotation clusters. The first consisted of genes involved in lipid metabolism and transport. An elevation of host lipid metabolism has also been observed in microarray analysis of caseating human tuberculous granulomas (Kim et al., 2010). The second cluster was a group of lectin genes, including members of the galactoside-binding lectin family, which in human macrophages have been shown to accumulate around mycobacterium-containing phagosomes (Beatty et al., 2002), and a member of the mannose-binding lectins, associated with human tuberculosis susceptibility (Denholm et al., 2010; Singla et al., 2012). Finally, the third cluster consisted of a group of three genes for immunosuppressive proteins, including two FK506-binding proteins and a vasointestinal peptide, which has been implicated in switching off activated macrophages during *M. tuberculosis* infection (Ma et al., 2003; Souza-Moreira et al., 2011). In conclusion, our analysis of *M. marinum*-infected cells showed a general down-regulation of macrophage marker genes accompanied by the induction of several specific gene groups that may be linked to processes of host defense and to the immunosuppressive effects of mycobacteria on host cells.

Ptpn6 knockdown effect on expression profiles of leukocyte populations and *M. marinum*-infected cells

Ptpn6, also known as Shp-1, is a hematopoietic SH2-domain containing non-receptor type protein tyrosine phosphatase and a well-known negative regulator of innate and adaptive immune responses in human and mice (Blanchette et al., 2009; Kumagai et al., 2012; Henshall et al., 2001; Oh et al., 2009). We have previously shown that morpholino knockdown of *ptpn6* in zebrafish leads to increased susceptibility to *M. marinum* infection (Chapter 2). In addition, uninfected *ptpn6* morphants developed an inflammation-associated phenotype at the late larval stage (Chapter 2). RNA-Seq analysis demonstrated *ptpn6* expression in both myeloid and lymphoid cells (Table 2), in agreement with previous in situ hybridization results (Zakrzewska et al., 2010). To gain a better understanding of the underlying causes of the *ptpn6* morpholino knockdown effects, we performed RNA-Seq analysis on FACS-sorted leukocyte populations from *ptpn6* morphants and controls at 5 dpf, including *M. marinum*-infected cells, macrophages, neutrophils and T-cells. In addition, we analyzed the eGFP-negative background from uninfected *mpeg1:egfp* larvae. The results of all comparisons are summarized in Table 5 and details of expression levels are given in Supplementary Table 5.

In agreement with previous qPCR analysis (Chapter 2), RNA-Seq analysis revealed increased *mmp9* expression in *ptpn6* morphants. Notably, in Mma20-infected cells from *ptpn6* morphants not only *mmp9*, but also *mmp2*, *mmp13a* and the *mmp* (matrix metalloproteinase) inhibitor gene *timp2b* were increased compared with infected cells of control embryos. The *mmp9* and *mmp13a* genes were also specifically up-regulated in macrophages from *ptpn6* morphants, while *mmp2* was also up-regulated in neutrophils and in the eGFP-negative background of *ptpn6* morphants. There was no general up-regulation of pro-inflammatory genes in any of the *ptpn6* morphant cell types. Therefore, the effect of *ptpn6* knockdown on the immune response of zebrafish embryos towards *M. marinum* infection is markedly different from the effect of *ptpn6* knockdown during *S. typhimurium* infection, where the up-regulation of *mmp9* and *mmp13a* genes goes together with a general up-regulation of pro-inflammatory cytokines and immune-related transcription factors (Chapter 2).

In addition to the up-regulation of *mmp* pathway genes, Mma20-infected cells of *ptpn6* morphants showed strongly increased expression of *serum amyloid A* (*saa*). This apolipoprotein family gene encodes a major acute phase protein that is known to be highly expressed in response to inflammation and tissue injury. Interestingly, several reports have suggested a link between expression of *saa* and *mmp* genes, which may also underlie connection between chronic inflammation and cancer (Malle et al., 2009). Transcription of *MMP9* was found to be induced by SAA in human monocytic cells via the formyl methionine receptor (Lee et al., 2005). Furthermore, an SAA-activating factor (SAF-1) was shown to function cooperatively with the AP-1 transcription factor complex to activate *MMP9* transcription (Ray et al., 2005). SAA has also been linked to granulomatous inflammation (Chen et al., 2010). Increased expression of *mmp9* has been shown to be essential for granuloma formation in zebrafish embryos (Volkman et al., 2010). In this study, *mmp9* secretion by epithelial cells nearby granulomas was proposed to drive macrophage recruitment. Our RNA-Seq data do not exclude *mmp9* expression by epithelial cells, but do show that the major induction of *mmp9* occurs directly in Mma20-infected cells and is enhanced by *ptpn6* knockdown together with a strong induction of *mmp13a* in these cells. As granuloma formation is accelerated in *ptpn6* morphants, we propose that Saa-dependent induction of *mmp9* and *mmp13a* in Mma20-infected cells plays a major role in the formation and expansion of granulomas (Fig. 3).

Another gene that showed increased up-regulated in Mma20-infected cells under *ptpn6* knockdown conditions was *hamp1*, and a *ptpn6*-dependent increase of *hamp1* expression was also observed in *lck:egfp*-positive T-cells and in eGFP-negative background cells. Hepcidin, the product of *hamp1*, regulates iron homeostasis by mediating the degradation of the iron export protein ferroportin 1, and is known to inhibit growth of *M. tuberculosis* (Sow et al., 2007, Sow et al., 2011). Three other genes connected with responses to nutrients also showed increased up-regulation in Mma20-infected cells of *ptpn6* morphants. These included the genes for the peptide hormones leptin b (*lepb*) and glucagon a (*gcga*) and the *steap4* gene, which encodes a plasma membrane metalloreductase involved in iron and copper transport. Leptin b has been

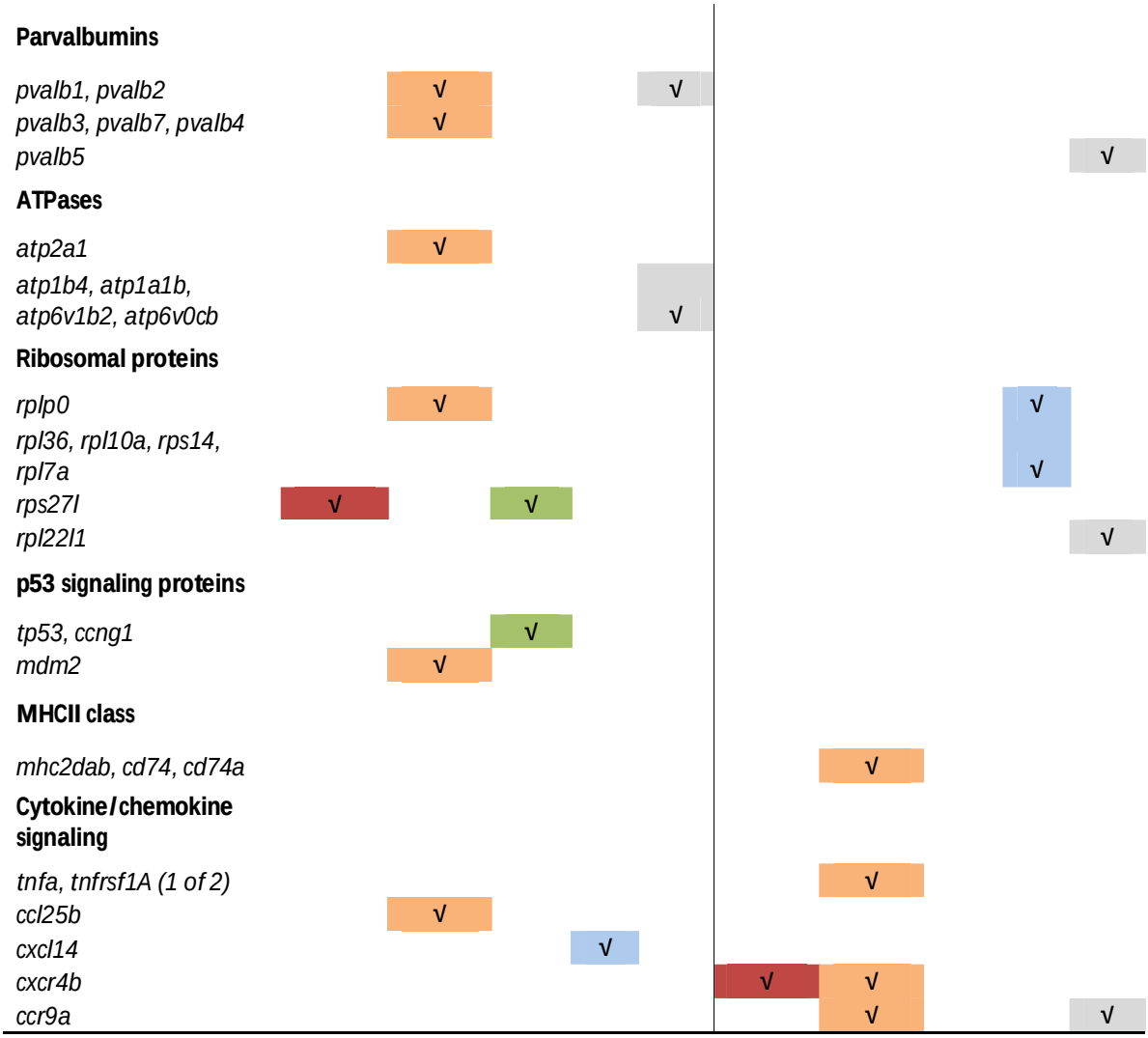
suggested to play a role in the early immune response to pulmonary tuberculosis, and mice deficient in leptin b or in the leptin receptor displayed disorganized granulomas (Wieland et al., 2005, Lemos et al., 2011). The *steap* 4 gene is homologous to the human tumor necrosis factor alpha-induced protein 9 gene (*TNFAIP9*, also known as *STEAP4* or *STAMP2*), which has been proposed to play an important role in integrating inflammatory and metabolic responses and to act as an anti-inflammatory protein in macrophages (Wellen et al., 2007; Ten Freyhaus et al., 2012). We have previously shown that that an alternative splice variant of this gene is induced by *M. marinum* infection in adult zebrafish (Hegedus et al., 2009). Any possible antimicrobial effects of *hamp1*, *lepb*, *gcga* or *steap4* were not sufficient to prevent increased *M. marinum* Mma20 infection in *ptpn6* morphants.

Knockdown of *ptpn6* also led to down-regulation of genes in Mma20-infected cells in comparison with infected cells of control embryos, for example, a chemokine gene (*si:ch211-149o7.4*) and the chemokine receptor gene *cxcr4b*. Both these genes are highly expressed in macrophages and other immune cell types of zebrafish larvae (Table 3, Supplementary Table 2). As we have shown that *M. marinum* infection lead to a general down-regulation of macrophage markers, the down-regulation of *si:ch211-149o7.4* and *cxcr4b* might reflect the advanced stage of infection in *ptpn6* morphants. Both genes were also down-regulated in macrophages of uninfected *ptpn6* morphants, along with other macrophage-expressed genes, such as *tnfa*, *tnfrsf1a*, *mbp*, *grn2*, *mhc2dab*, *cd74* and *cd74a*. In contrast, *ptpn6* knockdown in uninfected macrophages led to up-regulation of among others actin, myosin and parvalbumin genes, several immune-related proteases (*cpa5*, *npsn*, *cpn1* in addition to the *mmp9* and *mmp13a* genes discussed above), and of typical neutrophil markers like *lyz* and *mpx*. These data suggest a complex role of *ptpn6* in regulating the activation status of macrophages.

