Autophagy and Lc3-associated phagocytosis in host defense against Salmonella

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Chapter 2

Modelling infectious diseases in the context of a developing immune system

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Abstract

Zebrafish has been used for over a decade to study the mechanisms of a wide variety of inflammatory disorders and infections, with models ranging from bacterial, viral, to fungal pathogens. Zebrafish has been especially relevant to study the differentiation, specialization and polarization of the two main innate immune cell types, the macrophages and neutrophils. The optical accessibility and the early appearance of myeloid cells that can be tracked with fluorescent labels in zebrafish embryos and the ability to use genetics to selectively ablate or expand immune cell populations have permitted studying the interaction between infection, development and metabolism. Additionally, zebrafish embryos are readily colonized by a commensal flora, which facilitated studies that emphasize the requirement for immune training by the natural microbiota to properly respond to pathogens. The remarkable conservation of core mechanisms required for the recognition of microbial and danger signals and for the activation of the immune defenses illustrates the high potential of the zebrafish model for biomedical research. This review will highlight recent insight that the developing zebrafish has contributed to our understanding of host responses to invading microbes and the involvement of the microbiome in several physiological processes. These studies are providing a mechanistic basis for developing novel therapeutic approaches to control infectious diseases.

Introduction

Infectious diseases remain a major global health problem, with tuberculosis (TB) and HIV/AIDS as the biggest killers, each responsible for over a million deaths annually according to reports of the World Health Organization (www.who.int). The increasing occurrence of multidrug-resistant strains of *Mycobacterium tuberculosis*, the bacterial pathogen causing TB, indicates that current antibiotic treatment regimens are ineffective. Antibiotic resistances represent a serious problem also in hospital settings, with methicillin-resistant *Staphylococcus aureus* as a notable example of a pathogen causing opportunistic infections in immunocompromised patients. Despite intense research efforts, there are no effective vaccines against some of the major human bacterial pathogens, including *M. tuberculosis* and *S. aureus*. Furthermore, vaccines are not yet available for newly emerging viral diseases, which can spread rapidly due to transmission by insect vectors, as exemplified by the recent Zika virus outbreak. Development of novel therapeutic approaches for the treatment of infectious diseases requires detailed understanding of the mechanisms by which pathogens subvert the immune system of the infected host. As we discuss in this review, the zebrafish is a valuable addition to the range of animal models used for preclinical research into infectious disease biology.

The immune system of vertebrates functions by cooperative mechanisms of innate and adaptive immunity. During infection, innate immunity is activated by the recognition of microbial molecules and danger signals released by damaged host cells. Across species, innate immunity is mediated primarily by phagocytic cells, including macrophages, neutrophils and dendritic cells. Activated innate immune cells represent an important line of defense against a large spectrum of pathogens as they provide an immediate response to invading microbes. Additionally, cells of the innate immune system, by functioning as antigen presenting cells and by providing stimulatory signals, are essential to alert the adaptive immune system to mount a more specific immune response mediated by antibody-producing B-lymphocytes and cytotoxic T-lymphocytes. These cells collaborate to target, isolate or kill infected cells to prevent infection spreading throughout the organism.

Developing organisms rely more heavily on innate immunity, because the adaptive immune system takes longer to mature. For instance, it is well known that human neonates depend on maternal antibodies for adequate protection against infectious diseases. In zebrafish larvae, the first immature T-cell precursors are the first signs of an adaptive immunity, detected by 3 days post fertilization (dpf) (Langenau et al., 2004), however, functional phagocytes are present earlier, at 1 dpf **(Figure 1)** (Herbomel et al., 1999). B cells emerge from the pronephros of juvenile zebrafish only at 19 dpf and (Langenau et al., 2004) and antibody production does not occur until at least 21 dpf (Page et al., 2013). As a result, the zebrafish embryo and early larval stages have become widely used as an in vivo model to study innate immunity in separation from adaptive immunity (Harvie & Huttenlocher, 2015; Levraud et al., 2014; Meijer & Spaink, 2011; Ramakrishnan, 2013; Renshaw & Trede, 2012).

Figure 1: Development of zebrafish immune system. *(figure on next page).* In zebrafish, immune cells are generated via a primitive, intermediate and definitive wave of hematopoiesis, which are active in the indicated tissues in the developmental windows reported on the timeline. The figure also indicates the key transcriptional regulators controlling the differentiation fate and the distinctive markers expressed by each cell type (described in more detail in the main text). Abbreviations: Anterior lateral mesoderm (ALM), Posterior lateral mesoderm (PLM), Rostral blood island (RBI), Intermediate cell mass (ICM), Posterior blood island (PBI), Aorta-gonad-mesonephros (AGM), Ventral wall of dorsal aorta (VDA), Caudal hematopoietic tissue (CHT), Head kidney (HK), Myeloid progenitor cell(MPC), Erythromyeloid progenitor (EMP), Hematopoietic stem cells (HSC), Common myeloid progenitor (CMP).

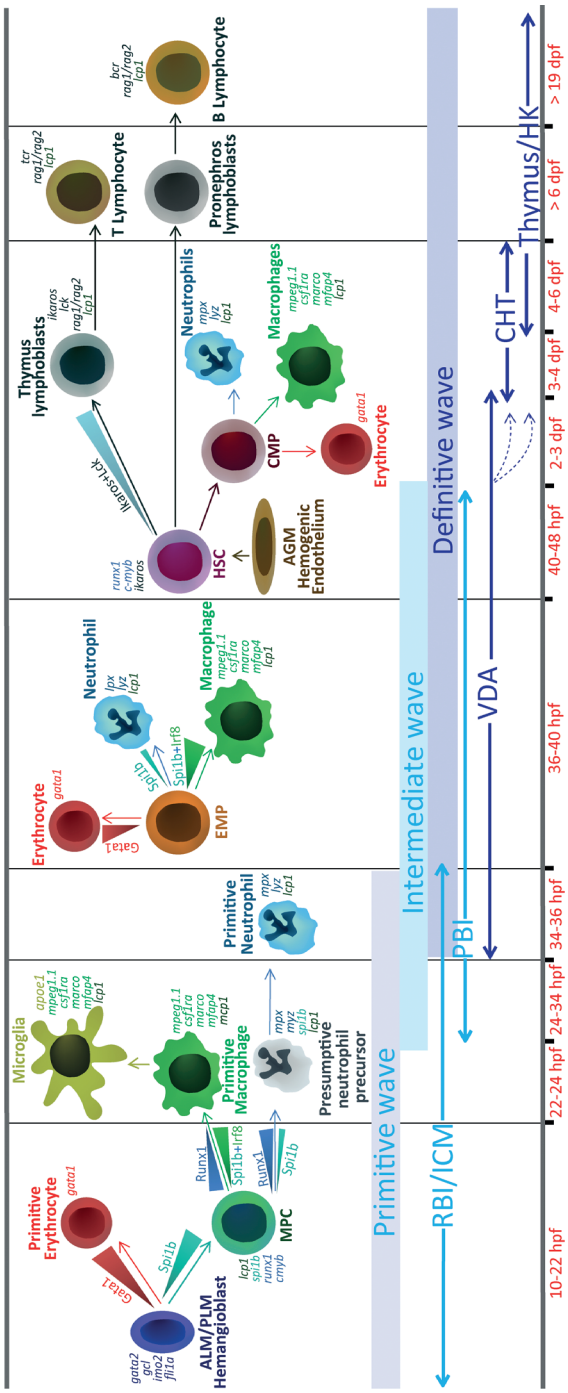

Figure 1: Development of zebrafish immune system. *(figure legend on previous page).*

The different cell types of the immune system are generated by hematopoiesis, defined as the differentiation of multipotent, self-renewing stem cells into all cellular components of the blood (Davidson & Zon, 2004; Jagannathan-Bogdan & Zon, 2013). In all vertebrates, hematopoiesis is a highly conserved process that involves successive waves of primitive, intermediate, and definitive generation of hematopoietic progenitor cells during ontogeny **(Figure 1)** (Bertrand et al., 2007; Galloway & Zon, 2003). Hematopoiesis can be further differentiated into erythropoiesis (the development of red blood cells), myelopoiesis (the development of leukocytes mediating innate immunity), and lymphopoiesis (the generation of the leukocytes (lymphocytes) of the adaptive immune system). Myeloid cells consist of two main categories based on cellular contents: (i) granulocytes and (ii) agranulated cells. Granulocytes (including neutrophils, eosinophils, basophils, and mast cells) display characteristic secretory granules in the cytoplasm containing antimicrobial molecules and inflammatory mediators. Furthermore, granulocytes can be recognized by a polymorphic nucleus, while agranulated cells, including monocytes and macrophages, are mononuclear.

In zebrafish embryos and early larval stages, all mononuclear cells are commonly referred to as (primitive) macrophages, irrespective of whether these cells are circulating in the blood or have invaded tissues (Herbomel et al., 1999; Herbomel et al., 2001). The specialized macrophages resident in the brain (microglia) are also already present in the early life stages of zebrafish and their progenitors can be distinguished as early as 1 dpf **(Figure 1)**. Neutrophils are the main granulocyte cell type in embryos and larvae (Lieschke et al., 2002). Mast cells can also be distinguished, but eosinophils are only described in adult zebrafish and basophils have not been identified (Balla et al., 2010; Dobson et al., 2008).

In this review, we describe how innate immune cell types arise during the normal course of zebrafish embryo and larval development, and how the production, differentiation and function of these cells can be affected by infection, inflammation and the presence of the gut microbiota. We discuss recent studies that show how innate immune responses are intricately linked with the regulation of energy metabolism and homeostasis, in which autophagy plays a major role. Furthermore, we review work that contributed to develop zebrafish infection models **(Table 1)**, which has

been particularly helpful to dissect the specific implications of different innate immune cell types in infectious disease pathologies. To illustrate this, we highlight recent studies of bacterial infections, including causative agents of human infectious diseases or opportunistic infections, such as Mycobacteria, Listeria, Shigella, Staphylococci and a range of viral, and fungal pathogens. These studies are providing new insight into host-pathogen interaction mechanisms that hold promise for translation into novel therapeutic strategies for human infectious diseases.

