

# **1** | **Introduction to the vertebrate innate immune system**



The immune system of animals is a complex composition of cellular and humoral components that protects the host against infectious diseases and cancer by identifying and killing pathogens and detrimental cells. To successfully protect the host, the immune system must be able to distinguish self from non-self and recognize danger signals. The concept of protecting self from non-self is already present in unicellular organisms, demonstrated by a repertoire of mechanisms ranging from the production of antimicrobial peptides to the employment of specific molecular systems protecting against foreign nucleic acids. With the appearance of multicellular organisms an increasingly complex immune system evolved. Distinct cells of the organism adopted specialized immune functions showing the ability to detect invading pathogens, migrate to sites of infection and eventually engulf and eliminate the encountered microorganisms. At the same time, soluble factors such as antimicrobial peptides and acute-phase proteins are used to combat the infection. To be able to recognize a wide variety of pathogens and detrimental cells various classes of receptors have evolved and diversified in different organisms. The most sophisticated immune system today exists in all higher vertebrates and combines a wide range of receptors with the development of immunological memory. This thesis focuses on the use of the zebrafish (*Danio rerio*) as a model to study the vertebrate immune system.

## The vertebrate immune system

Traditionally, the complex defence mechanisms of vertebrates are categorized into the evolutionary ancient innate immune system, that is present in all multicellular organisms, and the relatively young adaptive (acquired) immune system that arose with the appearance of jawed fish (*Chondrichthyes*) (1, 2). Both systems comprise humoral as well as cellular components that synergistically act upon infection. The innate immune system forms the first line of defence against pathogens. The elicited immune responses are rapid, occurring usually within minutes to hours. However, the innate immune mechanisms are considered relatively nonspecific and are mediated by a fixed set of germline-encoded receptors. These receptors are referred to as pattern recognition receptors (PRRs), showing a broad specificity against microbial-derived molecules such as lipopolysaccharide (LPS), flagellin or viral RNA. PRRs are mainly expressed by the cells from the myeloid lineage such as macrophages and dendritic cells (DCs). Activation of PRRs initiates signal transduction pathways that culminate in the production of inflammatory mediators to attract and communicate with other cells of the immune system (T-helper cells, for instance). In contrast, the adaptive immune system takes days to weeks to mount a response mediated by cytotoxic T-lymphocytes, which can recognize and kill infected cells or cancer cells, and B-lymphocytes (plasma cells), producing highly specific antibodies. T- and B-cells, presenting a diverse array of receptors on their surface, are generated by recombi-

nation of gene segments and clonal selection (3). The high affinity of antibodies to specific antigens is further achieved by somatic hypermutation in the gene segments encoding the variable regions of the antibody (4). Subsets of the activated B- and T-lymphocytes will be retained in lymphoid organs as memory cells. These cells can be reactivated by a recurring infection of a particular pathogen leading to a faster and highly specific immune response, thereby generating a long-lasting immunity against previously encountered pathogens. Although the two systems show obvious differences in the molecular tools used to battle infections, one should keep in mind that the innate and the adaptive immune systems in vertebrates are closely linked in their response to infectious microbes and that the innate immune system is pivotal for an accurate adaptive response. Given that the major focus of this thesis is on innate immunity, the components (both humoral and cellular) and the functions of the vertebrate innate immune system will be described in more detail in the following sections.

## Humoral components of the vertebrate innate immune system

### Acute-phase proteins

Sensing and killing of microbes can be achieved by several molecules of the innate immune system. A group of proteins, collectively termed acute-phase proteins (APPs), are greatly increased or decreased in the blood upon infection. Production and secretion of such proteins occurs in hepatocytes activated by cytokines such as TNF $\alpha$ , IL-6 and IL-1 (5). Acute phase proteins are a heterogenic group of proteins with either pro- or anti-inflammatory functions. A well known factor of the acute-phase response is the C-reactive protein (CRP), a member of the pentraxin family. CRP was shown to bind, among others, phosphocholine and phosphoethanolamine, which can be found on the cell surface of bacteria. CRP bound to macromolecules can lead either to opsonisation of bacteria (or apoptotic cells) or to the activation of the classical complement pathway described below (6).