Under uninfected conditions, the *ptpn6* knockdown effects on different cell populations were very specific. The up-regulation of *mmp* and other protease genes was observed in *ptpn6*-deficient macrophages but not in *ptpn6*-deficient neutrophils or T-cells. An up-regulation of genes involved in p53 signaling (*tp53*, *mdm2*, *ccng1*) was only observed in *ptpn6*-deficient neutrophils. Furthermore, neutrophils showed specific down-regulation of apolipoprotein and protease genes under *ptpn6* knockdown conditions, while down-regulation of ribosomal protein genes was specific for T-cells. There was no effect on p53 signaling genes in the eGFP-negative background cells of *ptpn6* morphants and no indication of a general toxicity effect in the RNA-Seq data. Therefore, enhanced *mmp* genes secretion by macrophages might be a major cause of the inflammation-associated phenotype that develops at the late larval stage in *ptpn6* morphants.

Table 5. Effect of *ptpn6* knockdown on gene expression in M. marinum-infected cells and different leukocyte populations*

	ptpn6 MO1 up-regulated					ptpn6 MO1 down-regulated				
	Mma20 +	mpeg1 +	mpx +	lck +	GFP -	Mma20 +	mpeg1 +	mpx +	lck +	GFP -
Total no. of genes	22	58	23	39	88	38	40	25	12	37
Matrix metalloproteinases/ inhibitors										
mmp2	√		√		√					
mmp9, mmp13a	√	√								
timp2b	√									
Other proteases										
try					√			√		
cpa5		√			√					
npsn, cpn1		√								
cela1, cpb1, ela2, cpa4								√		
adamts1					√					
Iron transport										
hamp1	√			√	√					
steap4	√				√					
tfa						√				√
hbm						√				
Peptide hormones										
lepb	√			√						
gcga	√									
prl, adm					√					
lepa				√						
Acute phase proteins										
saa	√									
apcs						√				
Apolipoproteins					√					
apom, apoc2						√				
apoc1l						√		√		
apoa2								√	√	√
apod (3 of 3)					√			√		
Actin/myosin/tropomyosin										
tnnc2, myl3		√								
mylz2		√		√	√					
mylz3, tnni2a.4		√			√					
acta2	√									
acta1a, acta1b, actc1b, myhz1.1, myl10, tnnc1b, tnni2b.2, tnnt3b		√								



*Embryos injected with a morpholino against ptpn6 (ptpn6 MO1) or control embryos were infected with mCherry-labelled M. marinum Mma20 bacteria at 1 dpf and RNA-Seq libraries were prepared from mCherry-positive (+) and negative (-) cell fractions at 4 dpi (5 dpf) as in Supplementary Table 4. Likewise, ptpn6 MO1 was injected in embryos from mpeg1:egfp, mpx:egfp, and lck:egfp transgenic embryos to compare macrophage (mpeg1+), neutrophil (mpx+), and T-lymphoblast (lck+) expression levels at 5 dpf with those from control larvae. The eGFP-negative cell fractions from mpeg1:egfp larvae were used to determine the effect of ptpn6 MO1 on gene expression levels in the background without macrophages. Two biological replicates were used for all comparisons. Genes are ordered by annotation groups to show up- or down-regulation by ptpn6 MO1 in the different cell fractions. Significance cut-offs in DESeq analysis were log2 fold change >2 and P <0.1. As an additional criterion, genes up-regulated by ptpn6 MO1 showed a minimum RPKM of 30 in both replicates of the respective ptpn6 MO1 groups of each FACS-sorting, and genes down-regulated by ptpn6 MO1 showed a minimum RPKM of 30 in both replicates of the respective control groups. The full list of up- or down-regulated genes with fold change, P-value, and RPKM values of the biological replicates in given in Supplementary table 5.

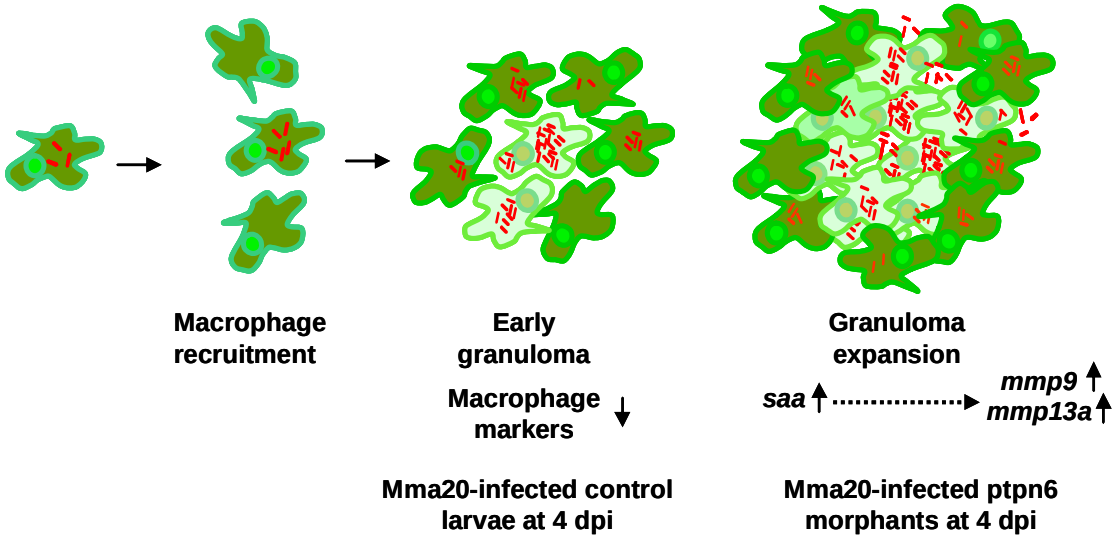


Figure 3. Model for granuloma expansion in wild type zebrafish larvae and ptpn6 morphants. Intravenously injected *M. marinum* bacteria are rapidly phagocytosed by embryonic macrophages in the blood circulation of 1 day-old embryos. Infected macrophages migrate into tissue and attract other uninfected macrophages to form granuloma-like aggregates (Davis et al., 2002). These early stages of granulomas are exploited by mycobacteria for spreading to other macrophages that facilitate tissue dissemination of the infection (Clay et al., 2007, Davis et al., 2009) and granuloma formation has been shown to require the function of *mmp9* (Volkman et al., 2010). In this study we show that the expression of macrophage marker genes is down-regulated upon proliferation of *M. marinum* inside these cells (indicated by a lighter color green of macrophages in the figure). As the infection progresses, infected cells up-regulate expression of serum amyloid A (saa) and matrix metalloproteinase (*mmp*) genes. Up-regulation of *mmp9* and *mmp13a* is proposed to be regulated by *saa*. Under conditions of *ptpn6* morpholino knockdown an increased bacterial burden and size of granuloma-like aggregates is observed. In the present study, *ptpn6* morphants showed increased expression of *saa*, *mmp9*, and *mmp13a* at 4 days post infection compared to control infected larvae, which had smaller granulomas that were not yet at the advanced stage of development where these genes are up-regulated.

Conclusions

By means of RNA-Seq analysis of FACS-sorted leukocyte and lymphoid subpopulations from fluorescent reporter lines we could determine gene expression signatures of macrophages, neutrophils and early T-cells in zebrafish larvae. Commonly used markers, including *mpeg1* for macrophages, *mpx* for neutrophils, and *lck* for T-cells, faithfully distinguished the different leukocyte subtypes in the RNA-Seq data. In addition, our RNA-Seq analysis identified other more abundant or more specific markers for macrophages, neutrophils, and T-cells, which will be highly useful for developing new reporter lines and lineage-specific antibodies for zebrafish. For example, the granulin gene *grn2* was highly abundant and almost exclusively detectable in macrophages, whereas *mpeg1* expression showed some overlap with neutrophils and was expressed at 10-fold lower level than *grn2*. MHC class II genes were also highly

specific for macrophages. The macrophage gene signature was markedly changed by *M. marinum* infection. This infection led to a general down-regulation of macrophage marker genes accompanied by an induction of ribosomal protein genes and genes involved in oxidative phosphorylation, proteolysis, transport of iron and other ions, chromatin assembly, lipid metabolism, carbohydrate binding, and immunosuppression. Knockdown of the protein tyrosine phosphatase gene *ptpn6*, which is known as a negative regulator of immune responses, also had specific effects on the macrophage expression profile of zebrafish larvae. Notably, expression of matrix metalloproteinase genes *mmp9* and *mmp13a* was specifically up-regulated in *ptpn6*-deficient macrophages. Likewise, these matrix metalloproteinase genes were also strongly up-regulated in *M. marinum*-infected cells of *ptpn6* morphants, along with the expression of the serum amyloid A gene (*saa*), which has been implicated in regulating matrix metalloproteinase gene expression. The induction of these genes at high levels in *M. marinum*-infected cells is not beneficial to the host, as the bacterial burden is strongly enhanced in *ptpn6* morphants. Increased expression of these genes in infected *ptpn6*-deficient macrophages may accelerate the formation of granulomas, which are exploited by mycobacteria for their expansion and tissue dissemination (Fig. 3).

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Supplementary data

Supplementary Table 1. Overlap in significant genes in biological replicates of FACS-sorted macrophages from mpeg1:egfp transgenic embryos*

replicates	#2	#3	#4
#1	51%	42%	68%
#2		47%	62%
#3			56%

*Four independent pools of approximately 250 5-day-old (#1, #2) or 6-day-old (#3, #4) larvae from *mpeg1:egfp* transgenic zebrafish were subjected to FACS-sorting on different days. For each replicate the eGFP-positive cell fraction was compared against the eGFP-negative background by DESeq analysis to determine the number of genes showing significant enrichment in eGFP-positive macrophages. The overlap in significant genes between replicates is shown as the percentage of the total number of genes in the smaller group of the replicate pair.