Table 1: Human infection diseases modelled in zebrafish.

Infectious agents	Human disease	Zebrafish infection model	First description
Bacteria	Tuberculosis	*Mycobacterium marinum* surrogate model for *Mycobacterium tuberculosis*	Davis et al., 2002
	Salmonellosis	*Salmonella enterica* serovar Typhimurium	van der Sar et al., 2003
	Shigellosis	*Shigella flexneri*	Mostowy et al., 2013
	Listeriosis	*Listeria monocytogenes*	Levraud et al., 2009
	Opportunistic infections	*Burkholderia cenocepacia*	Vergunst et al., 2010
		Pseudomonas aeruginosa	Clatworthy et al., 2009
		Staphylococcus aureus	Prajsnar et al., 2008
Viruses	Influenza	Influenza A virus	Gabor et al., 2014
	Herpes Simplex	Herpes simplex virus type 1	Burgos et al., 2008
	Chikungunya fever	Chikungunya virus	Palha et al., 2013
Fungi	Candidiasis	*Candida albicans*	Chao et al., 2010
	Aspergillosis	*Aspergillus fumigatus*	Knox et al., 2014

Infectious agents	Human disease	Zebrafish infection model	First description
	Mucormycosis	*Mucor circinelloides*	Voelz et al., 2015
	Cryptococcosis	*Cryptococus neoformes*	Tenor et al., 2015

Development of the cell types of the innate immune system

To understand how the immune system works, we must first understand how the cells in the innate immune system form, and zebrafish have provided an outstanding system for such studies. This is covered in depth elsewhere (Kawan & Trista, 2017). Here, we review the developmental aspects of innate immunity that are relevant to understanding the response to infection.

Generation of primitive myeloid cells

The development of the zebrafish immune system mirrors processes observed in other vertebrates, including mammals, but at an accelerated scale **(Figure 1)**. The first innate immune cells of the zebrafish embryo are generated during primitive hematopoiesis, which occurs in two locations of the zebrafish embryo: the anterior lateral mesoderm (ALM) and posterior lateral mesoderm (PLM). As the development proceeds, the ALM and PLM differentiate into the rostral blood island (RBI) and intermediate cell mass (ICM), respectively (Bertrand et al., 2007). The primitive myeloid cells develop from the RBI, while primitive erythrocytes originate from the ICM. By the 6-somite stage, expression of *spi1b* (*pu.1*) is detected, which encodes Pu.1, a master transcriptional regulator of myelopoiesis (Lieschke et al., 2002; Rhodes et al., 2005). By 16 hours post fertilization (hpf), Pu.1 positive myeloid progenitors originating from the RBI start to migrate over the yolk sac **(Figure 1)** (Bennett et al., 2001; Lieschke et al., 2002). This process requires granulocyte colony-stimulating factor receptor (Gcsfr) signaling (Liongue et al., 2009). During migration, these myeloid progenitors turn on the pan-leukocyte marker L-plastin (*lcp1*) (Bennett et al., 2001; Herbomel et al., 1999; Herbomel at al., 2001; Liu & Wen, 2002). Morphologically distinguishable macrophages are observed as

early as 22 hpf on the yolk sac and enter the blood circulation by 26 hpf. Some macrophages migrate into the cephalic mesenchyme from 22 hpf onwards in a *csf1ra* dependent manner and can eventually develop into microglia (Herbomel et al., 2001; Peri & Nusslein-Volhard, 2008). These macrophages are functional, and are capable of phagocytosing apoptotic debris, senescent red blood cells and experimentally injected bacteria (Herbomel et al., 1999). Thus, as early as 1 dpf, zebrafish embryos can be used to study the response to infection.

The genes *csf1ra*, *mpeg1.1*, *marco*, and *mfap4* are marker genes that are predominantly expressed in macrophages in comparison with other leukocytes (Benard et al., 2014; Ellett et al., 2011; Walton et al., 2015; Zakrzewska et al., 2010). Several of these markers have been used to generate transgenic reporter lines that are frequently used in infectious disease research **(Table 2)** (Ellett et al., 2011; Gray et al., 2011; Walton et al., 2015).

Morphologically distinguishable neutrophils appear later than macrophages (Le Guyader et al., 2008). Using an *in vivo* photoactivatable cell tracer, it has been demonstrated that primitive neutrophils originate from the RBI-derived hemangioblasts, the same lineage as the primitive macrophages, after the dispersal of the progenitors into the tissues **(Figure 1)** (Le Guyader et al., 2008). At 34 hpf, differentiated neutrophils are detectable by electron microscopy (Willett et al., 1999). In agreement, granules are observed under video-enhanced differential interference contrast microscopy around 35 hpf, a time when neutrophils can also be detected by staining with Sudan Black, a lipid marker for granules (Le Guyader et al., 2008). Sudan Black-positive neutrophils also stain positive for myeloperoxidase (Mpx) enzyme activity (Le Guyader et al., 2008; Lieschke et al., 2001) as early as 24 hpf, along with expression of the other neutrophil marker lysosome C (*lyz*) (Le Guyader et al., 2008; Meijer et al., 2008). Transgenic reporter lines for the *mpx* and *lyz* marker genes are widely used to study neutrophil behavior **(Table 2)**, (Hall et al., 2007; Renshaw et al., 2006). The *mpx*/*lyz*-positive phagocytes first appear as migrating cells on the yolk sac, and these are most likely progenitors of the neutrophils that can be detected in tissues of older embryos using Sudan Black staining (Harvie & Huttenlocher, 2015; Le Guyader et al., 2008)

Table 2: Markers for cell types of the zebrafish innate immune system.

Cell type	Transgenic marker[1]	Gene marker	Antibody/Cell staining	Functional assay
Pan-leukocytic	-	*lcp1*	anti-L-plastin	Morphological and functional characterization of macrophages and neutrophils.
	Tg(coro1a:EGFP)	*coro1a*	-	
Myeloid cell precursors	*Tg(-5.3spi1b:EGFP)* *Tg(-9.0spi1b:EGFP)* *Tg(-4spi1b:Gal4)* *Tg(-4spi1:LY-EGFP)*	*spi1b/pu.1*	-	Marker of macrophage and neutrophil precursors
Macrophages	*Tg(mpeg1:EGFP)* *Tg(mpeg1:Gal4-VP16)* *Tg(mpeg1:mCherry-F)* *Tg(mpeg1:Dendra2)*	*mpeg1.1*	-	Specific marker of macrophages, but down-regulated by several infections; also labels microglia
	TgBAC(csf1ra:Gal4-VP16)	*csf1ra/fms*	-	Specific marker of macrophages; also labels non-motile pigment cells (xanthophores)
	Tg(mfap4:dLanYFP-CAAX) *Tg(mfap4:mTurquoise)*	*mfap4*	-	Specific marker of macrophages; less sensitive to infection down-regulation than *mpeg1.1*
Neutrophils	*TgBAC(mpx:EGFP)* *Tg(mpx:GFP)* *Tg(mpx:mCherry)* *Tg(mpx:EGFP-F)* *Tg(mpx:DsRed-F)* *Tg(mpx:Dendra2)*	*mpx*	anti-Mpx/Mpx enzyme activity staining	Specific marker of neutrophils

Cell type	Transgenic marker[1]	Gene marker	Antibody/Cell staining	Functional assay
	Tg(lyz:EGFP) *Tg(lyz:DsRed2)* *Tg(lyz:Gal4-VP16)*	*lyz/lysc*	-	Specific marker of neutrophils; some overlap with macrophages at early developmental stages
	-	-	Sudan black	Staining of neutrophil granules
Activated macrophages/ neutrophils	*Tg(il1b:GFP-F)*	*il1b*	anti-Il1b	Reporter to distinguish inflammatory phenotypes of macrophages (M1) and neutrophils
	Tg(tnfa:eGFP-F)	*tnfa*	-	Marker for activated macrophages (M1)
	Tg(irg1:EGFP)	*irg1*	-	Marker for activated macrophages (M1)
	Tg(CMV:EGFP-map1lc3b)	*map1lc3b*	-	Marker for autophagy activation

Cell type	Transgenic marker[1]	Gene marker	Anti-body/Cell staining	Functional assay
	Tg(Myd88:EGFP) *Tg(Myd88:Dsred2)*	*myd88*	-	Marker for TLR signaling potential
	Tg(NFκB:EGFP)	*nfκB*	-	Marker for transcriptional induction of innate immune response
Microglia	*Tg(apoeb:lynEGFP)*	*apoeb*	-	Specifically marker of microglia
	-	-	Neutral red	Efficient staining of microglia; partially effective staining of macrophages
Mast cells	-	*cpa5*	-	Marks a subpopulation of L-plastin positive myeloid cells by in situ hybridization

[1] Only the most frequently used transgenic lines are indicated; for additional lines and references we refer to the Zebrafish Model Organism Database (http://zfin.org/).

An important study in zebrafish has revealed previously underappreciated differences in phagocytic behavior between macrophages and neutrophils that are very relevant for the design of infection models (Colucci-Guyon et al., 2011). This study showed that, in contrast to macrophages, neutrophils possess limited ability to phagocytose fluid-borne bacteria, but can quickly migrate to wounded or infected tissues and efficiently re-

move surface-associated bacteria (Colucci-Guyon et al., 2011). A previous study describes a similar "surface phagocytosis" behavior for mammalian neutrophils (Wood, 1960). This property is likely to be relevant for human infectious disease, since the first encounter of microbes with phagocytes is critical for the outcome of infection (Colucci-Guyon et al., 2011). In zebrafish embryos and larvae, phagocytosis by macrophages is favored when microbes are injected into the blood or into a body cavity such as the hindbrain ventricle, whereas sub-cutaneous, muscle or tail fin injections will provide the conditions for efficient engagement of neutrophils (Colucci-Guyon et al., 2011). The technical options allowed by using zebrafish, where the initial infection site can be varied to investigate how macrophages and neutrophils respond differently, is a strength of zebrafish infection models.