### Complement system

Although some of the proteins of the complement pathway are also considered acute phase proteins, the complement system is a complex humoral mechanism on its own. Core proteins of the complement system are present as inactive enzyme precursors in the blood. Initial activation of the proteolytic complement cascade can be achieved via three independent pathways: the classical, the lectin and the alternative. The classical pathway is activated through C1q, a member of the collectin family, which can bind directly to the surface of a pathogen. In addition, activation of this pathway can also occur through binding of C1q to an antibody-antigen complex, linking this pathway to the adaptive immune system. A similar mode of complement activation is followed by the lectin pathway, where recognition of infectious microorganisms is mediated by the mannan-binding lectin protein. Conversely, the alternative pathway

is mediated by the spontaneous hydrolysis of complement component 3 (C<sub>3</sub>) and the subsequent formation of the C<sub>3</sub>bBb complex without participation of a specific pathogen-recognizing protein. Activation by any one of these pathways will lead to cleavage of C<sub>3</sub> to C<sub>3</sub>a and C<sub>3</sub>b by a C<sub>3</sub> convertase. The C<sub>3</sub> convertase has distinct compositions: a C<sub>4</sub>bC<sub>2</sub>b complex in the classical and lectin pathways, and a complex of C<sub>3</sub>b and activated factor B (Bb) in the alternative pathway (3).

The C<sub>3</sub>a fragment is a potent inflammatory mediator triggering vasodilatation and increasing permeability of small blood vessels. In addition C<sub>3</sub>a can induce oxidative burst in macrophages, neutrophils and eosinophils and leads to degranulation of mast cells and basophils, thereby sustaining the inflammation. The second product of C<sub>3</sub> cleavage, C<sub>3</sub>b, can bind to the cell surface of pathogens and either form additional C<sub>3</sub>bBb complexes that amplify the complement signal in the close proximity of the pathogen, or function as opsonin, enhancing phagocytosis. The latter is mediated by complement receptors on the surface of phagocytes (3).

Subsequent to C<sub>3</sub> cleavage, C<sub>3</sub>b can complex with C<sub>4</sub>bC<sub>2</sub>b to form a C<sub>5</sub> convertase leading to the release of C<sub>5</sub>a and C<sub>5</sub>b. The C<sub>5</sub>a fragment functions as a pro-inflammatory mediator in the same fashion as C<sub>3</sub>a. On the other hand, C<sub>5</sub>b leads to the assembly of the membrane attack complex. Essentially, this complex is composed of C<sub>9</sub> molecules that form a pore in the cell membrane of the pathogen, resulting in a loss of cellular homeostasis and free passage of host enzymes such as lysozyme, ultimately causing cell lysis (3).

### Antimicrobial peptides

The diverse group of small molecules (<100 amino acids) that are involved in the elimination of pathogenic microbes and enveloped viruses are collectively named antimicrobial peptides (AMPs) (7-9). In vertebrates AMPs can be classified into three groups: defensins, histatins and cathelicidins (10, 11). They are produced in various tissues and cell types such as Paneth cells of the intestine, lung epithelial cells and leukocytes (12-14). The precise mode of action of antimicrobial peptides is not fully understood, but involves membrane permeabilization and/or inhibition of protein and RNA synthesis (15).

### Cell-mediated vertebrate innate immunity

Various cell types of the myeloid lineage are responsible for the detection and clearance of infectious microorganisms, apoptotic cells and tumour cells. Furthermore, they possess an instructive role towards the adaptive immune system. In addition to the various myeloid cells, the natural killer (NK) cells, derived from a lymphoid precursor, are also considered part of the cellular innate immune system. In the following paragraphs, the function of individual cell-types, predominantly studied in rodents, will be discussed in more detail.

