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).

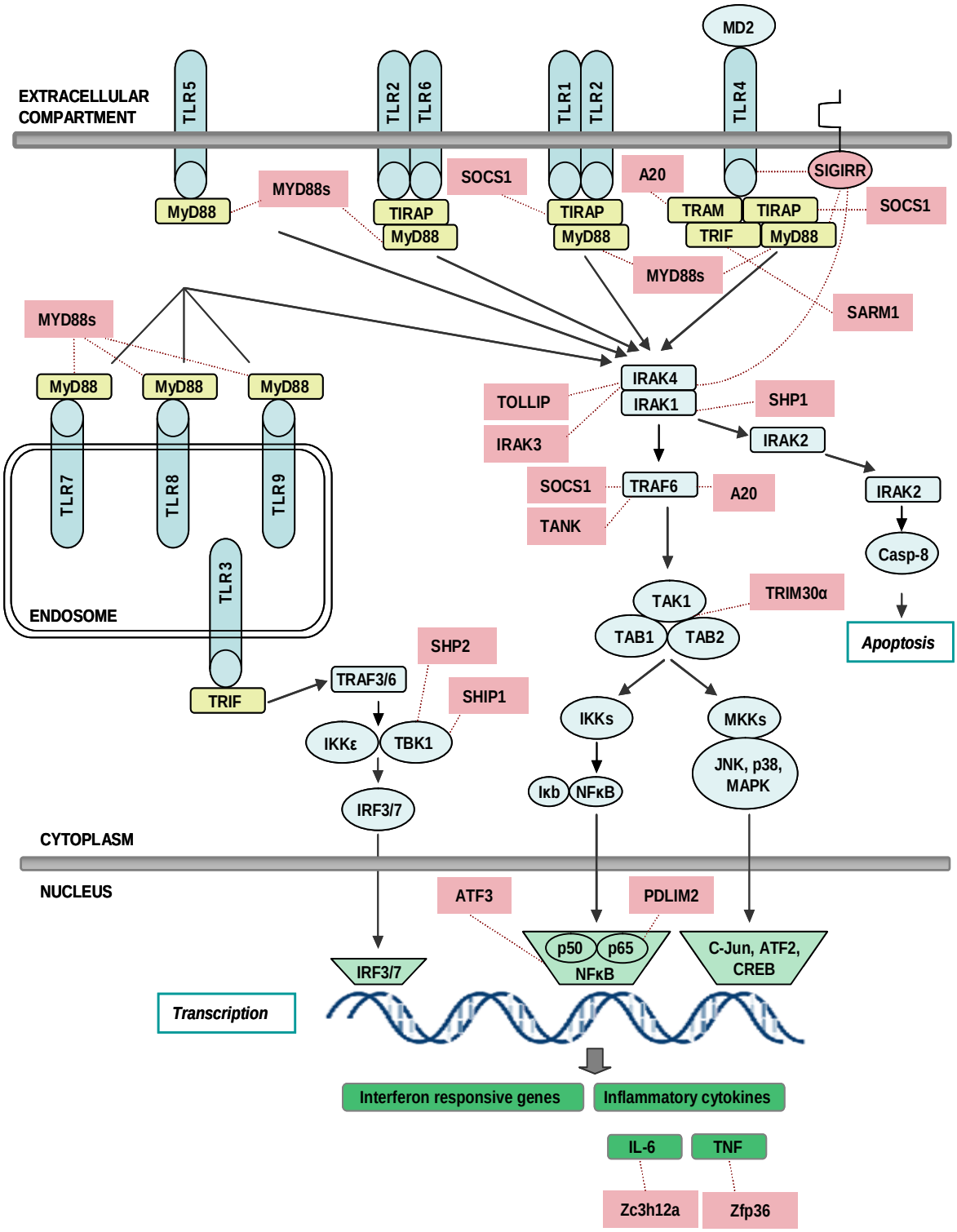

Figure 1. Negative regulation of the TLR pathway. Different negative regulators of the TLR pathway are shown in pink color around their target proteins. See text for details.

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.