In addition to neutrophil and macrophage lineages, also mast cells are thought to be generated from the RBI (Dobson et al., 2008). The activation of mast cells at sites of infection can have direct effector functions or contribute to the regulation of innate and adaptive immune responses (Prykhozhij & Berman, 2014). As the gene encoding carboxypeptidase A5 (*cpa5*), a marker for mast cells, is expressed as early as 24 hpf (Dobson et al., 2008), zebrafish embryos could become a valuable model to study the function of mast cells in context of infection. However, to date, studies in zebrafish infection models have concentrated on macrophage and neutrophil functions, where work has uncovered novel insights into how these cells respond to infection, and into the genes required for mounting an immune response, as further discussed below.

Generation of myeloid cells by the intermediate and definitive waves of hematopoiesis

As in all vertebrates, hematopoiesis in zebrafish occurs in waves (Jagannathan-Bogdan & Zon, 2013). The second wave of hematopoiesis is identified as an intermediate wave **(Figure 1)**, occurring at the posterior blood island (PBI) at the most posterior part of the ICM. The PBI is a temporary location of hematopoiesis in zebrafish (24-48 hpf), analogous with the mammalian fetal liver. The intermediate wave of hematopoiesis generates the first committed erythromyeloid progenitors (EMPs) which are capable of giving rise to both erythroid and myeloid lineage cells (Ber-

trand et al., 2007), including macrophages, neutrophils and mast cells **(Figure 1)** (Bertrand et al., 2007). The primitive and intermediate waves cannot sustain hematopoiesis for a long time. Only the final wave that occurs during embryogenesis, namely definitive hematopoiesis, is able to produce hematopoietic stem cells (HSCs) that can generate all types of hematopoietic cells for the whole life span. The development of HSCs is dependent on transcription factor Runx1 (Lam et al., 2009). In zebrafish, HSCs are generated from about 1 dpf to 2.5 dpf in the ventral wall of the dorsal aorta (VDA) **(Figure 1)**. This hematopoietic site derives from the aorta-gonad-mesonephros (AGM), which is also the origin of HSC in mammals. HSCs emerging from the VDA migrate to and colonize the three sites of definitive hematopoiesis: the caudal hematopoietic tissue (CHT) the thymus and the anterior part of the kidney (pronephros). From 3 to 6 dpf, the CHT is the main hematopoietic tissue of the larvae. However, the CHT does not produce lymphoid progenitors and is readily exhausted. From approximately 4 dpf, the thymus and the pronephros (which will later develop into the adult head kidney) start to contribute to hematopoiesis and only these organs will maintain erythroid, myeloid and lymphoid hematopoiesis throughout the life span of the fish (Jin et al., 2007; Kissa et al., 2008; Murayama et al., 2006; Willett et al., 1999).

In the VDA, HSCs are shown to originate from hemogenic endothelial cells via a developmental process termed endothelial hematopoietic transition (EHT) (Bertrand et al., 2010; Kissa & Herbomel, 2010). The hemogenic cells are bipotential precursors that can differentiate into both hematopoietic and endothelial cells (Vogeli et al., 2006). These HSCs undergo limited divisions to either maintain the stem cell pool throughout the life of the host, or give rise to multipotent and lineage-committed hematopoietic progenitor cells (HSCs) that generate all mature blood cell lineages (Takizawa et al., 2012). Macrophages originating from the primitive and the intermediate wave play a decisive role in the expansion and specification of definitive HSCs. They colonize the AGM during the HSCs emergence stage, start patrolling between the dorsal aorta and the posterior caudal vein, and intimately interact with the HSCs. Genetic or chemical depletion of macrophages derived from the non-definitive waves impairs the accumulation of the definitive HSCs in the AGM and their colonization of the CHT (Travnickova et al., 2015). Furthermore, it has been shown that the mobilization of HSCs and the intravasation and colonization of tis-

sues is dependent on the function of matrix metalloproteinases (MMPs), in particular Mmp9, which can be produced by myeloid and surrounding tissue cells (Travnickova et al., 2015). Mmp9 is known as a strongly inducible component of the pro-inflammatory response to infections, facilitating leukocyte migration and cytokine processing (Stockhammer et al., 2009; Van Lint & Libert, 2007; Volkman et al., 2010). Therefore, the role of Mmp9 in HSC mobilization is likely to be significant also under conditions of infection, which demand enhanced hematopoiesis.

Functional diversification of myeloid subtypes

It is not precisely known to what extent the zebrafish macrophages or neutrophils generated by primitive, intermediate, or definitive hematopoiesis have different functional competencies when dealing with infections. It is clear, however, that zebrafish embryos are less competent to combat infections at 1 dpf than at later stages, which likely can be attributed for a major part to the fact that neutrophils are still undergoing differentiation between 1 and 2 dpf **(Figure 1)** (Clatworthy et al., 2009). Indeed, these early neutrophils have been shown to phagocytose less well than neutrophils at later developmental stages (Le Guyader et al., 2008). Nevertheless, zebrafish embryos infected at 1 dpf are already capable of inducing a robust innate immune response with expression of genes for cytokines, complement factors, proteases, and other mediators of pathogen defense (Stockhammer et al., 2009; Van der Vaart et al., 2012).

A pioneering study using zebrafish showed, for the first time in a living vertebrate, that macrophages undergo polarization to develop into functional M1 (classically activated) and M2-like (alternatively activated) subtypes (Nguyen-Chi et al., 2015). M1 macrophages promote inflammation, while M2 macrophages are involved in the resolution of inflammation and wound healing. Therefore, in many diseases, the persistence of M1 macrophages signifies an inflammatory state that can promote a range of negative outcomes, including inflammatory disorders (Mills, 2012). On the other hand, tumor-associated macrophages often display an M2 phenotype linked with properties that stimulate tumor growth, angiogenesis, tissue invasion, and metastasis (Noy & Pollard, 2014). Nguyen-Chi et al. used live imaging of a zebrafish fluorescent reporter line for tumor necrosis factor alpha (Tnfα), a distinctive proinflammatory marker for M1 mac-

rophages. They showed that a subset of macrophages start to express the *tnfa* reporter in response to wounding, or in response to a tissue infection with *E. coli*. Moreover, these *tnfa* positive macrophages revert back to an M2-like phenotype when the inflammation is resolving (Nguyen-Chi et al., 2015). By separating *tnfa*-expressing and *tnfa*-negative macrophages using fluorescent cell sorting, it was found that *tnfa* positive cells express other typical M1 markers, such as interleukin 1β and 6 (*il1b* and *il*6), while negative cells express M2 markers, such as tumor growth factor β *(tgfb)*, CC-motif chemokine receptor 2 *(ccr2)* and CXC-motif chemokine receptor 4b (*cxcr4b*).

Macrophage activation has also been demonstrated using a fluorescent reporter fish line **(Table 2)** for immunoresponsive gene 1 (*irg1*), which is strongly induced by injection of bacterial lipopolysaccharide (LPS) (Sanderson et al., 2015). Arginase-2 (*arg2*) is considered to be a reliable M2 marker for teleost fish and a reporter line for this gene would thus be a valuable addition to further study M1/M2 polarization in zebrafish (Wiegertjes et al., 2016).

There is increasing interest also in neutrophil subtypes, which by analogy with macrophage subtypes are referred to as N1 and N2 (Mantovani, 2009). With new transgenic lines being generated by several labs **(Table 2)**, zebrafish embryos and larvae provide a unique opportunity to carry out live imaging of such possible neutrophil polarization and of neutrophil-specific defense mechanisms, like the formation of neutrophil extracellular traps (NETs) (Palic et al., 2007). The release of NETs coincides with a specific type of neutrophil cell death, named NETosis, resulting in an extracellular network of chromatin and granular proteins that can entrap and kill microbes. Besides this direct antimicrobial function, NETosis is thought to deliver danger signals that alert the innate immune system, and, if not properly controlled, NETosis may contribute to inflammatory and autoimmune diseases (Brinkmann & Zychlinsky, 2012). A newly established zebrafish notochord infection model is very useful to address neutrophil-specific defenses (Nguyen-Chi et al., 2014). The notochord is the developmental precursor of the vertebral column and this structure is inaccessible to phagocytes. However, injection of *E. coli* bacteria into this tissue induces massive macrophage and neutrophil accumulation in the surrounding area. The accumulating neutrophils are polarized to express

high levels of *il1b* and a significant proportion of them show release of the Mpx-containing granules. This response results in rapid elimination of the bacterial infection, but the inflammatory reaction is persistent and has long term consequences leading to notochord damage and vertebral column malformations (Nguyen-Chi et al., 2014). This study provided the first *in vivo* evidence that neutrophils can degranulate without making direct contact with a pathogen. Furthermore, the zebrafish notochord model developed in this study provides a new tool to study human inflammatory and infectious diseases of cartilage and bone, such as osteomyelitis and septic arthritis.

Genetic control and experimental manipulation of the zebrafish innate immune system

Development and differentiation of innate immune cells

Primitive myelopoiesis in zebrafish is genetically controlled by two parallel pathways, the *cloche-estrp-scl* pathway and the *bmp*/*alk*8 pathway (Hogan et al., 2006; Liao et al., 1998). Cloche is required very early for development of normal hemangioblasts as *cloche* mutants have defects in both endothelial and hematopoietic (erythroid and myeloid) lineages . The *estrp* and *scl* genes act downstream of *cloche* to regulate hematopoietic and endothelial development (Liao et al., 1998; Liu & Patient, 2008; Sumanas et al., 2008; Sumanas & Lin, 2006). The Bmp receptor Alk8 specifically regulates primitive myelopoiesis in the RBI but is not required for erythropoiesis. In agreement with an instructive role of the *bmp/alk8* pathway in myelopoiesis, the expression of *pu.*1 is lost in the absence of *alk8* while constitutively expressed *alk8* can increase *pu.1* expression (Hogan et al., 2006). The differentiation of EMPs is controlled by the orchestrated expression of transcription factors, where Pu.1 is the master regulator of the myelopoiesis and Gata1 is the key regulator of the erythroid cell lineage. Pu.1 and Gata1 negatively regulate each other and an interplay between these two transcription factors determines myeloid versus erythroid cell fate **(Figure 1)** (Galloway et al., 2005; Rhodes et al., 2005).