**Neutrophils**, also named neutrophil granulocytes, are the most abundant myeloid

cells in mammals and are major effectors of innate immunity. Together with basophil and eosinophil granulocytes they form the polymorphonuclear cell family. Neutrophils can efficiently phagocytose and kill internalized microbes in phagosomes through reactive oxygen species (ROS) and proteolytic enzymes. In addition, by exocytosis of their granules (degranulation), neutrophils can release a multitude of antimicrobial proteins and proteases that destroy pathogens extracellularly. Another (phagocytosis-independent) mechanism that is used by neutrophils to kill pathogens is the activation of neutrophil extracellular traps (NETs) (16). NETs are web-like structures composed of DNA, histone proteins and neutrophil elastase (a serine protease) that can trap and kill microbes. Both anti-bacterial and anti-fungal properties of NETs have been described (16, 17).

**Macrophages** are the predominant phagocytic cells of mammals. Immature macrophages derived from bone marrow circulate as monocytes through the blood. A subpopulation of these monocytes leave blood-circulation and migrate into the surrounding tissue where they can develop into resident macrophages such as osteoclasts (bone), microglia (CNS) or Kupffer cells (liver) depending on the tissue they inhabit. Monocytes and macrophages express various types of pattern recognition receptors on their cell surface and intracellularly, that allow these cells to respond effectively to diverse classes of pathogens. Cytokines produced by NK cells or tissue macrophages upon infection or tissue damage can trigger migration of additional monocytes from the blood to the site of the infection, where they differentiate into mature macrophages. Depending on the mode of activation triggered by diverse combinations of cytokines, macrophages can promote inflammation or even participate in wound healing (18). During the adaptive immune response macrophages are activated by T-cells, leading to increased production of ROS and activation of the autophagy pathway. Macroautophagy is a major defence mechanism against intracellular pathogens (19). Beside their function during inflammation, macrophages also contribute to maintaining homeostasis by removal of cell debris, apoptotic cells and erythrocytes (18).

**Dendritic cells** form, together with macrophages, the major antigen presenting cells (APC) of the mammalian immune system. Immature DCs are constantly patrolling the tissue, taking up pathogens and apoptotic cell fragments by macropinocytosis, eventually leading to antigen presentation on the cell surface. Encounter of pathogen-derived molecules by immature DCs leads to DC activation, maturation and pro-inflammatory cytokine secretion. Mature DCs migrate subsequently to nearby lymph nodes where antigen presentation takes place, inducing primary T-cell mediated immune responses (20).

**Mast cells** are predominantly found in mucosal and connective tissues of skin, gut and airways. Present as immature progenitors in the blood, they undergo final matu-

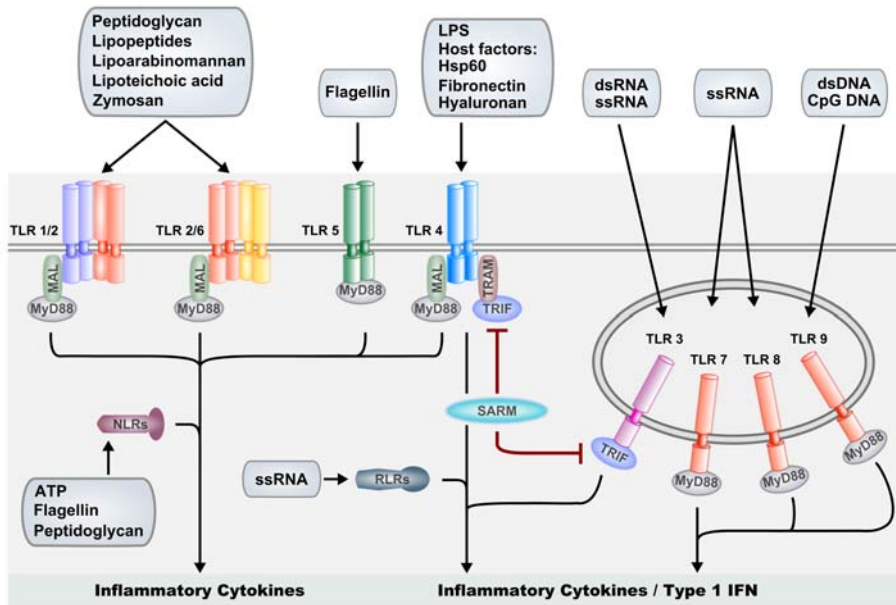
ration after tissue migration (21). Several pattern-recognition receptors are expressed on the cell surface of mast cells allowing direct sensing of invading pathogens (22). In addition to this direct activation, mast cells can also be activated indirectly via the complement system, leading to cytokine release and degranulation (22). Mast cell activation leads to a variety of effects, such as degradation of endogenous toxins, bactericidal activity, vasodilatation, T-cell activation and recruitment of neutrophils and DCs to the site of infection (23, 24). On top of their function in innate immune responses, mast cells have been extensively studied in the context of allergies (25).