Supplementary Table 2. Genes enriched in eGFP-positive cell fractions from mpeg1:egfp, mpx:egfp, and lck:egfp larvae. Supplementary table can be found online at: https://www.dropbox.com/s/g06wjz5zqm324xn/Chapter3suppl.table2.xlsx

Supplementary Table 3. Overview of 148 genes enriched in *M. marinum*-infected cells. Supplementary table can be found online at: https://www.dropbox.com/s/zp95a6c8v2mes9w/Chapter3suppl.table3.xlsx

Supplementary Table 4. Genes enriched in *M. marinum*-infected cells*

Annotation group	Description	Genes in group
macrophage	actin, myosin, troponin	*acta1b, myl1, mylpf (2 of 2), mylz3, tnni2a.4, tpma*
	cathepsin L	*ctsl.1*
	colony stimulating factor 3	*csf3*
	granulins	*grn1, grn2*
	lectins	*hbl4, lgals1l1*
	leukocyte cell-derived chemotaxin 2 like	*lect2l*
	lysozyme g-like	*lygl1*
	mediator complex subunit	*med11(2 of 2)*
	MHC class II	*cd74*
	microfibrillar-associated proteins	*mfap4, zgc:173915, si:zfos-2330d3.3*
	nephrosin	*npsn*
	parvalbumins	*pvalb2, pvalb4*
	riboflavin kinase	*rfk*
	S100 calcium binding protein A11	*s100a11*
	transducin beta-like	*tbl2(1 of 2)*
	ubiquitin-like with PHD and ring finger domains 1	*uhrf1(1 of 2)*
translation	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed a	*faua*
	histones	*h3f3c, hist2h4b (7 of 7)*
	ribosomal proteins	*mrpl11, rpl13, rpl24, rpl28, rpl37, rps10, rps14, RPS17, rps19, rps21, rps26l, rps3*
	translation initiation factors	*eif2b2, eif3k*
	ubiquitin A-52 residue ribosomal protein fusion product 1 [Source:ZFIN;Acc:ZDB-GENE-051023-7]	*uba52*
	VAMP (vesicle-associated membrane protein)-associated protein A, like	*vapal*
oxidative phosphorylation	ATP synthases, H+ transporting, mitochondrial	*atp5d, atp5j*
	cytochrome c oxidase subunits	*cox5a (1 of 3), cox5ab, cox6a1*
	NADH dehydrogenase (ubiquinone)	*ndufa13, ndufa2, ndufa5, ndufa6, ndufb3, ndufb7, ndufb8, ndufb9, ndufs5, ndufs7, ndufv2, ndufv3*
	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	*sdhb*
proteolysis	elastases	*ela2, ela2l, ela3l*
	lon peptidase 1, mitochondrial	*lonp1*
	proteasome subunits	*psma1, psmb1, psmb2, psmb6, psmd3*
	trypsins	*ctrb1, ctrl, try*

ion transport/ion binding	ATP synthases, H+ transporting, mitochondrial	*atp5d, atp5j*
	betaine-homocysteine methyltransferase	*bhmt*
	hemoglobin zeta	*hbz*
	hepcidin antimicrobial peptide 1	*hamp1*
	myeloid cell leukemia sequence 1a	*mcl1a*
	potassium channel, subfamily K, member 10a	*kcnk10a*
	ribosomal protein L37	*rpl37*
	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	*sdhb*
	zinc finger-like gene 2a	*znfl2a*
chromatin organization	histones	*h3f3c, hist2h4b (7 of 7)*
	single-minded homolog 1	*sim1 (3 of 3)*
	SRY-box containing gene 19b	*sox19b*
lipid metabolism	apolipoproteins	*apoa1, apoa1 (2 of 2), apoc2*
	enolase 3	*eno3*
	fatty acid binding proteins	*fabp10a, fabp1b.1*
	phospholipase D family, member 3	*pld3*
lectins	galactoside-binding lectins	*lgals2b, lgals1l1*
	hexose-binding lectin 4	*hbl4*
	intelectin 3	*itln3*
	mannose-binding lectin 2-like	*lman2l (2 of 2)*
immunosuppression	FK506 (fkbp5) binding proteins	*fkbp11, fkbp3*
	vasoactive intestinal peptide	*vip (2 of 2)*

*Embryos were infected by intravenous injection of mCherry-labeled *M. marinum* Mma20 bacteria at 1 dpf, and at 4 dpi (5dpf) RNA-Seq libraries were prepared from mCherry-positive (+) and negative (-) cell fractions obtained by FACS-sorting. Read counts from two biological replicates of infected embryos were compared by DESeq analysis against the eGFP-negative cell fractions of two biological replicates of uninfected *mpeg1:egfp* larvae (5 dpf). Ensembl ID codes of 148 significantly enriched genes (log2 fold change > 2, adjusted P-value <0.1) in mCherry-positive Mma20-infected cells were analyzed using DAVID, which showed enrichment of five annotation clusters: translation, oxidative phosphorylation, proteolysis, ion transport/binding, and chromatin assembly. Furthermore, overlap with genes enriched in *mpeg1:egfp*-positive cells identified a macrophage gene group, and manual annotation identified three additional gene groups: lipid metabolism, lectins, and genes involved in immunosuppression. The full list of significantly enriched genes with fold change, P-value, and RPKM values of the biological replicates in given in Supplementary table 3.

Supplementary Table 5. Genes affected by *ptpn6* knockdown in *M. marinum*-infected cells and different leukocyte populations**.** Supplementary table can be found online at: https://www.dropbox.com/s/n4bjcjtfseraljp/Chapter3suppl.table5.xlsx

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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, apoliprotein-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 $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 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'GACACCCTTGGAATTCAGTTCTCAA3') 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, except146b-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

(hpi). 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).

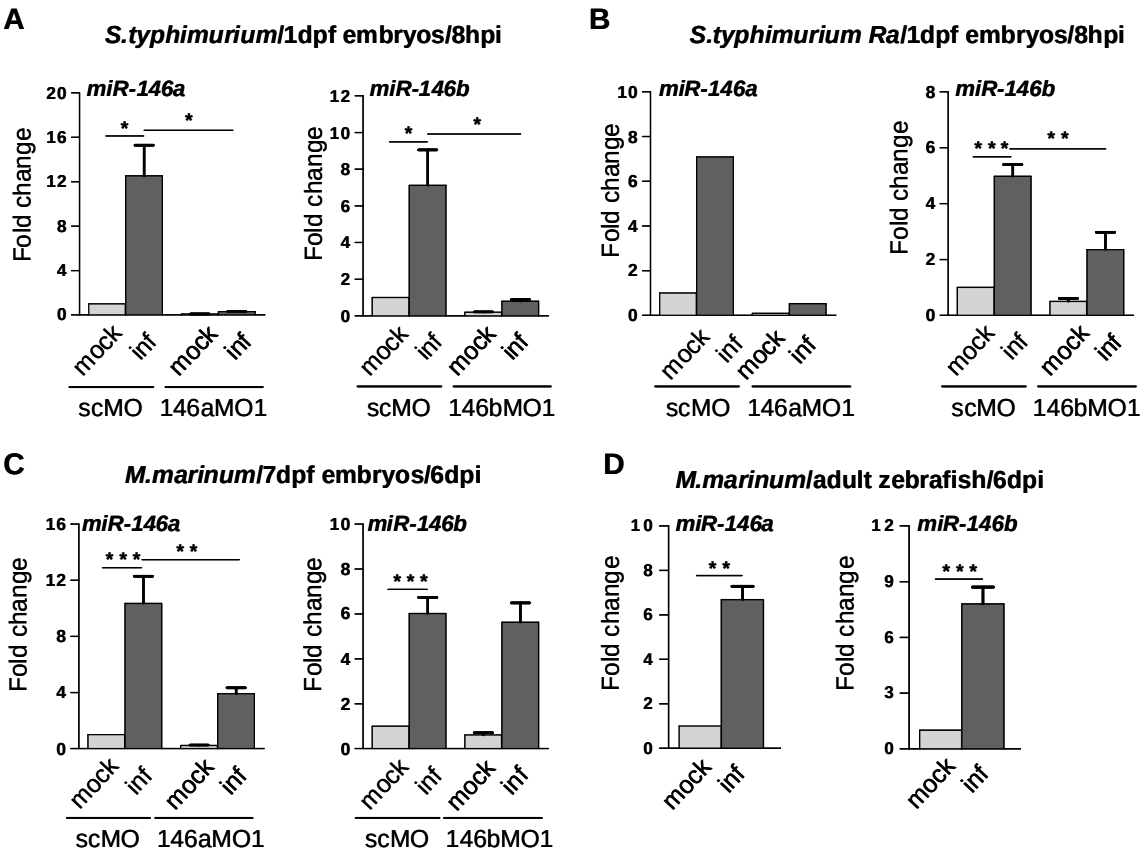

Figure 1. Expression of miR-146a/b is enhanced upon bacterial infections. (A) MiR-146a/b induction in zebrafish embryos by *S. typhimurium*. Embryos were infected with *S. typhimurium* wild type bacteria (inf) or injected with PBS (mock) at 28 hpf and expression of miR-146a/b was analyzed at 8 hpi. Infection-dependent 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.

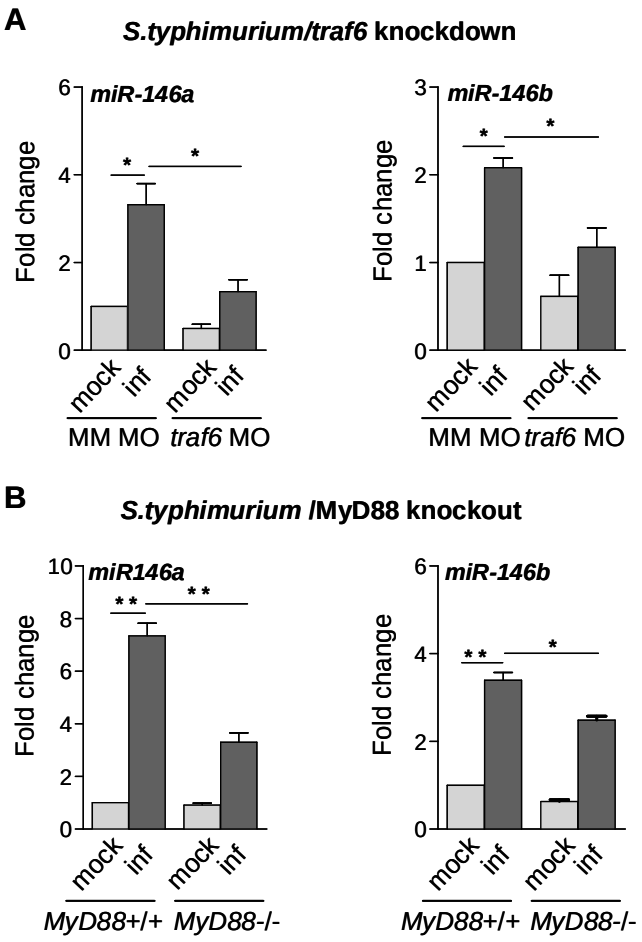

Figure 2. The Traf6-MyD88 pathway is involved in up-regulation of miR-146a/b expression levels upon infection. (A) Traf6-dependent miR-146a/b induction. Embryos were injected with *traf6* morpholino (MO) or a mismatch morpholino (MM) as a control**.** Embryos were infected at 28 hpf with *S. typhimurium* (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