Myeloid progenitors need additional factors to differentiate into any of the innate immune cell type populations. Some of these factors are required for pan-myeloid development, while some are required for a specific lineage development. The *spi1l* gene encodes an ETS transcription factor, closely related to Pu.1. It functions downstream of Pu.1 and promotes myeloid development (Bukrinsky et al., 2009). Extrinsic factors like granulocyte-colony stimulating factor (Gcsf) also play a critical role in myeloid cell development (Liongue et al., 2009). Pu.1, Runx1, and Irf8 are important for the cell fate determination between macrophages and neutrophils. High levels of Pu.1 promote macrophage fate whereas low levels promote neutrophil fate during primitive myelopoiesis (Jin et al., 2012; Su et al., 2007). Increased levels of Runx1 promote the expansion of the neutrophil population, whereas low levels of Runx1 result in more macrophages at the expense of the neutrophil progeny (Jin et al., 2012). In contrast to Runx1, Irf8 is necessary for macrophage fate determination. Suppressing *irf8* leads to reduced macrophage and increased neutrophil numbers, while increased *irf8* expression has the opposite effect (Li et al., 2011). The regulation of mast cell fate is less well understood, but it has recently been shown to be influenced by Gata2, which functions downstream of the Notch pathway. Pu.1 is also required for mast cell development, independent from Gata2 and the Notch pathway (Da'as et al., 2012). As discussed below, the knowledge of the genetic pathways that control myeloid development can be exploited in infection studies to determine the specific roles of macrophages and neutrophils in host defense and pathology.

Genetic and chemical approaches to manipulating the zebrafish innate immune system

The transcription factor, Pu.1 is essential for development of both macrophages and neutrophils. A low dose of a *pu.1* morpholino can block macrophage development up to 3 dpf, and can also block neutrophil development when injected at a higher dose (Su et al., 2007). *pu.1* morphants are more susceptible to various pathogens such as *Mycobacterium marinum*, *Salmonella enterica* Typhimurium, *Staphylococcus aureus*, and Chikungunya virus (CHIKV), indicating that macrophages are essential for defense against these. Additionally, similar experiments demonstrated that mac-

rophages are critical vectors for dissemination of *M. marinum* (Clay et al., 2007; Palha et al., 2013; Prajsnar et al., 2012; van der Vaart et al., 2012).

Not only macrophages, but also neutrophils are critical for the defense against *M. marinum*, which has been shown using a transgenic zebrafish line which mimics the WHIM (Warts, Hypogammaglobulinemia, Immunodeficiency, and Myelokathexis) syndrome. In the WHIM zebrafish line, the neutrophil specific *mpx* promoter is used to overexpress a constitutively active form of *cxcr4b*, which is an important retention factor for myeloid progenitors that permits their maintenance in the hematopoietic tissues. As a result, mature neutrophils are retained in the hematopoietic tissues that express Cxcl12a, the chemotactic ligand of Cxcr4b. Thus, neutrophils are unable to reach the tissue infection sites, resulting in increased growth of *M. marinum* (Yang et al., 2012). However, neutrophils cannot control *M. marinum* infection in the absence of macrophages, as shown by using *irf8* morpholino to expand neutrophils at the expense of macrophages (Elks et al., 2015; Pagan et al., 2015). In contrast, the essential role for neutrophils in controlling viral infection was shown by knockdown of *csf3r* (*gcsfr*) which mostly depletes the neutrophil population and renders embryos more susceptible to CHIKV infection ((Liongue et al., 2009; Palha et al., 2013). The selective depletion of neutrophils can also be achieved with *cebp1* morpholino, an approach used in a study demonstrating the importance of neutrophils as a source for inflammatory cytokines promoting hematopoiesis (He et al., 2015).

Alternative to examples of genetic manipulation of macrophage/neutrophil ratios, transgenic drug-inducible cell ablation systems have been applied in zebrafish infection studies. For example, selective ablation of macrophages demonstrated that these cells are less important than neutrophils in defense against CHIKV (Palha et al., 2013). The same approach showed that both macrophages and neutrophils are required for defense against *S. aureus*, but that neutrophils also function as a potential reservoir where the pathogen find a protected niche that enables it to subsequently cause a disseminated and fatal infection (Prajsnar et al., 2012). Finally, macrophages have been selectively depleted using clodronate-containing liposomes, showing their essential role in control of *Mycobacterium abscessus* and *Cryptococcus neoformans* infections (Bernut et al., 2014; Bojarczuk et al., 2016). Together, these examples demon-

strate the advantage of zebrafish infection models for in vivo dissection of innate immune cell functions, due to the ease of genetic and chemical manipulation of macrophage versus neutrophil ratios in this model.

Pathways required for pathogen recognition and activation of the innate immune response

Cells composing the innate immune system can recognize invading microbes by expressing a series of pattern recognition receptors (PRRs). PRRs were evolved to sense and respond to recurrent molecular patterns that are found in microbes (e.g. LPS, peptidoglycan, lipoprotein, flagellin, exogenous nucleic acids) or that are derived from the host as a consequence of the infection (e.g. heat shock proteins and aberrantly processed, exposed or localized cell components). These signals are collectively referred to as Pathogen/Damage Associated Molecular Patterns, P/DAMPs) (Akira et al., 2006). PRRs belong to different families, which comprise membrane proteins on the cell surface or endosomal compartments, cytosolic proteins as well as secreted proteins. PPRs are not only essential for innate immune responses, but also for the activation of adaptive immunity, and defects or polymorphisms in these receptors have been linked to numerous immune-related diseases in human (Caruso et al., 2014; Netea et al., 2012). The major families of PRRs are well conserved between mammals and zebrafish. However, as reviewed below, the current knowledge of PRRs and downstream signaling in zebrafish is still relatively limited.

Families of PRRs

Scavenger receptors

Scavenger receptors represent a heterogeneous group of surface PRRs receptors, able to recognize a broad spectrum of molecules from bacterial/fungal wall, viral capsid parasite glycocalyx as well as host derived ligands. The interaction of these receptors with their ligands can directly mediate phagocytosis of the pathogen or can contribute as co-stimulatory signal for the activation of downstream signaling pathways, such as cytokine responses mediated by NFκB signaling (Bowdish et al., 2009). The zebrafish homologs of human macrophage receptor with collagen structure (Mar-

co) and Cd36 were recently characterized (Benard et al., 2014; Fink et al., 2015). Marco expression by macrophages is important for rapid phagocytosis of *M. marinum* and mediates an initial transient proinflammatory response to this pathogen (Benard et al., 2014). Consequently, knockdown of this receptor impairs bacterial growth control. Although not highly expressed by macrophage and neutrophils, also the knockdown of Cd36 in zebrafish larvae led to higher bacterial burden upon *M. marinum* infection (Fink et al., 2015).

C-type lectin receptors

The mammalian C-type lectin receptors (CLRs) include cell surface as well as secreted proteins (collectins) that are able to bind to different surface carbohydrate moieties from viruses, bacteria, fungi or eukaryotic parasites and similarly to scavenger receptors, they can guide phagocytosis of non-opsonized bacteria, and their destruction in acidified phagolysosomes. Several homologs of CLRs have been detected in zebrafish, but a real functional characterization of this class of receptors in zebrafish is still missing. Only recently the zebrafish mannose receptor was cloned and found to be highly induced upon infection with *Aereomonas sobria* (Fink et al., 2015). In addition to this cell surface receptor for mannose-rich glycans, mannose recognition is also mediated extracellularly by the mannose binding lectin (MBL).

Zebrafish embryos express a homolog of mammalian MBL and this molecule can opsonize both Gram-negative and Gram-positive bacteria, promoting their phagocytosis by macrophages, like its mammalian counterpart (Yang et al., 2014). Neutralization of this molecule could also increase mortality of embryos infected with *Aereomonas hydrophila*, while injection of the recombinant protein promotes resistance to this pathogen. This study also suggests that the lectin pathway may be already functional in the early embryos in zebrafish before their cell-mediated innate immunity is fully matured, and largely contributes to the protection of the developing embryos.

Toll-like receptors

Toll-like receptors (TLRs) are a family of PRRs located on the plasma membrane or on the endosome/phagosome membranes that can sense a wide variety of PAMPs and DAMPs. Their extracellular ligand binding domain contains conserved leucine-rich repeat motifs and their cytoplasmic signaling domain consists of a TIR (Toll-Interleukin-1 Receptor) homology domain. TLRs are known to essentially signal as hetero- or homo-dimers, via coupling with downstream adaptor molecules (Akira et al., 2006). In mammals, five adaptors have been identified, namely MYD88 (myeloid differentiation factor 88), TIRAP, TRIF, TRAM and SARM1 (Akira et al., 2006). Among these, MYD88 represents the most central mediator, since most of the TLRs rely heavily on MYD88 to activate their downstream signaling pathway. This consists mostly of modulation of gene expression via activation and translocation of transcription factors such as NFκB, ATFs, IRFs, AP-1 and STATs (Akira et al., 2006). Stimulation of these factors triggers profound modification of gene expression, especially upregulation of an array of proinflammatory effector molecules, including cytokines, chemokines, antimicrobials and activators of adaptive immunity (Kanwal et al., 2014).