**Natural Killer cells**, unlike the aforementioned cell types, derive from a common lymphoid precursor that also generates B- and T- lymphocytes of the cellular adaptive immune system (26). NK cells are able to target both virally infected and tumor cells, and destroy them by releasing cytotoxic molecules such as perforin and granzymes (27). NK cell function is orchestrated by various cell surface receptors that, upon activation, can either lead to inhibition or activation of exocytosis of the cytotoxic granules and lysis of the targeted cell. Inhibitory signals can be mediated via the CD94/NKG2A and CD94/NKG2B receptors that recognize specific major histocompatibility complex (MHC) class I surface proteins (28, 29). Abnormal or virally infected cells tend to down-regulate MHC class I proteins on the cell surface and hence lack an inhibitory signal (30, 31). At the same time these cells present activating signals such as MHC class I chain-related (MIC) molecule MICA that facilitate NK cell activation via NKG2D, a C-type lectin receptor (32, 33). Intercalation of the various signals eventually leads to the formation of the lytic immunological synapse between NK and target cells, cytotoxin release and lysis of the infected cell (34).

### Pathogen monitoring by pattern-recognition receptors

Recognizing potentially harmful microorganisms is an essential first step in the initiation of an immune response. The innate immune system relies on the recognition of highly conserved structural components of microbes, often referred to as pathogen-associated molecular patterns (PAMPs) or microbial-associated molecular patterns (MAMPs). PAMPs or MAMPs are usually essential for microbial survival, hence a constant factor for the host to detect. Examples are bacterial cell-wall components such as LPS and peptidoglycan, flagellin from bacterial flagella or viral RNAs. The receptors involved in PAMP recognition, PRRs, are widely expressed on the cells of the innate immune system such as macrophages and DCs, and on non-immune cells that are likely to encounter pathogens, such as epithelial cells. Several families of PRRs have been described in vertebrates, including the Toll-like receptor (TLRs), NOD-like receptor (NLR) and RIG-I-like (RLRs) receptor families (35-37).

The TLRs are the best studied and probably most essential receptors of the vertebrate innate immune system. The TLRs are named after the *Toll* receptor from *Drosophila melanogaster*, which in 1985 was described as having an essential role in



**FIGURE 1.** Schematic overview of mammalian PRRs and their associated ligands. TLRs are expressed on the cell surface and on endosomal membranes and act cooperatively with cytoplasmic receptors of the NLR and RLR families to induce expression of inflammatory cytokines and type I interferons. Differential use of adaptor proteins (Myd88, Mal, TRIF, TRAM and SARM) is indicated for the TLR family members. Figure adapted from T. Mogensen (54).

dorsal-ventral polarity determination (38). More than a decade later Lemaître *et al.* unravelled a function of *Toll* in the antifungal immune response of *D. melanogaster* and shortly afterwards mammalian TLR4 was identified as the receptor that mediates LPS signalling (39, 40).