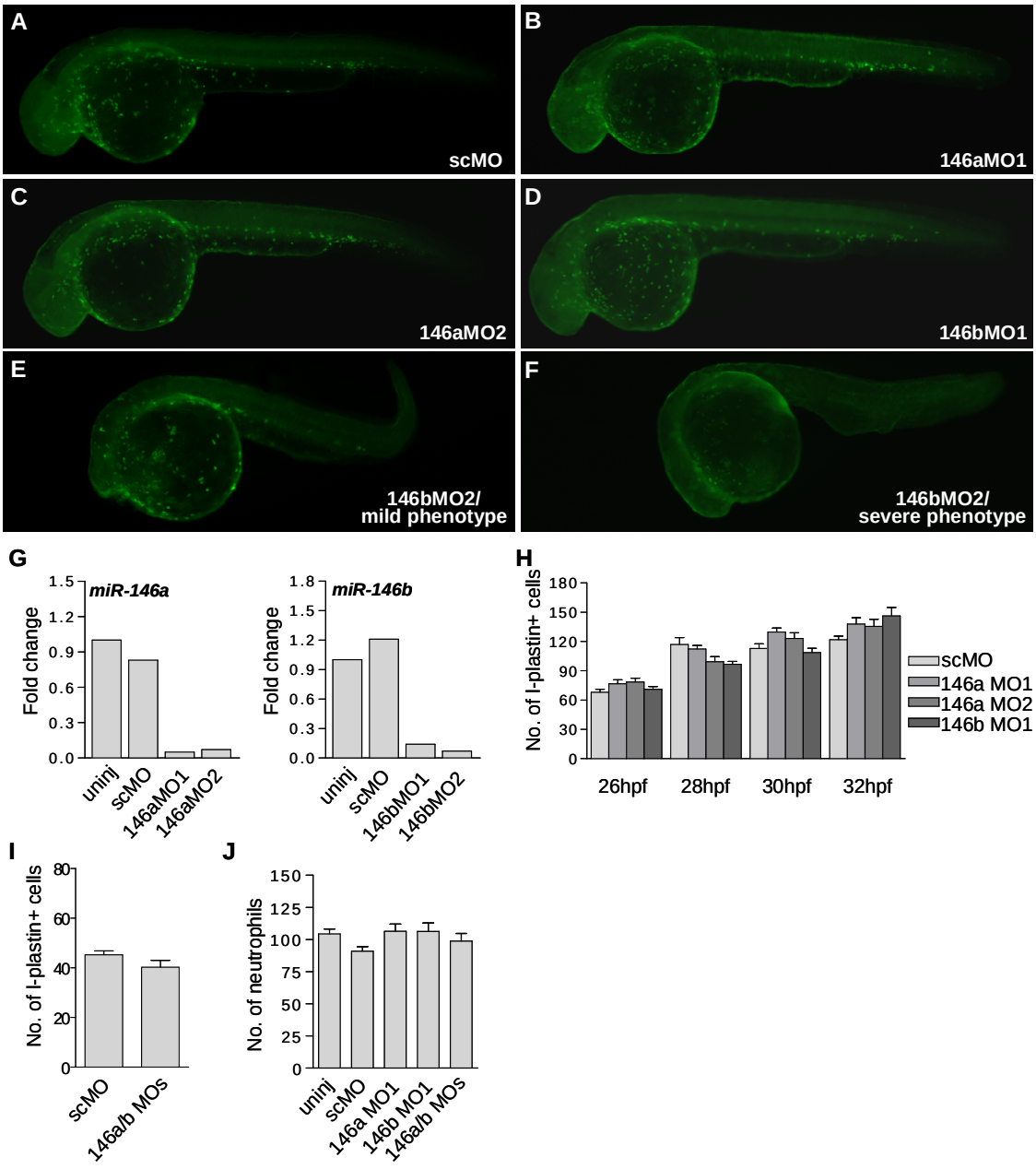

Figure. 3 Knockdown of miR-146a/b does not affect leukocyte development. Embryos were injected at the 1-cell stage with morpholinos targeting miR-146a (146aMO1 and 146aMO2) or miR-146b (146bMO1 and 146bMO2) or were injected with standard control morpholino (scMO). (A-F) Representative images of L-plastin 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 neutrophis 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.

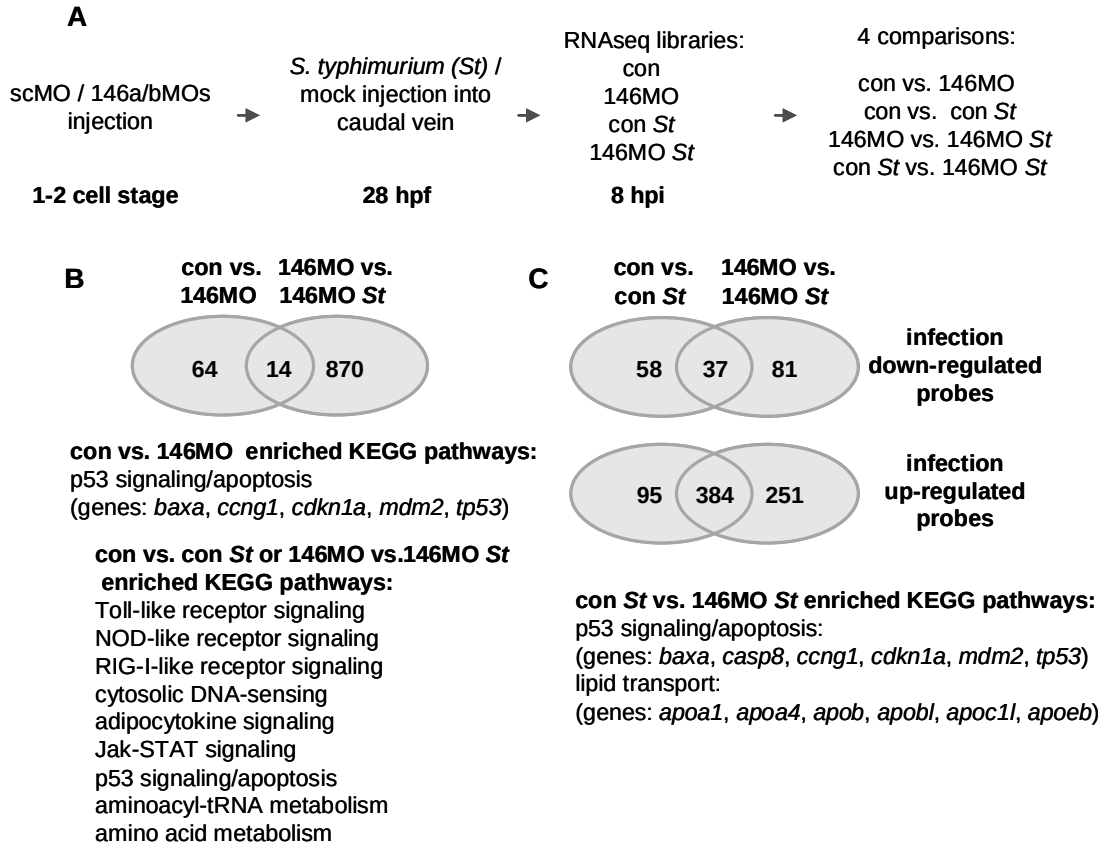

Figure 4. Transcriptome response of miR-146a/b morphants to *S. typhimurium* infection. (A) Experimental set-up of the deep sequencing methodology. Embryos were injected at the 1-2 cell stage with a combination of 146aMO1 and 146bMO1 (146a/bMOs) or with the standard control morpholino (scMO). Approximately 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.

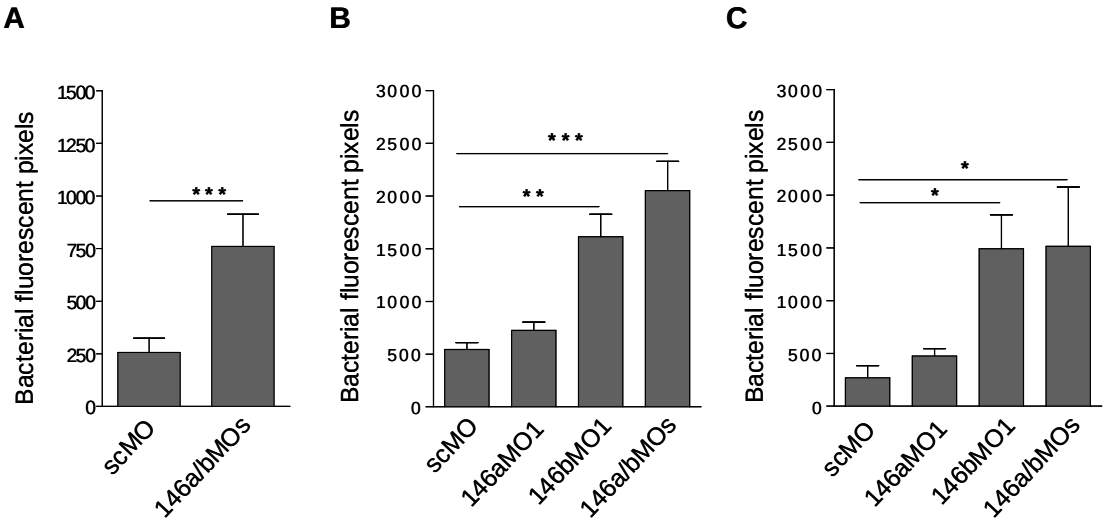

Figure 5. Knockdown of miR-146b increases bacterial burden of *M. marinum* infection. (A) Increased *M. marinum* infection by combined miR-146a/b knockdown. Embryos were injected at the 1-cell stage with a combination of 146aMO1 and miR-146bMO1 (146a/bMOs) or with standard control morpholino (scMO). At 28 hpf the embryos were infected with *M. marinum* Mma20 strain by injecting into the caudal vein. Bacterial burden was determined at 3 dpi by fluorescent pixel quantification of mCherry-labeled bacteria. Data are the mean ± SEM of cumulative data of 3 independent experiments. (B,C) Knockdown of miR-146b as the major contributor to increased *M. marinum* infection. Embryos were injected at the 1-cell stage with 146aMO1, 146bMO1, with the combination of both (146a/bMOs), or with standard control morpholino (scMO). Infection and quantification of bacterial burden was performed as in (A) in two independent experiments (B,C). Due to differences in the injection dose, bacterial burden was analyzed at different time points in these experiments, at 3dpi (B, ≥ 39 embryos per group) and at 4dpi (C ≥ 30 embryos per group). Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) tested by unpaired t-test (A) or one-way ANOVA analysis with Tukey's method as post-hoc test (B, C).