Orthologs of TLR1-2-3-4-5-7-8-9 and of their adaptor intermediates (Myd88, Tirap, Trif and Sarm1) and other downstream signaling intermediates (e.g. Traf6) have been identified and studied in zebrafish too (Kanwal et al., 2014). However, for some of them it is still unclear what ligands they respond to. The zebrafish Tlr2-3-5-9 maintain ligand-specificity consistent with their mammalian counterparts, yet the closest orthologs to mammalian TLR4 in zebrafish are unable to respond to LPS, its ligand in mammals (Kanwal et al., 2014). Several functional and fish-specific Tlrs also exist, such a Tlr21 and Tlr22, which can respond to dsRNA and CpG-oligodeoxynucleotides respectively (Kanwal et al., 2014). Another fish specific Tlr cluster is represented by Tlr20, which phylogenetically seems related to mammalian Tlr11-12 (Kanwal et al., 2014). In agreement with studies in mammalian models, transcriptional analysis of the responses to bacterial infections has demonstrated that activation of downstream transcription factors and proinflammatory immune response genes is largely dependent on the function of the Myd88, which serves as an adaptor in

both Tlr and Interleukin 1 receptor signaling (Gay et al., 2011; van der vaart et al., 2013).

A reporter zebrafish line **(Table 2)** containing promoter elements of the zebrafish *myd88* gene (Hall et al., 2009) has helped to define that the innate immune cells, have the highest potential for MyD88-dependent/ TLR-mediated signaling. *Myd88*:GFP labelled cells include a set of myeloid leukocytes which not only are highly responsive to wounding and infections, but also express a full battery of Tlrs and other Tlr-downstream adaptors together with *myd88*.

Application of the zebrafish model has recently also contributed to define common and specific downstream signaling targets controlled by several Tlrs. While a large part of well-defined inflammatory markers such as *il1 b, tnfa, mmp9* and *Cxcl18b/Cxcl-c1c* were inducible by either Tlr2 and Tlr5 stimulation at a similar extents, other infection-responsive genes, especially transcription factors (e.g. *fosb*, *egr3*, *cebpb*, *hnf4a*) but also some effector molecules, including *il6* and *il10* were found to rely more heavily on one or the other signaling system. Comparative studies of Tlr signaling in zebrafish with other teleost and mammalian species have been more comprehensively reviewed in (Kanwal et al., 2014) and these studies, in summary, demonstrate how zebrafish genetics can be used to dissect the specific molecules that contribute to a robust immune response.

Nod-like receptors

Differently from scavenger receptors and TLRs, Nucleotide-binding-oligomerization-domain (NOD) like receptors (NLRs) are soluble receptors and can detect PAMPs and DAMPs in the cytosol, such as those deriving from pathogens escaping from phagosomes (Akira et al., 2006). NOD1 and NOD2 have been implicated in the recognition of bacterial cell wall, although several studies suggest a broader range of ligands for these NLRs, since they seemed implicated also into recognition of intracellular eukaryotic parasites (Silva et al., 2010). Other NLR include IPAF, NALP1, and NALP3, which can assemble in the inflammasome, a cytosolic multicomponent complex which is involved in the activation of procaspase 1 to caspase 1 (Martinon et al., 2002). The active form of caspase 1, in turn, can process pro-IL1β and pro-IL18 into IL1β and IL18 (Martinon et

al., 2002). Most of NLRs are conserved in zebrafish in addition to another large teleost-specific subfamily of NLRs (Stein et al., 2007). The functional conservation of NOD1-2 was demonstrated by depletion of these genes during *S. enterica* Typhimuruim infection, which resulted in increased burden, and decreased host survival (Oehlers et al., 2011). Investigation of the NLR-dependent inflammasome activation and Il1β processing still requires a more detailed characterization in this species (Ogryzko et al., 2014; Varela et al., 2014).

RIG-I-like receptors

RIG-I-like receptors (RLRs) are another family of cytosolic PRRs that activate the inflammasome (Kell & Gale, 2015). RLRs can detect the presence of RNA from a broad range of viruses. The downstream signaling cascade is cooperative with Tlr signaling and induces activation of transcription factors like IRF3, IRF7 and NFκB, leading to high production of interferons (IFN) and interferon-stimulated genes (ISGs) (Kell & Gale, 2015). Both type I and type II interferons exist in zebrafish, and like in humans, these molecules are key for the antiviral response. However, direct homologies with the mammalian systems cannot be definitively traced. Zebrafish Ifnγ1 and Ifnγ2 are the type II homologs, while Ifnϕ1 and Ifnϕ2, members of a large Ifnϕ family in zebrafish, represent a fish-specific type of interferons that more closely resemble the mammalian type I interferon molecules (Aggad et al., 2009; Langevin et al., 2013).The zebrafish homologs for RIG-I and other members of RLRs are predicted in the zebrafish genome but functional characterization in zebrafish is still incomplete. The RLRs were shown to be involved in IFN gene induction in zebrafish by overexpression of the key RLR-adaptor IPS-1/MAVS. This led to massive induction of ISGs, similar to what was found in mammalian models (Biacchesi et al., 2009). This role in IFN induction places RLRs as a central factor in containing viral infections. Studies in zebrafish suggest that they might also have a significant function in defense against bacterial infections (Zou et al., 2013).

Inflammatory signaling initiated by PRRs

The downstream mediators activated by most PRR signaling include pro- and anti-inflammatory protein and lipid molecules secreted at the infection site. Cytokines are small secreted proteins exerting central modula-

tory activities in both adaptive and innate immunity. This heterogeneous group of peptides includes TNF, interleukins, and chemokines (CCLs, CXCLs, CX3CLs and XCLs). All these classes exist in zebrafish and other teleosts. However, expansions and diversifications have occurred (Nomiyama et al., 2008).

Similarly to mammalian models, a large number of these mediators is transcriptionally modulated by infection with different pathogens (Stockhammer et al., 2009; Veneman et al., 2013), or cleaved to their mature/active form. In zebrafish, functional similarities are proven for the Tnf, Il1β, Il8/Cxcl8, Cxcl11, Il6, and Il10 (Roca & Ramakrishnan, 2013). Knockdowns or full knockouts of several of these molecules or their cognate receptors led to significant aberrancies in the containment of infections (Roca & Ramakrishnan, 2013). For example, knockdown of the Tnfa receptor *tnfrsf1a* in mycobacterial infection revealed a key function of this axis to control the host inflammatory status (Roca & Ramakrishnan, 2013). The chemokines Il8/Cxcl8 and Cxcl11, like in mammalian species, were found to recruit neutrophils (via Cxcr2) and macrophages (via Cxcr3.2), respectively and impacted on the mobilization and response of phagocytes to infection.

The mechanisms for lipid inflammatory/anti-inflammatory mediators, including prostaglandins, leukotrienes and lypoxins are highly conserved from zebrafish to human. The importance and functional conservation of these molecules are exemplified by the results of a zebrafish genetic screen for genes causing hypersusceptibility to *M. marinum ,* which uncovered the gene encoding Lta4h (leukotriene A4 hydrolase) (Tobin et al., 2010). Lta4h catalyzes the final step of synthesis of the lipid mediator leukotriene B4 (LTB4) and its deficiency in zebrafish impairs the balance between anti-inflammatory and proinflammatory lipid mediators (Tobin et al., 2010). Similarly, polymorphisms in the human *LTA4H* locus have been reported to associate with susceptibility to *M. tuberculosis* (Tobin et al., 2010). LTB4 synergizes with Tnfα in order to maintain a balanced level of inflammation. Via its cognate receptor (Tnfr), Tnfα mediates activation of Rip1/2 kinases and release of reactive oxygen species (ROS) by increasing mitochondrion permeability (Roca & Ramakrishnan, 2013). ROS act as a double edged-sword, by both exerting a microbicidal function and mediating activation of necroptosis of the host cell. Therefore, impaired (too

high or too low) inflammatory statuses lead to increased susceptibility to mycobacterial infection in zebrafish (Roca & Ramakrishnan, 2013). A tight control of the inflammatory status is critically important also in human tuberculosis and other infectious diseases (Dorhoi & Kaufmann, 2014).

Complement system

In addition to the PRR-mediated cellular responses of the innate immune system, zebrafish embryos highly upregulate components of the complement system upon challenge with a variety of pathogens, indicating that soluble complement factors and complement receptors may be critical for opsonization, recognition and lysis of pathogens in this developmental window. In early zebrafish embryos, extracellular *S. enterica* Typhimurium LPS mutant and heat-killed bacteria are rapidly lysed, a phenomenon that was suggested to be complement-mediated, since LPS-mutants were found to be highly susceptible to complement killing in other models (van der Sar et al., 2003). Bacteriolytic mechanisms ascribed to complement are also proposed to contribute to the antibacterial activity in zebrafish egg cytosol (Wang & Zhang, 2010). Mostly complement components are known to derive from the liver. However, complement components are infection-inducible in the early embryos long before hepatic development (Wang et al., 2008). In line with these observations, we have found by transcriptional profiling of sorted phagocytes during infections that these cells can be a relevant source of extrahepatic production of complement components (unpublished results). Additionally, many of the complement factors in zebrafish can be transferred from mothers to eggs at either protein or mRNA level (Hu et al., 2010). Maternal immunization with *A. hydrophila* also resulted in increased protein transfer of complement factors to their offspring (Wang et al., 2009) and contributed to immunoprotection of the early embryo against this pathogen (Wang et al., 2008).

Effects of commensal microbes on development of the immune system

The impact of the gut microbiota on development of the mammalian immune system is well known (Kaplan et al., 2011). Following a large body of work in rodents, methods for growing zebrafish in a germ-free envi-

ronment or in the presence of defined microbial communities (gnotobiotic) are now well established (Pham et al., 2008). Comparison of studies in germ-free and gnotobiotic zebrafish and rodent models has revealed strong similarities among vertebrates in how microbes shape the development of the gut epithelium and the mucosal immune system, and influence the expression of genes involved in processes such as cell proliferation, metabolism, and inflammation (Cheesman & Guillemin, 2007; Rawls et al., 2004).