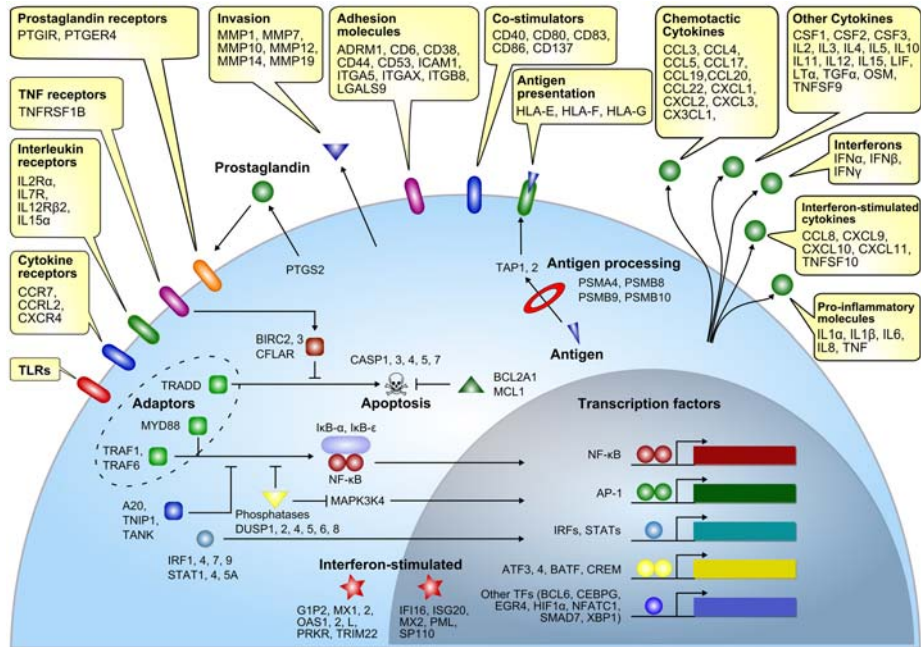
Today, a lot is known about TLR activation and downstream signalling events in mammals. All TLRs are germline-encoded type I transmembrane receptors characterized by a highly variable extracellular leucine-rich repeat (LRR) domain, involved in ligand recognition, and an intracellular tail, containing the conserved Toll/Interleukin-1 receptor (TIR) domain, mediating association of TLRs with downstream signalling intermediates. A set of 10 TLRs have been described in human so far, showing distinct specificity to various PAMPs. For instance, TLR3, -7, and -8 recognize double- and/or single-stranded RNA, whereas TLR-9 recognizes bacterial DNA. TLR4 has been shown to recognize LPS and TLR5 is specific for bacterial flagellin. Heterodimers of TLR2 with TLR1 or TLR6 are able to recognize various lipoproteins and glycolipids from gram-positive bacteria (Fig.1) (41-45). In accordance with their ligand specificity TLR3, -7, -8 and -9 are located in the endolysosomal



compartments, whereas the TLR<sub>1</sub>, -2, -4, -5 and -6 are located on the cell surface (46). Activation of the TLRs initiates distinct signalling pathways that result in the accumulation of pro-inflammatory cytokines and type I interferons (IFNs). A group of 5 adaptor proteins (MyD88, MAL, TRIF, TRAM and SARM), binding to the various TLRs upon activation, are differentially used by TLRs to mediate a tailored response. MyD88 is the most commonly used adaptor, and signalling through MyD88 has been reported for all human TLRs with the exception of TLR<sub>3</sub>, that instead shows a TRIF-dependent signalling route (47, 48). While TLR<sub>5</sub>, 7, 8 and 9 signal through MyD88 alone, TLR<sub>2</sub>/TLR<sub>1</sub> and TLR<sub>2</sub>/TLR<sub>6</sub> heterodimers require MAL as an additional adaptor to link MyD88 to the receptor complex. TLR<sub>4</sub> signalling can lead to activation of pro-inflammatory cytokine genes via a MyD88/MAL-dependent pathway, and can result in type I INF production via a MyD88-independent pathway that utilizes the adaptors TRAM and TRIF (49). The fifth TIR-domain adaptor, SARM, has been proposed to function as a negative regulator of TRIF, but was also shown to positively regulate the response to viral infection in brain cells (50, 51). Downstream of the TLR-adaptors, signals are relayed via TNF-receptor associated factors TRAF6 and TRAF3, activating downstream kinases which eventually lead to gene induction through the nuclear factor kappaB (NF- $\kappa$ B) and interferon response factor (IRF) families of transcription factors, and to MAP kinase (MAPK) signalling pathways activating the AP-1 (JUN/FOS) transcription factor complex (Fig.1) (52, 53).