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, apoliprotein-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* (*fgb*), 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 defence 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 zebrafsh 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

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Supplementary data

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

```
         -------U        CU  U  Cu          au            UGUUCA
GAGUUUGU         UUGAGCA   UU CC  gagaacuga   uccauagaugg       U
||||||||         |||||||   || ||  ||||||||||  ||||||||||||
CUUAAACA         AAUUUGU   AA GG  UUCUUGACU  GGGUAUCUACU       G
         UCGUCUGU        CU  C  UC          -C            UGAAAA
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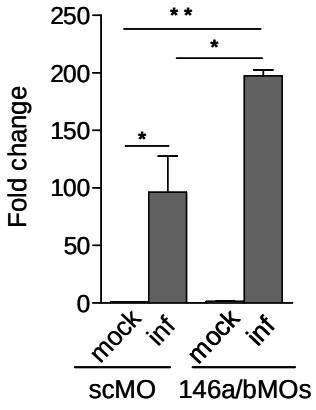
B. Stem-loop dre-miR-146b:

```
  -    UG     u                 aa     gUCUGCU
GC  UCU   GCUU gagaacugaauucc   gggu         U
||  |||   |||| ||||||||||||||   ||||
CG  AGG   UGAA UUCUUGACUUGAGG   CCCG         U
  U    UU     -                 CA     ACUUAUA
```

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 *mmp*9 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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Chapter 5

Summary and Discussion

Infectious diseases, caused by pathogenic microorganisms, continue to pose major health challenges in developed as well as developing countries. A proper functioning immune system is essential for defense against infectious agents. The immune system is functionally organized into two components: innate (natural) and adaptive (acquired) immunity. The innate immune system is inborn and provides immediate protection against invading microorganisms by recognizing important and conserved structural components, such as molecules of microbial cell walls. A variety of immune cells orchestrate responses of the innate immune system. These include: macrophages, neutrophils, eosinophils, basophils, dendritic cells, natural killer cells, and mast cells. Innate immune cells function cooperatively with T- and B-lymphocytes of the adaptive immune system, which execute a more specific response due to recognition of distinctive molecules (antigens) of different pathogens.

Upon detection of an infection or other indications of danger, the cells of innate immune system initiate the release of pro-inflammatory mediators, which are proteins and lipids that stimulate inflammation. Increased blood flow and attraction of immune cells during inflammation helps to limit the spread of infection. However, this process needs to be properly controlled, because excessive inflammation is harmful to the body's own tissues. Inappropriate regulation of the immune system may lead to auto-immune and auto-inflammatory diseases. Both groups of disorders result from the immune system attacking the body's own tissues, which in turn results in increased inflammation. In most auto-immune diseases, such as type 1 diabetes, coeliac disease, multiple sclerosis, systemic lupus erythematosus, Crohn's disease, and rheumatoid arthritis, defective responses of the adaptive immune system play a major role. Auto-inflammatory diseases are a relatively new category of diseases and are mainly caused by the defects in the innate immune system, causing inflammation for unknown reasons.

As reviewed in **Chapter 1**, both the innate and adaptive immune systems rely on many signaling molecules for their proper functioning. These molecules have roles in pathogen recognition (e.g., pattern recognition receptors such as TLRs, NLRs, and RLRs), signal propagation (e.g. adaptor proteins, kinases, phosphatases, and transcription factors) and control of gene expression (e.g. miRNAs). Functional defects in any of these molecules may increase susceptibility to infectious diseases or result in conditions like auto-immune and auto-inflammatory diseases. The zebrafish is a recent addition to biomedical research as a model for the study of inflammation and infectious diseases. The molecular components that orchestrate immune responses are well conserved between human and zebrafish. Infection and inflammation processes can be excellently visualized in zebrafish embryos and larvae, as these are optical transparent. The absence of functional adaptive immunity in embryos and larvae makes them highly suitable for modeling innate immune responses *in vivo*. In addition, high production rates and small size facilitate high-throughput assays using zebrafish. Several infection models in zebrafish have been well established, including *Salmonella typhimurium* and *Mycobacterium marinum* models for acute and chronic infections, respectively. In this

thesis we have made use of the zebrafish to study the importance of different regulatory pathways in innate immunity.

In **Chapter 2** we have demonstrated the importance of a negative regulator of the innate immune response, which is encoded by the protein tyrosine phosphatase non-receptor type 6 gene (*ptpn6)*. It is known that mutation of this gene in mice causes inflammatory lesions in multiple organs that become lethal at 3-5 weeks of age. Furthermore, dysfunction of human PTPN6, also known as SHP1, has been linked to auto-immune and auto-inflammatory disorders. PTPN6 is a hematopoietic phosphatase. In one day old zebrafish embryos, we found *ptpn6* to be specifically expressed in macrophages and at later stages of development we also observed expression in neutrophils and developing T-cells. We used a morpholino knockdown approach to study the role of *ptpn6* in the innate immune system of zebrafish embryos. Knockdown of *ptpn6* did not affect the early stages of development, but a late pleiotropic phenotype was observed with different morpholinos against this gene. At 5-6 days post fertilization (dpf), *ptpn6* morphant embryos showed more proliferation in the brain, contained more apoptotic cells, and they developed oedema and inflammatory lesions. An increased induction of the pro-inflammatory genes coding for interleukin 1b (*il1b*) and matrix metalloproteinase 9 (*mmp9*) was found in the *ptpn6* morphants. The induction of these genes was concomitant with the occurrence of inflammatory lesions that were highly infiltrated by leukocytes. This late phenotype was found to be independent of the presence of microbes, showing that an external trigger is not necessary for the development of inflammation in *ptpn6* morphants and the absence of this gene is sufficient to cause the inflammation-associated phenotype. The administration of anti-inflammatory glucocorticoids could not block the development of inflammation in *ptpn6* morphants. On the contrary, glucocorticoid treatment of *ptpn6* morphants intensified the development of inflammatory lesions as well as the induction of pro-inflammatory genes. These effects might be due to synergistic actions between the glucocortocoid receptor and several transcription factors which have been activated by *ptpn6* knockdown.

To analyze the function of *ptpn6* during infection we focused on the first days of embryonic development, prior to the manifestation of the spontaneous inflammatory lesions and the induction of pro-inflammatory genes. We challenged the *ptpn6* morphants at 1 dpf with *S. typhimurium* and *M. marinum* infections. A more rapid proliferation of *S. typhimurium* was observed in *ptpn6* morphants compared with control embryos. Furthermore, *ptpn6* morphants showed impaired control of the growth of an attenuated *S. typhimurium* strain, which could be more efficiently cleared by the immune system of control embryos. Infection with *M. marinum* also led to heavier bacterial loads and increased granuloma formation in *ptpn6* morphants compared with the controls. The infection-dependent induction of pro-inflammatory *il1b* and *mmp9* genes was higher in *S. typhimurium*-challenged *ptpn6* morphants than in the control embryos. Increased induction of *mmp9* was also observed in *M. marinum*-infected *ptpn6* morphants. Thus, hyperinduction of these genes proved to be disadvantageous for zebrafish embryos in combating infections.

We chose the *S. typhimurium* infection model to further investigate the specific effects of *ptpn6* knockdown on the innate immune response by microarray analysis. This analysis was performed 8 hours after challenge with *S. typhimurium* at 1 dpf, when no visible phenotypic effect of *ptpn6* knockdown was present. The total number of probes with a significant responsive to infection was approximately 2-fold larger in *ptpn6* morphants than in control embryos. Analysis of a group of 258 genes with higher up-regulation in infected *ptpn6* morphants showed significant overrepresentation of TLR, NLR, RIG-I, p53, MAPK, JAK-STAT and other immune-related KEGG pathways. More specifically, genes showing higher up-regulation in *ptpn6* morphants included cytokine/chemokine/interferon genes such as *il1b*, *il8*, *tnfa*, *tnfb*, and *ifnphi1*, matrix metalloproteinases such as *mmp9* and *mmp13*, and many transcriptional regulators of the ATF, CEBP, AP1, NFκB, and STAT families. Similar to the increased induction of pro-inflammatory genes, infected *ptpn6* morphants also showed increased induction levels of several negative regulators, such as *irak3*, *socs3a* and *socs3b,* and NFκB inhibitor genes (*nfkbiaa*, *nfkbiab*, *nfkbib*, *nfkbiz*). In contrast, the anti-inflammatory cytokine gene *il10* did not show increased induction in *ptpn6* morphants. The microarray results were confirmed by using an RT-MLPA assay that allows the simultaneous semi-quantitative PCR analysis of 34 innate immune genes. Thus, under *ptpn6* knockdown conditions, embryos responded to *S. typhimurium* infection by an enhanced gene induction profile of the innate immune response. Therefore, we could conclude that *ptpn6* functions as a key player regulating the inflammatory response to infection through a negative feedback mechanism. This function of *ptpn6* is crucial for preventing host-detrimental effects of inflammation and is essential for a successful defense mechanism against invading microbes.