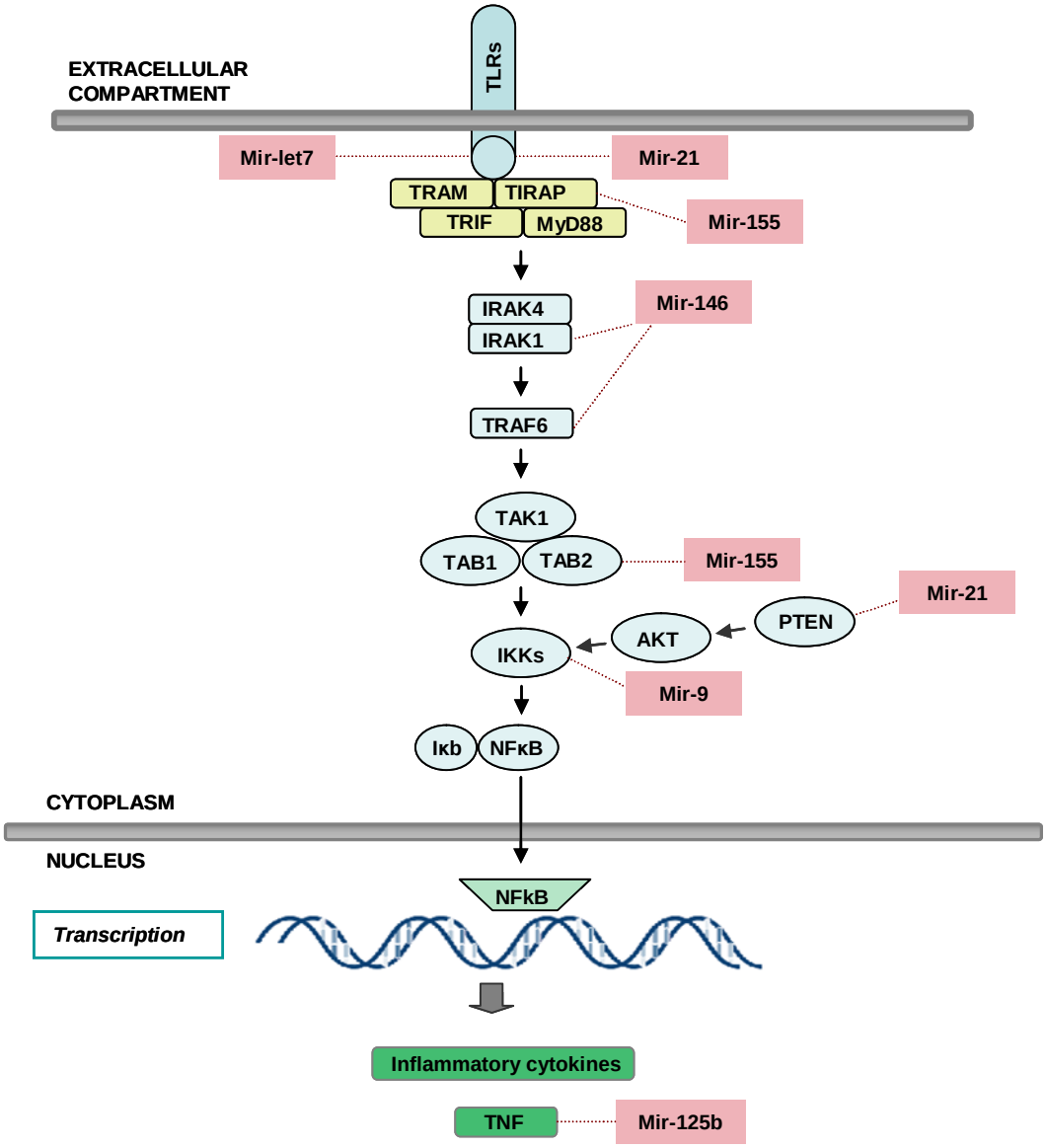

Figure 2. MiRNAs involved in negative regulation of the TLR Pathway. Immune-related miRNAs are shown in pink color around their target proteins. See text for details.

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).

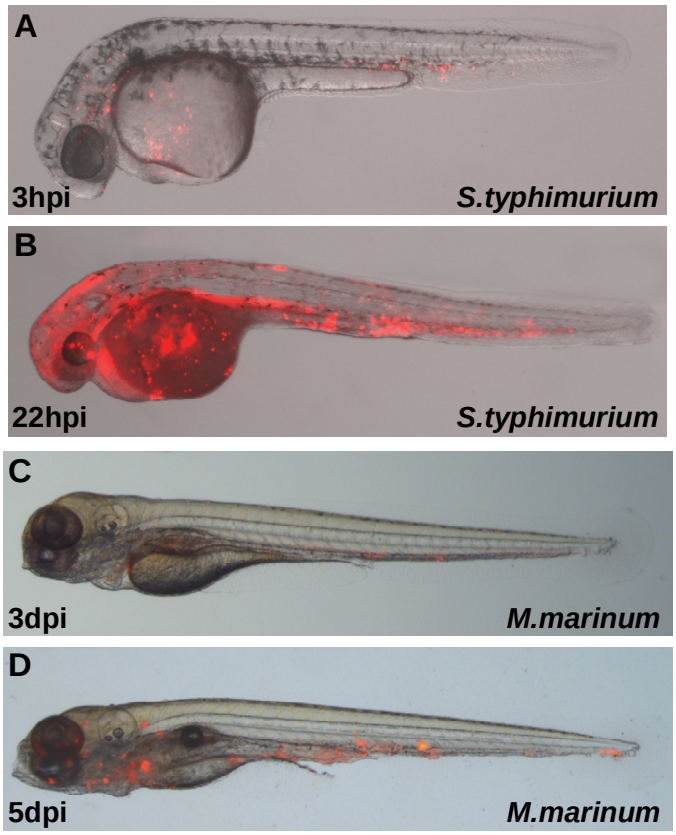

Figure 3. Disease progression in zebrafish larvae infected with *Salmonella typhimurium* and *Mycobacterium marinum.* Embryos were infected at 28 hpf with *S. typhimurium* (A, B) or *M. marinum* (C, D) by injection into the caudal vein. *S. typhimurium* rapidly proliferates and causes lethal infection 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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