Inside the chorion, the zebrafish embryo develops in an axenic environment, but the intestine of larvae hatching around 3 dpf is rapidly colonized by microbes (Kanther & Rawls, 2010). Zebrafish larvae reared in germ-free water were shown to express lower levels of the pro-inflammatory cytokine gene *il1b* compared to larvae reared under conventional conditions (Galindo-Villegas et al., 2012). This microbiota-induced *il1b* expression is mediated by the TLR/MyD88 signaling pathway described in section 4 (Galindo-Villegas et al., 2012). This microbial recognition pathway can also be activated before hatching under conditions of experimental infection with bacterial pathogens (Van der Vaart et al., 2013). Microbial colonization leads to activation of a reporter for NFκB **(Table 2)**, a master transcriptional regulator of the immune response downstream of Tlr/Myd88 signaling (Kanther et al., 2011). Furthermore, the presence of a microbiota has been shown to result in increased numbers of neutrophils and systemic alterations in neutrophil localization and migratory behavior, which were found to be dependent on the microbiota-induced acute phase protein serum amyloid A (Kanther et al., 2014). In another study, commensal microbes were not found to promote a higher rate of myelopoiesis, but did affect neutrophil activity in response to injury (Galindo-Villegas et al., 2012). In addition, this study showed that the presence of commensal microbes primes the innate immune system of zebrafish larvae resulting in an increased resistance to experimental infections.

Independent from the effect of commensal microbes, the expression of proinflammatory genes appears to be controlled by epigenetic mechanisms that likely serve to protect of zebrafish larvae against infectious agents before adaptive immunity has developed and prevent pathologies associated with excessive inflammation during development (Galindo-Villegas et al., 2012). This is corroborated by a recent study showing that

mutation in the epigenetic regulator *uhrf1* leads to a strong induction of the proinflammatory cytokine gene *tnfa* in zebrafish larvae (Marjoram et al., 2015). The *tnfa* induction in these *uhrf1* mutants is associated with severe damage of the intestinal epithelium and infiltration by neutrophils, mimicking the chronic inflammation seen in inflammatory bowel diseases (IBD). The development of zebrafish models for IBD provides new avenues to study the factors that contribute to the onset of these complex multifactorial diseases where inappropriate responses of the immune system to the intestinal microbiota are thought to play a major role (Marjoram & Bagnat, 2015).

Adaptation to infection and inflammation

In response to infection or inflammation, the hematopoietic system can mount an adaptive response that is known as demand-driven hematopoiesis or emergency hematopoiesis (Takizawa et al., 2012). This response serves in the first place to replenish neutrophils, which due to their short life span are rapidly consumed during infections. Both the expansion of HSCs and the skewing of myeloid cell specification into the direction of granulopoiesis play a role in demand driven adjustments of hematopoiesis in zebrafish larvae (Hall et al., 2016; Hall et al., 2012; Herbomel, 2012).

That zebrafish embryos can mount an emergency granulopoietic response was first recognized in as study showing that intravenous administration of LPS at 2 dpf led to a Gcsf/Gcsfr-dependent increase in the numbers of neutrophils within 8 hours (Liongue et al., 2009). A recent report shows that phagocyte numbers can be modulated by immune stimulation even at an earlier stage. In this case a host defense peptide, chicken cathelicidin-2, was injected into the yolk of embryos shortly after fertilization, resulting in a 30% increase of *lcp1* positive cells at 2 dpf and an increased resistance of embryos to bacterial infection (Schneider et al., 2016). We review recent work in zebrafish that has brought new insights into the molecular pathway underlying emergency hematopoiesis and has revealed roles for several proinflammatory mediators as well Tlr signaling in hematopoiesis.

Molecular mediators of emergency granulopoiesis

Embryos infected with *S. enterica* Typhimurium into the hindbrain at 2 dpf develop neutropenia within one day and counter this within 2 days by emergency granulopoiesis throughout the VDA/AGM and CHT regions (Hall et al., 2012). While this Gcsf/Gcsfr-dependent response is at the expense of lymphoid progenitors, it is not due only to an increased commitment of HSCs to myeloid rather than lymphoid fate but also due to increase in the number of Gcsfr-expressing HSCs (Hall et al., 2012). The zebrafish orthologue of CCAAT-enhancer binding protein (Cebpb), a well-known transcriptional regulator of emergency granulopoiesis in mammals, is required for the expansion of the HSC compartment (Hall et al., 2012). Importantly, the study in zebrafish revealed that inducible nitric oxide synthase (iNOS, Nos2a) functions downstream of Cebpb in the emergency granulopoiesis pathway (Hall et al., 2012). Knockdown of *nos2a* to block the infection-induced expansion of neutrophils was subsequently shown to be associated with increased viral replication and mortality of embryos during CHIKV infection (Palha et al., 2013). It is currently unknown if the role of nitric oxide in emergency hematopoiesis is conserved across species, but this is to be expected in view of the fact that nitric oxide is involved steady state hematopoiesis in both zebrafish and mouse (Hall et al., 2012). The newly discovered roles of Cebpb and nitric oxide therefore prompt further investigations into the possibilities of therapeutic targeting of these factors in human disease (Hall et al., 2012).

Through work in zebrafish, a highly conserved myeloid-specific microRNA, *miR-142a-3p*, has recently been linked with Gcsf/Gcsfr and nitric oxide (NO) dependent signaling (Lu et al., 2013). Depletion of *miR-142a-3p* was found to reduce the numbers of HSCs in the VDA/AGM and CHT, associated with reduced expression of *gcsfr* as well as decreased production of NO (Lu et al., 2013). The inflammatory transcription factor Interferon regulatory factor 7 (Irf7) is a potential target of this microRNA, suggesting that this pathway might also be relevant not only to steady state but also to infection-induced hematopoiesis. When *irf7* and *miR-142a-3p* were depleted simultaneously *gcsfr* expression and NO production could be restored, suggesting that Irf7 acts as a repressor of Gcsfr/NO signaling and that in turn *miR-142a-3p* can repress Irf7 function to promote HSC development (Lu et al., 2013). This mechanism is conserved in mouse

and therefore also of potential interest for therapeutic targeting (Lu et al., 2013).

Implication of cytokines and interferons in hematopoiesis

Macrophages are thought to be the source of Gcsf that promotes emergency granulopoiesis (Hall et al., 2012). It has recently been shown that also several proinflammatory cytokines that are produced by macrophages and neutrophils can influence the production of HSCs in the embryo. These cytokines include Tnfα, Ifng1-1, Ifng1-2 and Il1β (Espin-Palazon et al., 2014; He at al., 2015; Li et al., 2014). Tnfα in zebrafish is encoded by two genes, *tnfa* and *tnfb*, and the expression of both genes is inducible by infections (van der Vaart et al., 2013). Tnfα is expressed as a transmembrane protein functional on the cell surface and signals through two receptors, Tnfr1 (Tnfrsf1a) and Tnfr2 (Tnfrsf1b). Signaling through Tnfr1 is important for resistance to mycobacterial infection as it prevents necrosis of infected macrophages (Clay et al., 2008), whereas Tnfr2 is the receptor that has been implicated in hematopoiesis (Espin-Palazon et al., 2014). Primitive neutrophils were found to be the primary source of Tnfα, which was found to promote the specification and emergence of HSCs through Tnfr2 and the Notch and NFκB signaling pathways (Espin-Palazon et al., 2014).

Similar to Tnfα, interferon gamma (IFNγ) is another important activator of macrophages that has been implicated in hematopoiesis. Overexpression of a zebrafish homolog of IFNγ, ifng1-2, increases HSC counts in embryos with an intact Notch signaling pathway (Sawamiphak et al., 2014). Ifng1-2 specifically controls the endothelial to HSC transition by activating Signal transducer and activator of transcription 3 (Stat3) (Sawamiphak et al., 2014). In agreement, knockdown of Interferon regulatory factor 2 (Irf2) increases HSC production in zebrafish (Li et al., 2014). The other zebrafish homolog of IFNγ, Ifng1-1, and fish specific type I interferons (*ifnphi1-2-3* and *4*) also contribute to HSC development and expansion (Li et al., 2014). Thus, Ifns are key regulators of HSC behavior and this suggests that HSCs are a prime response to an infection that stimulates Ifns.

Role of Tlr signaling in hematopoiesis

The primary pathway of pathogen recognition, namely Tlr4-MyD88-NFκB signaling, has recently been linked to HSC development (He et al., 2015). Expression of *runx1* in the VDA/AGM at 1 dpf and *cmyb* in the CHT at 2 dpf is significantly reduced in *tlr4bb* or *myd88* deficient embryos when compared to controls (He et al., 2015). However, *myd88* mutant larvae at 3 dpf show no significant alterations in macrophage or neutrophil numbers (van der Vaart et al., 2013), suggesting that the defect in HSC development is compensated for by Myd88-independent mechanisms. Embryos deficient in *tlr4bb* or *myd88* show a reduction in the expression of Notch target genes, and overexpression of the intracellular domain of Notch in endothelial cells can rescue *runx1* expression in *tlr4bb* and *myd88* morphants (He et al., 2015). As discussed above, Notch signaling can regulate NFκB, and therefore it is likely that the Tlr4-MyD88-NFκB and Notch-NFκB signaling routes function cooperatively in HSC development (Espin-Palazon et al., 2014; He et al., 2015). The HSC defect in *tlr4bb* and *myd88* morphants can also be rescued by overexpression of the gene for Il1β, adding also this cytokine to the list of proinflammatory mediators that modulate hematopoiesis and the production of innate immune cells (He et al., 2015). Studies in *tlr4*$^{-/-}$ knockout mice confirmed that TLR-mediated inflammatory signaling plays an evolutionary conserved role in HSC development (He et al., 2015). In conclusion, a number of recent studies in zebrafish and mouse models support a previously unrecognized link between inflammatory signaling and hematopoiesis that might be translated into new approaches for treatment of immune-related diseases or to improve the success of HSC transplantations (Espin-Palazon et al., 2014; He et al., 2015; Li et al., 2014; Sawamiphak et al., 2014).