In **Chapter 3** we used Illumina RNA deep sequencing (RNA-Seq) technology to determine transcriptome profiles of different immune cell types and to further analyze the effects of *ptpn6* knockdown at a cellular level. Transgenic zebrafish lines with fluorescently marked macrophages (*mpeg1:egfp*), neutrophils (*mpx:egfp*), and early T-cells (*lck:egfp*) were used for this study. Zebrafish larvae were dissociated at 5 dpf and the fluorescent cells were isolated by fluorescence activated cell sorting (FACS). First, to develop a protocol for RNA-Seq analysis from FACS-sorted cells, we used the *mpeg1:egfp* macrophage marker line. From pools of approximately 250 dissociated larvae we could obtain 4000-10,000 GFP-labeled cells. Using the Ambion RNAqueous-Micro Kit for RNA isolation in combination with the Clontech SMARTer Ultra Low RNA Kit for Illumina Sequencing, we succeeded in obtaining good RNA-Seq libraries, even from samples with an estimated RNA input below 100 pg. We used the DEseq program for statistical analysis of libraries from the GFP-positive and GFP-negative cell fractions of four biological replicates with approximately 10 million paired-end reads per library. The GFP-positive cell fractions of all biological replicates showed specific enrichment of KEGG pathways and GO-terms related to the immune system and approximately 50% of significant genes were common between each pair of replicates. These results demonstrated the robustness of our assay.

Having established a reproducible procedure for RNA-Seq analysis from FACS-sorted cells, we compared the macrophage signature genes obtained from *mpeg:egfp*-positive cells with FACS-sorted neutrophils from an *mpx:egfp* transgenic line and early T-cells from an *lck:egfp* transgenic line. As expected, known macrophage markers were specifically enriched in *mpeg1:egfp*-positive cells, including *csf1r*, *cxcr3.2*, *irf8*, *marco*, *mfap4*, *mhc2dab*, and *mpeg1* itself. Neutrophils showed a higher expression of *mpx*, *lyz*, *mmp9* and *mmp13a* than macrophages. Furthermore, the expression profile of *lck:egfp*-positive cells showed very specific expression of T-cell markers including *cd2*, *cd28*, *cd4*, *ikzf1* (*ikaros*), *rag1*, and *rag2* along with *lck*. There was more overlap between the expression signatures of *mpx:egfp*-positive and *mpeg1:egfp*-positive cell fractions than there was between these myeloid cell fractions and *lck:egfp*-positive lymphoid cells. The RNA-Seq libraries gave us the opportunity to look for other specific and more abundant genes that may prove to be valuable markers for developing new transgenic lines and antibodies for distinguishing leukocyte lineages in the zebrafish model. Genes that showed higher abundance than *mpeg1* in macrophages were members of the families of chemokines, immunoglobulins, olfactomedins, granulins, cathepsins, fibrinogens, lectins, transmembrane receptors, complement factors, and MHC II class proteins. Among these, *granulin 2* (*grn2*) had a more than 10-fold higher expression level than *mpeg1* and was highly specific for macrophages. Several other genes, for example the MHC class II genes *cd74* and *mhc2dab,* were also detected almost exclusively in macrophages. In neutrophils, *lyz* and *npsn* showed higher abundance than *mpx*, and *il34* was identified as another good marker. Highly specific T-cell markers included *lck*, *rag1*, *ccr9b*, *il17r*, *p2rx1*, *rorc, foxp3a,* and several genes for T-cell specific immunoglobulins.

We also used the RNA-Seq technology to investigate the immune response-related gene expression pattern of zebrafish challenged with *M. marinum*. Following its uptake by macrophages, this pathogen causes a chronic infection in zebrafish with the formation of granuloma-like aggregates resembling the hallmark of tuberculosis in human. Infected cells carrying mCherry-labeled fluorescent *M. marinum* were isolated by FACS sorting from larvae at 4 days post infection (dpi). We compared the expression signature of the *M. marinum*-infected cells with that of uninfected *mpeg1:egfp*-positive macrophages. A set of 40 distinctive macrophage markers was detectable in the *M. marinum*-infected cells, but most of these genes were strongly down-regulated compared with uninfected macrophages. Furthermore, many genes that were expressed in uninfected macrophages, including *mpeg1*, were not detected in the infected cells. In addition to down-regulation of macrophage markers, the infected cells showed specific enrichment of genes that could be grouped into five main clusters of gene ontology terms: ribosome, oxidative phosphorylation, proteolysis, ion transport, and chromatin assembly. The induction of these gene groups may reflect activation of protein translation and defense mechanisms in the infected cells. Examples of these genes include the anti-microbial hepcidin gene (*hamp1*) and a ribosomal protein gene (*rps3*) for which an extra-ribosomal regulator function in innate immunity has been reported. It is an interesting possibility that the increased expression of chromatin assembly genes in infected cells may be associated with the *M. marinum*-induced

morphological changes that macrophages undergo during granuloma formation. In addition to the five main clusters of enriched genes, we found that *M. marinum*-infected cells also showed increased expression of lipid metabolism and transport genes, lectin genes, and genes with immunosuppressive functions. Some of these genes have also been implicated in human tuberculosis. Based on these results, we concluded that *M. marinum* infection has a major impact on the gene expression signature of macrophages, resulting in a general down-regulation of macrophage markers and an up-regulation of genes proposed to be involved in both host defense and immunosuppression.

Next, we decided to use the RNA-Seq profiling technology to gain further insight into the molecular processes responsible for increased granuloma formation in *ptpn6* morphants infected by *M. marinum*. Furthermore, by RNA-Seq analysis of different leukocyte populations we aimed at better understanding of the underlying causes of the inflammatory conditions that *ptpn6* morphants develop around 5 dpf. Notably, matrix metalloproteinase genes, including *mmp2*, *mmp9*, and *mmp13a*, and the *mmp* inhibitor gene *timp2b* were significantly up-regulated in *M. marinum*-infected cells from *ptpn6* morphants compared with infected cells from control embryos. Along with the *mmp* genes, *serum amyloid A* (*saa*) was up-regulated. Since this gene has previously been implicated in regulating *mmp* genes expression, we propose that increased levels of *saa* play a role in triggering increased *mmp* gene expression during *M. marinum* infection of *ptpn6* morphants. Some genes with possible functions in anti-microbial defense were also up-regulated, including *hamp1*, *lepb*, *gcga*, and *steap4.* These genes are connected with responses to nutrients and therefore may form links between the innate immune system and metabolic responses. However, the up-regulation of these possible anti-microbial genes was not sufficient to prevent the strong increase in bacterial burden of *M. marinum* infection in *ptpn6* morphants..

In contrast to what was observed during *S. typhimurium* infection of *ptpn6* morphants (chapter 2), there was no general up-regulation of pro-inflammatory genes in *M. marinum* infected *ptpn6* morphants. Therefore, we concluded that the increased bacterial burden in *ptpn6* morphants during *M. marinum* infection is mainly due to a specific hyperinduction of *mmp* genes, while in *S. typhimurium* infection a general hyperinduction of pro-inflammatory genes is the cause of the increased bacterial burden. A model summarizing our conclusions about the effect of *ptpn6* deficiency on the molecular processes involved in *S. typhimurium* and *M. marinum* infections is presented in Figure 1.

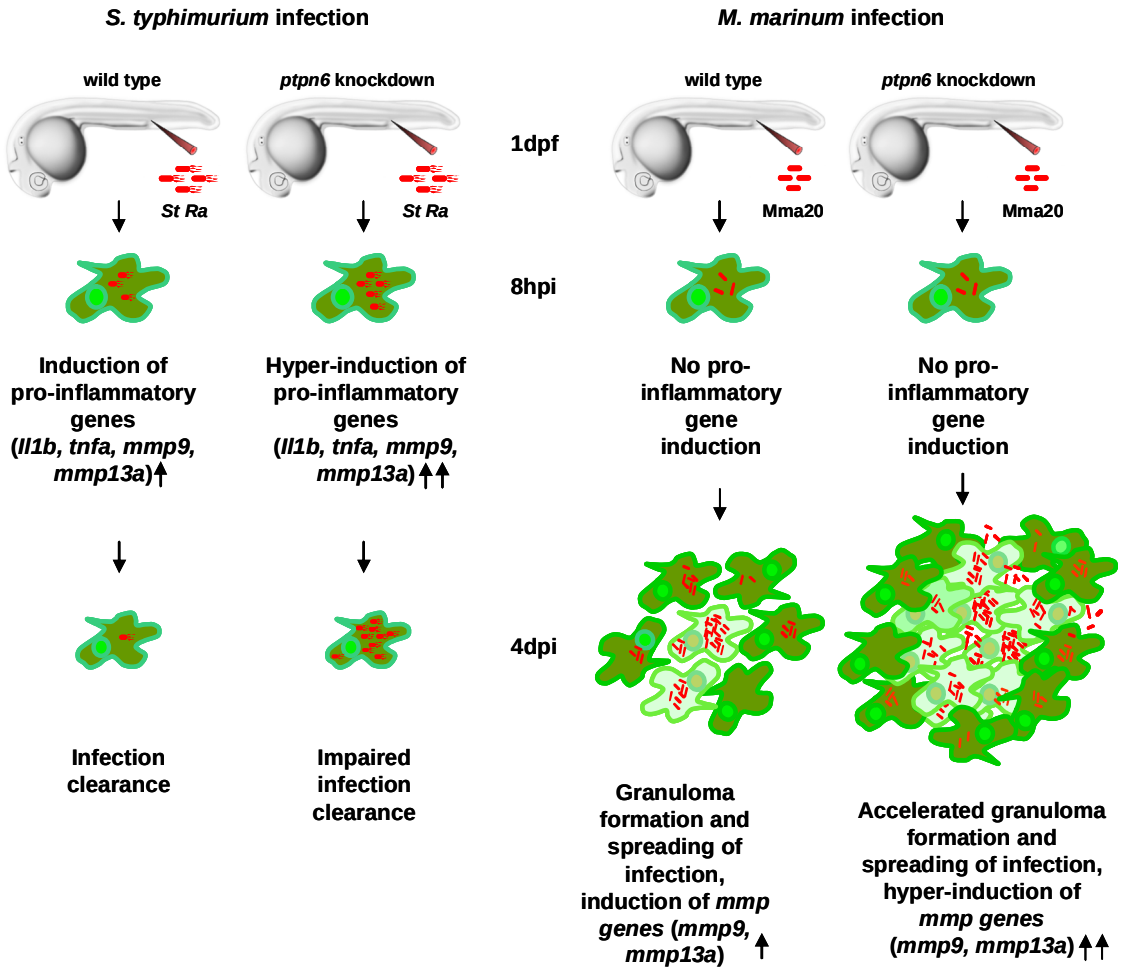

Figure 1. Schematic representation of the negative regulatory mechanisms of *ptpn6* during *S. typhimurium* and *M. marinum* infections in zebrafish embryos. Intravenous infection of wild type embryos at 1 day post fertilization (dpf) with *S. typhimurium* causes a general induction of pro-inflammatory genes (among others *il1b*, *tnfa*, *mmp9*, *mmp13a*) at 8 hours post injection (hpi), while this is not observed upon infection with *M. marinum* (Chapter 2, van der Vaart et al., 2012). In contrast, *M. marinum* infection is associated with the formation of granuloma-like aggregates of infected and uninfected macrophages. Infected macrophages in these granulomas show a down-regulation of macrophage marker genes (Chapter 3), indicated by a lighter color of green in the figure. The knockdown of *ptpn6* results in a hyperinduction of pro-inflammatory genes upon *S. typhimurium* infection. This increased inflammatory response, instead of helping the host controlling the infection, makes it more susceptible to disease progression. As a result, the host is no longer able to clear infections with the attenuated *S. typhimurium Ra* strain (Chapter 2). *M. marinum* infection with the Mma20 strain does not induce a strong pro-inflammatory response at 8 hpi in *ptpn6* knockdown embryos nor in control embryos. At later stages of infection, the morphants did not display a general hyperinduction of pro-inflammatory genes, but a specific hyperinduction of matrix metalloproteinase (*mmp*) genes was observed. This induction of *mmp* genes occurred specifically in infected and uninfected macrophages and was associated with an accelerated granuloma formation and increased bacterial burden (Chapters 2 and 3). These observations

show the importance of *ptpn6* for a balanced and productive immune response in zebrafish embryos against bacterial pathogens.

Interestingly, the *mmp9* and *mmp13a* genes were also up-regulated in macrophages of uninfected *ptpn6* morphants. These macrophages also showed up-regulation of actin, myosin and parvalbumin genes, of several immune-related proteases (e.g., *cpa5*, *npsn*, *cpn1*, *mmp9* and *mmp13a*), and of typical neutrophil markers like *lyz* and *mpx*. In contrast, several typical macrophage-expressed genes (e.g. *cxcr4b*, *tnfa*, *tnfrsf1a*, *mbp*, *grn2*, *mhc2dab*, *cd74*, *cd74a,* and the chemokine gene *si:ch211-149o7.4*) were down-regulated in macrophages of *ptpn6* morphants. These data suggest a complex role of *ptpn6* in regulating the activation status of the macrophages. The effects of *ptpn6* knockdown on different cell populations were very specific. The up-regulation of *mmp9, mmp13a* and several other protease genes was observed in macrophages but not in neutrophils, T-cells, or in the GFP-negative background of *ptpn6* morphants. Up-regulation of *mmp2* was detected in neutrophils in GFP-negative background cells. An up-regulation of genes involved in p53 signaling (*tp53*, *mdm2*, *ccng1*) was only observed in neutrophils. Furthermore, neutrophils showed specific down-regulation of apolipoprotein and protease genes under *ptpn6* knockdown conditions, while down-regulation of ribosomal protein genes was specific for T-cells. There was no effect on p53 signaling genes in the GFP-negative background cells of *ptpn6* morphants and no indication of a general toxicity effect in the RNA-Seq data. Based on these results, we suggest that enhanced *mmp* genes secretion by macrophages might be a major cause of the inflammation-associated phenotype that *ptpn6* morphants develop around 5 dpf.