Whereas the TLRs are located on the cell surface and endolysosomal membranes, the members of the NLR family are predominantly distributed in the cytosol of the cell, where they are primarily involved in bacterial recognition (35). The NLR proteins consist of an N-terminal effector domain, a central nucleotide-binding oligomerization domain (NOD) and a C-terminal LRR domain. The N-terminal domain facilitates signalling through downstream partners, whereas the LRR domain is necessary for PAMP detection. Similarly to the TLRs, NLRs can be activated by microbial compounds such as flagellin and peptidoglycan. In addition, NLRs are responsive to bacterial toxins and crystals as well as to endogenous danger signals. By analogy with PAMPs, these danger signals, of which extracellular ATP is a good example, are referred to as DAMPs (danger associated molecular patterns). As is the case of TLRs, triggering of NLRs can lead to NF- $\kappa$ B and MAPK pathway activation that in turn leads to production of cytokines and anti-microbial proteins. Furthermore, NLR signalling can lead to activation of the inflammasome that is required for secretion of active IL-1 $\beta$  and IL-18. These potent pro-inflammatory cytokines are produced as inactive precursors through TLR pathway activation, and caspase-1 mediated processing in the inflammasome is required for their activation (35). Thus, a robust inflammatory response is dependent on the cooperative action of TLRs and NLRs (Fig.1).

Similarly to NLRs, RLRs are also located in the cytoplasm. Together with TLR<sub>3</sub>, -7 and -8 the RLRs provide viral recognition and mount a robust induction of type I INF. Activation of RLRs initiates the activation of NF- $\kappa$ B and IRF3 (36).



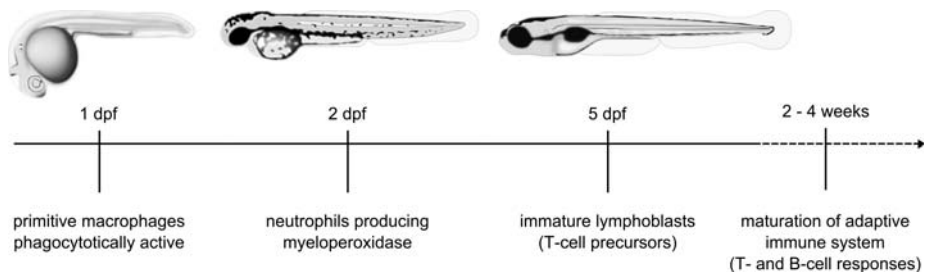
**FIGURE 2.** The common host immune response. Expression of genes indicated in the figure is induced by a variety of pathogens in human cell cultures. Figure adapted from Jenner and Young (57).

In addition to the above mentioned PRRs, a role in the innate immune defence is also played by the C-type lectin receptor (CLRs) family and the scavenger receptor family. C-type lectin receptors are expressed on such cells as DCs, where they can detect fungi, bacteria and viruses through the recognition of mannose, fucose and glucan carbohydrates. Several CLRs, such as DC-specific ICAM<sub>3</sub>-grabbing non integrin (DC-SIGN), have been shown to modulate TLR signalling. Dependent on the pathogen involved, both cooperative and antagonizing interactions between CLR and TLR signalling have been found. CLR signalling has furthermore been implicated in the tailored activation of T-cell subsets (55). Finally, scavenger receptors have been shown to participate in TLR signalling as TLR co-receptors; these mediate, in addition, non-opsonic phagocytosis of pathogenic microbes (56).