The interface of immunity and metabolism

During the first five days of development the zebrafish embryo/larva derives all its nutrients from the yolk and it has to adapt its metabolism to switch to external feeding when yolk proteins become limiting. How this metabolic adaptation might affect the immune system is currently unknown and worthy of exploration, especially considering new links between immunity and metabolism that have recently been revealed in

zebrafish (Hall et al., 2013; Marin-juez et al., 2014; van der Vaart et al., 2013). The relevance of immunometabolism for human disease is emerging strongly from recent studies that have revealed extensive metabolic reprogramming of human macrophages and dendritic cells in response to environmental conditions and during activation of innate and adaptive immune responses (O'Neill & Pearce, 2016).

Lipid and glucose metabolism as fuels for fighting infection

Fatty acid metabolism has been shown to fuel the production of mitochondrial ROS in zebrafish macrophages following infection of embryos with *S. enterica* Typhimurium (Hall et al., 2013). Immunoresponsive gene 1 (*irg1*), an infection-inducible and macrophage-specific gene encoding a homolog of bacterial 2-methylcitrate dehydratase, was found to be required for the utilization of fatty acids during this response, and knockdown of this gene increased the susceptibility to infection (Hall et al., 2013). This study showed that also murine macrophages require fatty acid β-oxidation for infection-induced mitochondrial ROS production and bactericidal activity. ROS production is also dependent on glucose metabolism and overproduction of ROS, which can have tissue damaging effects, has been associated with diabetes(Coughlan & Sharma, 2016). Studies in a zebrafish model for hyperinsulinemia suggest that the metabolic switch between insulin-sensitive and insulin-resistant states is mediated by protein tyrosine phosphatase non-receptor type 6 (Ptpn6), which is well known as a negative regulator of the innate immune response (Kanwal et al., 2013; Marin-juez et al., 2014). The dual role of this phosphatase in the regulation of glucose metabolism and immunity is particularly interesting in the light of the emerging co-epidemic of tuberculosis and diabetes (Pizzol et al., 2016). There are many ongoing efforts to develop zebrafish models for metabolic diseases, including diabetic complications, providing new opportunities to study the relation with infectious diseases (Schlegel & Gut, 2015).

Autophagy

The process of autophagy might be considered as the most important link between metabolism and immune function. Autophagy is a cellular process of self-degradation that functions to regulate energy metabolism

and it can be activated by nutrient stress, such as the depletion of the yolk during zebrafish larval development (Varga et al., 2015). During autophagy (or strictly macroautophagy), the cytosolic material is entrapped in double membrane structures (autophagosomes) and delivered to lysosomes for degradation. Autophagy has an important housekeeping function in removing and recycling aggregates of misfolded proteins and damaged organelles (Levine et al., 2011). The same machinery can also target intracellular microbes to lysosomal degradation and therefore several pathogens are thought to have evolved mechanisms to counteract the autophagic defenses (Huang & Brumell, 2014). Autophagy also controls inflammation, cytokine secretion, antigen presentation, and the regulation of innate and adaptive immune responses (Deretic et al., 2013).

Some recent work showing that autophagy is important in infection has used knockdown of the autophagy receptor p62 (Sqstm1) which mediates selective autophagy of ubiquitinated cargo. Sqstm1 morphants have an impaired defense against *Shigella* and *Mycobacterium* infections (Mostowy et al., 2013; van der Vaart et al., 2014). These bacterial pathogens have the ability to escape from phagosomes into the cytosol, where they can be tagged by ubiquitin (Ub) ligation and subsequently targeted to autophagy by p62 (Huang & Brumell, 2014). The susceptibility of p62-deficient zebrafish larvae to these pathogens clearly shows that autophagy is an essential cellular process for effective immunity against some deadly bacteria. Similarly, many studies in human cells have shown increased replication of *M. tuberculosis* under conditions of autophagy inhibition. In contrast, loss of p62 and other essential autophagy genes did not correlate with susceptibility to *M. tuberculosis* in mice (Kimmey et al., 2015). This suggests that, in the context of full adaptive immunity, *M. tuberculosis* might be less subject to phagosomal escape and autophagic targeting and that this pathogen is capable of effectively inhibiting the anti-bacterial function of the autophagy process.

The microtubule-associated light chain 3 protein (Lc3) is widely used as a marker of autophagosomes and the generation of a zebrafish reporter line **(Table 2)** expressing a GFP-Lc3 fusion protein *Tg(CMV:GFP-Lc3)* allows to monitor the process of autophagy *in vivo* (He et al., 2009). The zebrafish GFP-Lc3 reporter is activated by autophagy-inducing drugs (such as rapamycin), in different tissues of the developing embryo (for example

the heart), and in response to infections with *Shigella* and *Mycobacterium* (He et al., 2009; Hosseini et al., 2014; Lee et al., 2014; Mostowy et al., 2013; van der Vaart et al., 2014). The autophagic morphology of *M. marinum*-containing GFP-Lc3 positive vesicles in zebrafish has been confirmed by correlative light and electron microscopy (Hosseini et al., 2014). Furthermore, small GFP-Lc3 vesicles are frequently seen to accumulate around mycobacterial aggregates in infected zebrafish hosts (Hosseini et al., 2014; van der Vaart et al., 2014). These autophagosomes might serve to deliver neo-antimicrobial peptides and enhance the bactericidal properties of the autolysosomal compartment (Ponpuak et al., 2010).

From studies in human and mammalian cells, autophagy is known to be induced downstream of pathogen recognition by TLR signaling (Deretic et al., 2013). The DNA-damage regulated autophagy modulator 1 (Dram1) was discovered in zebrafish as a novel mechanistic link between autophagy induction and the TLR/Il1R-MydD88-NFκB innate immune sensing pathway (van der Vaart et al., 2014). Dram1 overexpression in the zebrafish host promotes the formation of autophagosomes and the p62-dependent selective autophagy targeting of *M. marinum*. Although the molecular mechanism remains to be elucidated, this host protective role of Dram1 might be exploited as a therapeutic strategy for treatment of mycobacterial disease in humans (van der Vaart et al., 2014). In further support of autophagy modulation as a therapeutic approach, a clinically approved anticonvulsant drug, carbamazepine, was recently shown to trigger autophagy in zebrafish embryos and protect against *M. marinum* infection (Schiebler et al., 2015). This drug was also shown to be effective against *M. tuberculosis* within primary human macrophages and in a mouse model of TB. Therefore, despite recent findings that deficiency in essential autophagy genes did not correlate with *M. tuberculosis* deficiency in mice, pharmacological activation of autophagy still remains a promising therapeutic strategy to be further explored (Kimmey et al., 2015).

Recent insights from modeling infectious diseases in developing embryos and larvae

Bacterial infections

Zebrafish infection models have been established for a wide variety of bacterial pathogens that are the causative agents of human infectious diseases or opportunistic infections, including species of the Mycobacteria, *Listeria*, *Shigella*, *Salmonella*, Streptococci, *Burkholderia* and other genera **(Table 1**). Since most of these models have been reviewed elsewhere (Cronan & Tobin, 2014; Meijer, 2016; Ramakrishnan, 2013; Saralahti & Ramet, 2015; Torraca et al., 2014; Vergunst et al., 2010), we focus here on some examples of recent work showing how these models are contributing to a better understanding of macrophage and neutrophil functions in the containment or the promotion of specific disease features.

Listeria and *Shigella* infections

Listeria monocytogenes and *Shigella flexneri* are two human pathogens that can cause serious gastrointestinal infections (food poisoning), especially in infants, the elderly, and immunocompromised patients. These bacteria share the capability to extensively manipulate the host cytoskeleton. Despite not being natural fish pathogens, these species were seen to escape into the cytosol after phagocytosis and to induce in the heterologous host the same cytoskeleton rearrangements, including actin tails and septin cages (Levraud et al., 2009; Mostowy et al., 2013). Mechanistically, *Shigella* and *Listeria* models in zebrafish mimic the main disease-causing feature of human shigellosis and listeriosis. Shigella bacteria are phagocytized by both neutrophils and macrophages, but while well contained by the first cell type, they rapidly induce cell death in the second. Both Shigella and Listeria, in human and in zebrafish tissue can largely exploit host actin polymerization to be propelled from the infected cell and invade new cells. These findings emphasize how these mechanisms of pathogenicity are shared across distant bacterial species and across vertebrates.

Similarly to *Shigella*, *Salmonella* (*S. enterica*) is an enterobacterial species that does not generally infect ectothermic animals. However, injection of

S. enterica Typhimurium establishes severe infection in zebrafish, which cannot be contained in most of the infected embryos and goes together with profound transcriptional induction of inflammatory genes (cytokine storm), a response that is largely dependent on Myd88-dependent signaling and negatively regulated by Ptpn6 phosphatase (Kanwal et al., 2013; van der Vaart et al., 2013). Deficiency in either of these signaling factors is detrimental to the resistance of zebrafish embryos to Salmonella infection (Kanwal et al., 2013; van der Vaart et al., 2013), indicating that the inflammatory response is protective when properly controlled but leads to lethality when inhibitory mechanisms are lost.

Staphylococcal infections

Staphylococcus aureus causes a range of serious infections in human and mammalian models, including skin ulceration, osteomyelitis, pneumonia and septicemia. Injections of large inoculums of this bacterium in zebrafish embryos also provoke septicemic death (Prajsnar et al., 2013; van der Vaart et al., 2013). Histologically, in zebrafish like in mammals, *S. aureus* determines formation of necrotizing lesions, the abscesses. Interestingly, the zebrafish model revealed that the bacteria forming the individual abscesses derive from clonal expansion of persistent bacteria. While the vast majority of the injected Staphylococci are cleared by macrophages and neutrophils, some bacteria gain the capability to avoid intracellular killing and will secondarily expand, forming the localized lesion (Prajsnar et al., 2012). This study identified the neutrophils as the main niche necessary to establish this immunological bottleneck and to determine the emergence of clonal infection foci.