In **Chapter 4** we studied the function of microRNAs (miRNAs) as another potential level of regulation of innate immune responses during infection of zebrafish embryos. MiRNAs are evolutionary conserved, genome-encoded small RNAs (~22 nucleotides) involved in post-transcriptional gene repression. We focused on the role of microRNA-146a and microRNA-146b (miR-146a/b), which are two closely related members of the miRNA-146 family that is well conserved between human and zebrafish. Dysregulation of miR-146a/b has been frequently linked to inflammatory diseases and malignant tumors, and therefore modulating its function might have a therapeutic potential. Previous studies in human cells and mouse models 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. The *IRAK1* and *TRAF6* homologs of both zebrafish and human contain putative target sites for miR-146a/b in their 3'UTRs, suggesting that miR-146 feedback control of TLR signaling is evolutionary conserved (Ordas et al., 2010). Using quantitative PCR (qPCR) analysis we found that the expression of miR-146a/b in zebrafish is inducible by infection with *S. typhimurium* and *M. marinum*, which was in line with previous microarray data from our group. Furthermore, we showed that infection-inducible expression of miR146a/b was partially dependent on the TLR pathway genes *traf6* and *myd88*. The induction of miR-146a/b during *S. typhimurium* infection was significantly reduced in *traf6* knockdown

embryos and in *myd88* knockout mutant embryos compared with the induction levels in control embryos. This demonstrated the requirement of a Myd88/Traf6 pathway for full-scale induction of miR-146a/b during infection in zebrafish. However, it is likely that a parallel Myd88/Traf6-independent induction route also exists, as neither *traf6* knockdown nor *myd88* knockout was sufficient to block the infection-induced expression of miR-146a/b completely.

We took advantage of the morpholino knockdown technology for functional analysis of miR-146a/b in zebrafish embryos by. designing two different morpholinos for each of the miR-146 family members. The efficiency of each knockdown was confirmed by Taqman qPCR analysis. We did not observe any effects of the loss of miR-146a/b on the development or differentiation of leukocytes in zebrafish embryos. Hypothesizing that miR-146a/b might be involved in negative regulation of the innate immune response, we were interested to test whether miR-146a/b knockdown leads to hyperinduction of pro-inflammatory genes following *S. typhimurium* infection, similar as was observed in our knockdown analysis of the negative regulator *ptpn6*. RNA-Seq analysis was performed for this purpose. First, we checked if miR-146a/b knockdown had an effect on gene expression in the absence of infection. We found that only 78 genes were affected by miR-146a/b knockdown, among which 5 genes in p53 signaling. This might reflect a non-specific effect of the miR-146 knockdown, since morpholino effects on the p53 pathway are relatively common. However, considering that miR-146 has been frequently linked with cancer, a direct effect on the p53 pathway is also conceivable. *S. typhimurium* infection led to slightly higher numbers of up- and downregulated genes in miR-146 morphants than in control embryos, but there was no general hyperinduction of pro-inflammatory genes in miR-146 morphants. The only pro-inflammatory marker that was induced at higher levels in miR-146 morphants was the *mmp9* gene. Consistent with this observation, human *MMP9* was down-regulated following overexpression of miR-146a/b in cultured cancer cells and macrophages. Given that there are no predicted target sites for miR-146a/b in *mmp9*, miR-146-mediated regulation of this gene may occur via the Myd88/Traf6 pathway that acts upstream of *mmp9* induction. The fact that *mmp9* is the most strongly induced pro-inflammatory marker in *S. typhimurium* infection may explain why differences in induction of other Myd88/Traf6-dependent pro-inflammatory markers were not seen in our RNA-Seq analysis of miR-146a/b morphants and control embryos.

Although the overall effect of miR-146a/b knockdown observed in our RNA-Seq analysis was relatively minor, KEGG pathway analysis revealed a possible effect on apolipoprotein-mediated lipid transport in *S. typhimurium*-infected miR-146 morphants. This is an interesting observation considering that many studies have linked apolipoproteins to immunoregulation and host defense and that several inflammatory disorders are associated with uncontrolled lipid accumulation. MiR-146a has been suggested to be involved in negative regulation of oxidized low-density lipoprotein accumulation in human macrophages. In future work, zebrafish infection models may prove useful to further explore the role of miR-146 family members in lipid-mediated inflammatory responses.

Knockdown of miR-146a/b did not affect bacterial burdens during infection with *S. typhimurium*, but infection with *M. marinum* under miR-146a/b knockdown conditions resulted in accelerated granuloma formation and led to significantly increased bacterial burdenscompared to control infected embryos. Knockdown experiments with morpholinos targeting each of the miR-146 family members separately, showed that loss of miR-146b rather than loss of miR-146a caused the increased bacterial burden during *M. marinum* infection. It remains to be further investigated if accelerated granuloma formation under miR-146b knockdown conditions is linked with increased *mmp* gene expression, similar as in the *ptpn6* knockdown situation.

Conclusion: The work presented in this thesis has provided new insights into the mechanisms involved in the regulation of innate immune responses to infection in zebrafish embryos. Furthermore, cell-specific transcriptome profiling studies in this thesis have identified novel marker genes for distinguishing immune cell types, which is highly useful information to fulfill the demand for new fluorescent reporter lines and lineage-specific antibodies in the zebrafish model. Our functional studies of Ptpn6 show that the role of this protein-tyrosine phosphatase as a negative regulator is of critical importance to a properly balanced innate immune response and for the control of infections with *S. typhimurium* and *M. marinum*. We found that Ptpn6 has a specific regulatory function in responses to each of these bacterial pathogens. In *S. typhimurium* infection, *ptpn6* deficiency caused a general hyperinduction of pro-inflammatory genes, which was contra-productive as it impaired the control of infection. In *M. marinum* infection, a more specific effect of *ptpn6* deficiency on matrix metalloproteinase gene expression is likely to be a major underlying cause of accelerated granuloma formation and increased bacterial burden. We further concluded that Ptpn6 functions as a much stronger negative regulator than infection-inducible miRNAs of the miR-146 family, which may be involved in more subtle fine-tuning of the innate immune response. Knowledge about the distinct roles of Ptpn6 and miR-146 miRNAs has practical applicability in regard to their potential as therapeutic targets for inflammatory diseases and cancer. Our zebrafish knockdown models for these negative regulators may contribute to anti-inflammatory and anti-microbial drug discovery, for which high-throughput assays in zebrafish embryos are very powerful.

Samenvatting

Infectieziekten vormen een serieuze bedreiging voor de gezondheid, niet alleen in ontwikkelingslanden maar ook in de westerse wereld. Een goed functionerend immuunsysteem is onmisbaar voor de afweer tegen ziekteverwekkende micro-organismen. In reactie op een infectie produceren de cellen van het immuunsysteem signaalmoleculen die een ontstekingsbevorderende werking hebben. Een toename van de bloedtoevoer en aantrekking van immuuncellen tijdens een ontstekingreactie helpt om verdere verspreiding van de infectie te beperken. Dit proces moet echter zeer precies gereguleerd worden, omdat een ontsteking ook schadelijk is voor de eigen lichaamscellen. Een verstoorde regulatie van dit proces kan leiden tot auto-immuunziekten en chronische ontstekingsziekten.

Hoofdstuk 1 geeft een overzicht van de signaalmoleculen die een belangrijke rol spelen bij de regulatie van het immuunsysteem. Deze signaalmoleculen zijn sterk evolutionair geconserveerd tussen de mens en de zebravis, het modelsysteem dat wij gebruikt hebben voor het onderzoek in dit proefschrift. De transparante embryo's van de zebravis zijn zeer geschikt om de functie te bestuderen van macrofagen en neutrofielen, de eerste celtypen van het immuunsysteem die reageren op een infectie. In ons onderzoek werden zebravisembryo's geïnfecteerd met *Salmonella typhimurium* and *Mycobacterium marinum*, als model voor respectievelijk acute and chronische bacteriële infecties. Met behulp van deze infectiemodellen hebben wij verschillende regulerende factoren van het immuunsysteem onderzocht.

In **hoofdstuk 2** hebben wij het belang aangetoond van een negatieve regulator van het immuunsysteem: Ptpn6, een eiwit dat behoort tot de familie van fosfatases. Het was al bekend dat functionele defecten van dit fosfatase in de muis gepaard gaan met hevige ontstekingen en dat er in de mens een verband is met auto-immuunziekten en ontstekingsziekten. Net als bij de mens, komt het gen voor Ptpn6 in de zebravis specifiek tot expressie in de cellen van het immuunsysteem (leukocyten). Uitschakeling van dit gen in zebravisembryo's (door knockdown met morpholino's) had geen zichtbaar effect tijdens de vroege ontwikkeling, maar leidde tot een laat fenotype in larven van vijf tot zes dagen oud. Deze larven ontwikkelden oedeem en huidlaesies met een sterke infiltratie door leukocyten. Bovendien vertoonden ze een sterk verhoogde expressie van ontstekingbevorderende (pro-inflammatoire) genen, zoals *interleukine 1b* (*il1b*) en *matrix metalloproteinase 9* (*mmp9*). Dit fenotype was onafhankelijk van de aanwezigheid van micro-organismen en kon niet onderdrukt worden door behandeling met glucocorticoïde ontstekingsremmers. Om de functie van Ptpn6 tijdens infecties te onderzoeken hebben wij ons gericht op de eerste dagen van de embryo-ontwikkeling, voorafgaand aan het optreden van het latere fenotype in de larven. In deze periode vertoonden Ptpn6-deficiënte embryo's een grotere vatbaarheid voor experimentele infecties met *S. typhimurium* en *M. marinum*. Tijdens *S. typhimurium*-infectie trad er in de Ptpn6-deficiënte embryo's een sterkere inductie op van de pro-inflammatoire genen *il1b* en *mmp9* dan bij geïnfecteerde controle-embryo's. Tijdens *M. marinum*-infectie werd eenzelfde effect van Ptpn6-deficiëntie op expressie van *mmp9* waargenomen. De