### Common factors of the host response to infection

Activation of PRRs leads to the induction of a transcriptional response, priming the host to adequately respond to the encountered pathogen. In a comprehensive review study, Jenner and Young performed a meta-analysis on transcriptome data from various infection studies of different cells types with different pathogens, leading to the identification of a gene set that is commonly regulated upon infection. Among

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**FIGURE 3.** Schematic overview of the appearance of myeloid and lymphoid cells along zebrafish embryogenesis.

the identified genes were pro-inflammatory mediators (TNF, IL1 $\beta$ , IL6 and IL8), chemotactic and interferon stimulated cytokines (CCL3, CXCL1, CCL8), tissue invasion proteins (MMP1, MMP14), cell adhesion proteins (CD6, ICAM1), signalling adaptors (MyD88, TRAF6) and various transcription factors (NF- $\kappa$ B, AP1, STATs and IRFs), as shown in figure 2 (57).

### Zebrafish embryos as a model to study vertebrate immunity

In recent years the zebrafish (*Danio rerio*) embryo system has emerged as a new model to study vertebrate innate immunity, offering several advantages that complement mammalian model systems. The transparent character of the externally fertilized zebrafish embryo in combination with fluorescently-labelled immune cells and bacteria facilitate the study of host-microbe interaction and inflammation processes in the living organism (58-64). The efficiency at which infections and chemical treatments in zebrafish can be performed at a large-scale allows identification of novel microbial virulence factors and high-throughput compound screens to investigate disease mechanisms (65, 66). Moreover, the zebrafish system is particularly suitable for large-scale forward and reverse genetic screens aimed at the identification of genes with novel functions in the development of the immune system or in the immune response (67-69).

Like other vertebrates the zebrafish has a primitive and definitive wave of hematopoiesis giving rise to various cell types of the erythroid, lymphoid and myeloid lineage. During development, hematopoiesis occurs at several temporal locations in the embryos, finally shifting to the kidney marrow, which is equivalent to the mammalian bone marrow. Similarly to other vertebrates, adult zebrafish possess T- and B-cells, macrophages, neutrophils, eosinophils, basophils, mast cells and probably NK cells (70). Cells of the innate immune system are detectable as early as the first day of zebrafish development (71). These primitive macrophages are able to phagocytose bacteria and foreign material (72, 73). Functional neutrophils, producing the myeloperoxidase enzyme, are present from the second day of embryogenesis. By contrast, a functionally mature adaptive immune system is not active during the first

three weeks of zebrafish development (58, 74, 75). This clear temporal separation in zebrafish embryos provides a convenient system for *in vivo* study of the vertebrate innate immune response to infection, independently from the adaptive immune response (Fig.3). In recent years, numerous bacterial and viral infection models have been established for the zebrafish to study host-pathogen interaction, chemotactic responses and inflammation processes (62, 76-78).

### Zebrafish pattern-recognition receptors and innate immune response activation

Genome analysis revealed one or more homologs of the human *TLR* genes (*TLR1*, -2, -3, -4, -5, -7, -8 and -9) to be present in zebrafish, as well as a group of fish-specific TLRs (79, 80). The zebrafish genome is also known to contain four of the downstream adaptor protein genes (*MyD88*, *TRIF*, *MAL* and *SARM*), while the fifth TLR adaptor TRAM remains to be identified (79, 80). Several other genes of the TLR signalling cascade have been identified in the zebrafish genome, as well as members of the NLR family and the downstream adaptor of the RLR family IPS-1 (81-83). TLR and adaptor genes are broadly expressed during zebrafish embryogenesis, even prior to the appearance of the first innate immune cells (84). Challenge of zebrafish embryos with different pathogens activates the expression of a wide range of innate immune response genes strongly conserved with those in mammals (85). In addition to the cell-mediated innate immune response, zebrafish have a well developed complement system and produce acute-phase response proteins such as hepcidin and fibrinogen (78).