hyperinductie van deze genen had dus een negatief effect op de capaciteit van de embryo's om zich te verdedigen tegen de bacteriële infecties. Vervolgens hebben wij het *S. typhimurium*-infectiemodel gebruikt om het effect van Ptpn6-deficiëntie verder te onderzoek met behulp van een microarray-analyse en een multiplex PCR-methode (RT-MLPA). Dit liet zien dat er bij infectie van Ptnp6-deficiënte embryo's een hyperinductie is van een grote groep van pro-inflammatoire genen en activerende transcriptiefactoren van de immuunrespons. Deze resultaten leidden tot de conclusie dat Ptpn6 functioneert als een negatieve feedback-regulator van de immuunrespons van zebravisembryo's tegen infecties. Deze functie van Ptpn6 blijkt cruciaal te zijn voor het afweermechanisme van de embryo's.

In **hoofdstuk 3** hebben wij RNA-Seq-technologie (Illumina RNA sequencing) toegepast om de expressieprofielen te bepalen van verschillende celtypen van het immuunsysteem en om de effecten van Ptpn6-deficiëntie verder te analyseren op cellulair niveau. Hiervoor hebben wij gebruik gemaakt van transgene zebravislijnen met fluorescent-gemarkeerde macrofagen (*mpeg1:egfp*), neutrofielen (*mpx:egfp*), en vroege T-cellen (*lck:egfp*). De fluorescerende cellen werden geïsoleerd uit vijf dagen oude larven met behulp van FACS-sortering (fluorescence activated cell sorting). Eerst hebben wij een robuust protocol opgezet voor RNA-isolatie, cDNA-amplificatie en RNA-Seq-analyse van zeer kleine hoeveelheden uitgangsmateriaal (4000-10000 cellen en RNA-hoeveelheden rond 100 pg). Hiermee konden wij vervolgens sets van specifieke merkers bepalen voor macrofagen, neutrofielen, en T-cellen. Hierbij hebben wij nieuwe merkers ontdekt die een hoger expressieniveau hebben dan tot nu toe bekende merkers en die daarom zeer geschikt zijn voor de ontwikkeling van nieuwe transgene lijnen of antilichamen voor de onderscheiding van verschillende typen immuuncellen in de zebravis. We hebben de RNA-Seq-technologie tevens toegepast om de reactie van het immuunsysteem op een infectie met *M. marinum* te onderzoeken. Na opname door macrofagen, veroorzaakt deze pathogeen een chronische infectie in zebravisembryo's, waarbij de geïnfecteerde macrofagen opeenhopen in granulomen. De vorming van granulomen is ook karakteristiek voor tuberculose bij de mens, veroorzaakt door de nauw verwante pathogeen *Mycobacterium tuberculosis*. Wij vonden dat de infectie met *M. marinum* een groot effect had op het expressieprofiel van macrofagen, waarbij er een sterke verlaging was van de expressieniveau's van macrofaag-specfieke merkergenen en er tegelijkertijd verhoogde expressie was van genen die een mogelijke rol spelen bij de afweerreactie van de gastheer of juist bij immuunsuppressie. Onder condities van Ptpn6-deficiëntie vertoonden de geïnfecteerde cellen een extra verhoging van de expressie van genen voor matrix metalloproteinases, waaronder *mmp9* en *mmp13a*. Bovendien was er een verhoogde expressive van *serum amyloid A* (*saa*), een gen dat mogelijk de expressie van *mmp* genen reguleert. Er was ook verhoogde expressie van genen met een mogelijke anti-microbiële functie, maar dat kon niet verkomen dat Ptpn6-deficiënte embryos vatbaarder waren voor infectie met *M. marinum*. In tegenstelling tot wat we zagen bij *S. typhimurium*-infectie (hoofdstuk 2), was er geen algemeen verhoogde expressie van pro-inflammatoire genen. Daarom concludeerden wij dat de grotere vatbaarheid van Ptpn6-deficiënte embryo's voor *M. marinum*-infectie

met name wordt veroorzaakt door een specifieke hyperinductie van *mmp*-genen, terwijl dit bij *S. typhimurium*-infectie komt door een algemene hyperinductie van pro-inflammatoire genen. De expressie van *mmp*-genen was ook verhoogd in macrofagen van ongeïnfecteerde zebravislarven met Ptpn6-deficiëntie. Dit zou een belangrijke oorzaak kunnen zijn van de spontane onstekingsreacties die optreden in deze larven (hoofdstuk 2).

In **hoofdstuk 4** hebben we de functie van microRNAs (miRNAs) onderzocht die een moeglijke rol spelen bij post-transcriptionele regulatie van het immuunsysteem tijdens infecties. Het onderzoek was gericht op microRNA-146a and microRNA-146b (miR-146a/b), twee nauw verwante leden van de miRNA-146-familie, die sterk geconserveerd is tussen de mens en de zebravis. Een veranderde expressie van miR-146a en b is vaak in verband gebracht met ontstekingsziekten en kwaadaardige tumoren. Op grond van eerder onderzoek in humane cellen en muizen was gepostuleerd dat miR-146a en b functioneren als negatieve feedback-regulatoren van het immuunsysteem via afbraak van de mRNA's van twee centrale signaalmoleculen, IRAK1 and TRAF6. Overeenkomstig met eerder microarraydata van onze groep vonden wij met behulp van kwantitatieve PCR dat de expressie van miR-146a en b in zebravis induceerbaar is door infectie met *S. typhimurium* en *M. marinum*. Bovendien konden wij aantonen dat deze infectie-inductie grotendeels afhankelijk is van een Myd88/Traf6-signaalroute. Net als bij het onderzoek naar Ptpn6 hebben wij gebruik gemaakt van morpholino-knockdown om de functie van miR-146a/b in zebravisembryo's te onderzoeken. Deficiëntie van miR-146a/b had geen effect op de ontwikkeling of differentiatie van leukocyten in de zebravisembryo's. Ook bleek uit RNA-Seq-analyse dat er weinig effect was van miR-146a/b-deficiëntie op genexpressie, zowel in ongeïnfecteerde embryos als tijdens *S. typhimurium*-infectie. Een opvallend verschil met deficiëntie van Ptpn6 was dat er bij miR-146a/b-deficiëntie geen algemene hyperinductie van pro-inflammatoire genen optrad tijdens S. typhimurium-infectie. Het enige pro-inflammatoire gen met een enigszins verhoogde inductie bij miR-146a/b-deficiëntie was *mmp9*. Wel vonden we aanwijzingen in de RNA-Seq-analyse voor een effect van miR-146a/b-deficiëntie op lipidetransporters (apolipoproteïnen) tijdens *S. typhimurium*-infectie. Dit is een interessante observatie, aangezien veel studies een verband hebben gelegd tussen lipidetransport en immuniteit en aangezien verschillende onstekingsziekten zijn geassocieerd met een ongecontroleerde ophoping van lipiden. Toekomstig onderzoek in zebravis-infectiemodellen zou kunnen bijdragen om de functie van de miR-146-familie in lipide-afhankelijke ontstekingsprocessen verder op te helderen. Deficiëntie van miR-146a/b had geen meetbaar effect op de vatbaarheid van zebravisembryo's voor infectie met *S. typhimurium*, maar injectie van *M. marinum*-bacteriën resulteerde in een versnelde vorming van granulomen en een progressiever verloop van de infectie. Met gebruik van specifieke morpholino's voor miR-146a en voor miR-146b bleek dat de versnelde granuloomvorming vooral werd veroorzaakt door deficiëntie van miR-146b. Verder onderzoek zal moeten uitwijzen of de versnelde granuloomvorming onder condities van miR-146b-deficiëntie ook gepaard gaat met verhoogde *mmp9* expressie, net zoals bij Ptpn6-deficiëntie.

Conclusies: Het onderzoek in dit proefschrift heeft nieuwe inzichten opgeleverd in de mechanismen die betrokken zijn bij de regulatie van de immuunreactie tegen infecties in zebravisembryo's. Met cel-specifieke expressieprofileringen hebben wij bovendien nieuwe merkergenen ontdekt waarmee verschillende celtypen van het immuunsysteem onderscheiden kunnen worden. Dit is zeer nuttige informatie omdat er een grote behoefte is aan merkers voor de ontwikkeling van nieuwe transgene lijnen (fluorescente reporters) en antilichamen voor detectie van immuuncellen. Onze functionele analyses van Ptpn6 hebben laten zien dat dit leukocyt-specifieke fosfatase betrokken is bij negatieve regulatie van het immuunsysteem en dat deze rol cruciaal is voor een gebalanceerde reactie van het immuunsysteem op infecties met *S. typhimurium* and *M. marinum*. Het bleek dat Ptpn6-deficiëntie speficieke effecten heeft op deze verschillende infecties. Bij *S. typhimurium*-infectie veroorzaakte Ptpn6-deficiëntie een algemene hyperinductie van pro-inflammatoire genen, wat contraproductief was omdat het de afweer tegen de infectie verslechterde. Bij *M. marinum*-infectie was een specifiek effect van Ptpn6-deficiëntie op de expressie van genen voor matrix metalloproteinases de meest waarschijnlijke oorzaak van versnelde granuloomvorming en progressie van de infectie. Verder hebben we geconcludeerd dat Ptpn6 functioneert als een veel sterkere negatieve regulator dan infectie-induceerbare miRNAs van de miR-146-familie, die mogelijke betrokken zijn bij een subtielere "fine-tuning" van de immuunrespons. Kennis over de functies van Ptpn6 en miR-146-miRNAs heeft praktische toepasbaarheid gezien hun potentieel als therapeutische doelwitten vooor ontstekingsziekten en kanker. Onze knockdown-modellen van deze negatieve regulatoren kunnen bijdragen aan grootschalige screeningen voor nieuwe anti-inflammatoire en anti-microbiële medicijnen, waarvoor zebravisembryo's zeer geschikt zijn.

List of publications

Cui, C., E. L. Benard, **Z. Kanwal**, O. W. Stockhammer, M. van der Vaart, A. Zakrzewska, H. P. Spaink, and A. H. Meijer. 'Infectious Disease Modeling and Innate Immune Function in Zebrafish Embryos', *Methods in cell biology* Vol. 105, 273-308, 2011.

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Curriculum vitae

Zakia Kanwal was born on 19 June 1984 in Sialkot, Pakistan. She completed her secondary school education in 2002 at the Government College for Women in Daska and obtained her Bachelor of Science in 2004 at the Government College for Women in Sialkot. During her Bachelor education she studied Zoology, Chemistry, and Botany as majors. In 2006 she obtained her Master degree in Zoology at the University of Agriculture, Faisalabad, Pakistan. For her master thesis she performed a research project on feeding and roosting habits of rose-ringed parakeet and starling, under the supervision of Dr. H.A. Khan. In 2007 she finished the course program of the M.Phil in Zoology at the same university and obtained a scholarship from the Higher Education Commission of Pakistan to perform a PhD study at Leiden University, The Netherlands. In 2008 she obtained a Pre-PhD diploma in Biology from Leiden University, with a research training internship in the Molecular Cell Biology Group of the Institute of Biology, under the supervision of Dr. A.H. Meijer. During this internship she worked on transient expression of a fluorescent reporter construct for myeloid cells in zebrafish embryos. Subsequently, she started her PhD project in Nov. 2008 in the same group. Her PhD research, resulting in this thesis, was focused on regulatory mechanisms of innate immune signaling, employing the zebrafish embryo model.