A conserved TLR ligand specificity was demonstrated between human and zebrafish for TLR5 (Chapter 3). Stimulation of zebrafish embryos with purified flagellin led to transcriptional activation of distinct host defence genes such as interleukin-1 $\beta$  (*il1b*), matrix metalloproteinase 9 (*mmp9*) and cxc chemokine ligand C1c (*cxcl-C1c*) *in vivo* that were significantly impaired after knock-down of the *tlr5a* and *tlr5b* genes (85). In contrast, the zebrafish counterparts of TLR4 (Tlr4a and Tlr4b) were reported to be non-responsive to LPS, suggesting a rendered ligand specificity or function in zebrafish (86, 87). While the alternative ligand specificities of zebrafish Tlr4a and b remain unknown, Sullivan *et al.* demonstrated that the intracellular portions of Tlr4a and b have the capacity to activate NF- $\kappa$ B signalling. This is in contrast with data from Sepulcre *et al.*, who reported negative regulation of NF- $\kappa$ B by Tlr4b (87).

One of the best studied TLR adaptors in mammals is MyD88. Myd88 transcript was detected in cells of the myeloid lineage in the head, on the yolk sac, the trunk and the posterior blood island of zebrafish embryos. Myd88-positive leukocytes contribute to inflammatory responses and were able to phagocytose bacteria (88). Knock-down studies in zebrafish embryos revealed an essential function of Myd88 during *Salmonella* infection, showing a strongly impaired response to an otherwise non-pathogenic *Salmonella* strain (Chapter 2, 84). Furthermore, challenge of *myd88* morphants by a pathogenic *Salmonella* wild-type strain revealed activation

of Myd88-dependent and -independent signalling pathways, as are also present in mammals (Chapter 3, 85). In addition, like in mammals the zebrafish homolog of TRIF has been shown to play an essential role in antiviral immunity (89). However, the precise mechanisms of NF $\kappa$ B and IFN activation upon viral infection appear to have diverged between fish and mammals. Therefore, while there is a large similarity of TLR receptors and downstream mediators between fish and mammals, further studies should clarify to what extent the TLR ligand specificities and downstream signal transduction mechanisms are conserved.

## Outline of this thesis

In the work described in this thesis we make use of the zebrafish embryo to study vertebrate innate immune responses to systemic bacterial infections in general, and to assess the role of the TLR signalling pathway in the innate immune response in particular. To model systemic infections we use the Gram-negative enterobacteria *Salmonella enterica* Serovar Typhimurium (*S. typhimurium*), the cause of human salmonellosis.

In **Chapter 2** the fundamental and conserved role of Myd88 in the zebrafish embryonic innate immune response is demonstrated. Using a morpholino-based knock-down approach we show that Myd88-mediated signalling events are crucial in mounting a sufficiently strong immune response to clear an infection with a non-pathogenic *S. typhimurium* strain.

**Chapter 3** presents a time-resolved transcriptome analysis of the inflammatory and innate immune responses elicited by zebrafish embryos to a systemic infection with a pathogenic and non-pathogenic *S. typhimurium* strain. The transcriptional response to infection with both strains shows clear conservation with host responses detected in other vertebrate models and human cells, including induction of genes encoding cell surface receptors, signalling intermediates, transcription factors and inflammatory mediators. Extending the work of chapter 2 we show that *Salmonella* infection is mediated by Myd88-dependent and -independent signalling events. Additionally, we demonstrate that gene induction by flagellin is mediated by Tlr5 in zebrafish embryos, indicating that ligand specificity for this member of the TLR family is conserved between human and zebrafish.

**Chapter 4** is focused on the immune function of the zebrafish homolog of mammalian TRAF6, an important downstream mediator of the TLR pathway, demonstrating that *traf6* knock-down leads to a strongly decreased transcriptional immune response upon systemic *Salmonella* infection. Among the Traf6-dependent genes is not only a large set of well known anti-microbial and inflammatory genes but also several genes whose role in the immune system was not previously expected to be Traf6-dependent. One such example is the fertility hormone gene *GnRH*.

Finally, in **Chapter 5** an effort is made to define the role of Traf6 in the early zebrafish development using a microarray-based approach. Comparison of the genes

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that were dependent on Traf6 during early development with the Traf6-dependent immune genes from chapter 4 revealed only a minor overlap, indicating the diverse functions of Traf6 during infection and development.

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