Experimental *S. aureus* infection in zebrafish has also been instrumental in a recent study that revealed an unexpected role of the Spaetzle-like nerve growth factor β (NGFβ) in pathogen-specific host immunity to Staphylococcal infection (Hepburn et al., 2014). Spaetzle is a key mediator of the immune response to Gram-positive bacteria in *Drosophila*, and is required for the activation of the Toll signaling pathway (Lemaitre & Hoffmann, 2007) . While Spaetzle had always been thought to lack a vertebrate equivalent, chordate NGFβ in fact shares remarkable structure similarities to this mediator. It was found that Staphylococcal infection triggers release of NGFβ in human macrophages, a mechanism that de-

pends on recognition of pathogen exoproducts and on activation of the immune response via NOD-like receptor signaling. Subsequently, knockdown in zebrafish of tropomyosin-related kinase receptor A (TrkA), the corresponding receptor of NGFβ, was found to impair neutrophil recruitment and to increase susceptibility to *S. aureus* infection (Hepburn et al., 2014). This study supports an evolutionary conserved role for NGFβ acting as an alarm signal in the inflammatory response to *S. aureus* infection. Moreover, this work suggests that variation between individuals in secretion of NGFβ by macrophages might determine susceptibility to Staphylococcal disease.

Tuberculosis

One third of the world population carries *M. tuberculosis* and more than 10 million people fell ill with TB in 2015 (www.who.int). The zebrafish model for TB is by far the best studied zebrafish infection model, and has made great contributions to our understanding of TB pathology **(Figure 2)** (Cronan & Tobin, 2014; Meijer, 2016; Ramakrishnan, 2013). *M. marinum is* a natural pathogen of zebrafish and is phylogenetically very close to *M. tuberculosis,* the causative agent of human TB. The establishment of *M. marinum* pathogenesis in the zebrafish host is strikingly similar to human TB **(Figure 2)**. The disease hallmark in both host-pathogen systems consists of granulomas, essentially consisting of organized collections of immune cells that engulf and confine the bacteria.

Granulomas were previously considered relatively static structures generated by the host as a protective mechanism to restrict bacterial spread. Use of the zebrafish- *M. marinum* infection model demonstrated that these structures are instead highly dynamic **(Figure 2)** (Ramakrishnan, 2012). Non-invasive imaging in live zebrafish has shown that granulomas are characterized by a continuous trafficking of innate immune cells and that the pathogen takes advantage of infected macrophages to dissemi-

nate secondary lesions (Clay et al., 2007; Oehlers et al., 2015; Torraca et al., 2015). This model also helped to reconsider the contribution of the innate and the adaptive branches of the immune system in initiating the formation of granulomas. Imaging the earliest stages of granuloma formation in zebrafish embryos has shown that this process is initially driven by macrophages and occurs before lymphocyte differentiation, demonstrating that cells of the adaptive immune system are not required for granuloma formation (Davis et al., 2002).

Establishing TB infection in the form of granulomas depends on both pathogen and host factors, including mycobacterial virulence components, macrophage chemoattractants and inflammatory mediators. The Region of Difference 1 (*RD1*) is a virulence-associated locus covering the ESX-1 bacterial secretion system, and is notably shared between pathogenic mycobacteria, including *M. marinum*. ESX-1 is crucial for the establishment of granulomas and the zebrafish model helped to understand that the ESAT-6 virulence released via ESX-1 mediates macrophage aggregation in the early granulomas by stimulating production of Mmp9 in the epithelium surrounding the infection focus. By digesting the extracellular matrix, Mmp9 in turn facilitates infiltration of macrophages and establishment of chronic intracellular parasitosis **(Figure 2)** (Ramakrishnan, 2013). MMP9 is highly expressed in human TB and other inflammatory conditions; therefore, the observation that Mmp9 depletion confers resistance to mycobacterial infection in zebrafish highlights MMP9 as a potential therapeutic target (Volkman et al., 2010).

In addition to bacteria-driven mechanisms of granuloma expansion, chemokine signaling affecting macrophage recruitment is important to establish mycobacterial infection and to sustain granuloma expansion and secondary dissemination. Using the zebrafish model it was shown that deficiency in Ccr2/Ccl2 signaling reduces the chances of successful establishment of infection and that abrogation of Cxcr3-Cxcl11 signaling delays granuloma formation and attenuates seeding of the pathogen throughout the host **(Figure 2)** (Cambier et al., 2014; Torraca et al., 2015). However, the equilibrium controlling macrophage supply to the granuloma is very delicate, and while slight perturbations lead to host-beneficial effects, more drastic alterations can promote bacterial growth.

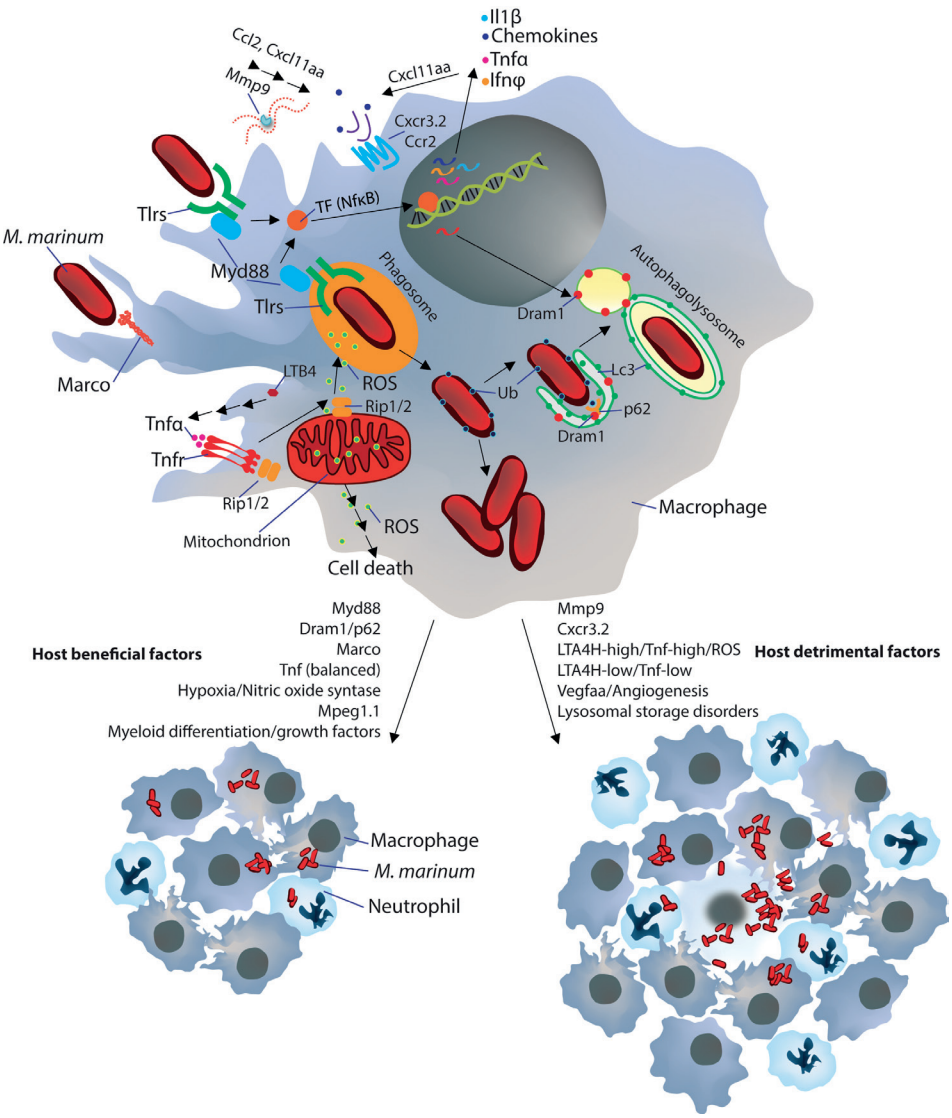

Figure 2: Mechanistic insight into mycobacterial pathogenesis provided by the zebrafish-*M. marinum* infection model. The host factors implicated in *M. marinum* infection of macrophages in the zebrafish host are summarized in this figure. The factors limiting (host beneficial) or promoting (host detrimental) the early expansion of granulomas are indicated below the schematic drawing of the macrophage. Macrophage-recruitment and tissue-inflammation mediators (such as Ccl2 and Mmp9) are also produced by neighboring cells as indicated by the arrows above the macrophage. Genes, pathways, and molecular functions depicted in

the figure: Marco, scavenger receptor important for efficient phagocytosis and immune recognition; Tlr/Myd88/NFκB signaling pathway, leading to induction of inflammatory cytokines (e.g. Tnfα, Il1β), interferons (e.g. Ifnϕ), chemokines (e.g. Cxcl11aa) and autophagy modulators (e.g. Dram 1); Mmp9, matrix metalloproteinase 9 facilitating macrophage migration; Ccl2/Ccr2 and Cxcl11aa/Cxcr3.2, chemokine ligand/receptor signaling axes implicated in macrophage migration; LTB4, lipid mediator of inflammation; Tnfr-Rip1/2 pathway, mediating release of reactive oxygen species (ROS) from mitochondria; Dram1, lysosomal/autophagosomal membrane protein stimulating autophagic flux; p62, pattern recognition receptor targeting ubiquitin-tagged (Ub) mycobacteria (escaped from the phagosomal compartment into the cytosol) to autophagy; Lc3, marker for autophagic activity. Vegfaa, angiogenesis promoting factor. See text for further details.

Macrophages that are engorged with undigested contents, such as in lysosomal storage disorder (LSD) patients and in smokers, display severe migratory aberrations, which can be mimicked in the zebrafish model by knockdown of LSD-associated genes (*gba*, *arsa*, *hexa*), by filling macrophages with indigestible particles or by compromising the levels of lysosomal cathepsins. These paralyzed macrophages cannot sufficiently contain the infection and will permit extracellular growth of the pathogen (Berg et al., 2016). Similarly, blockade of key macrophage differentiation regulators, such as *spi1*, *csf1ra* or *irf8,* leads to severe depletion of macrophages, with the consequent massive non-cellular bacterial growth (Clay et al., 2007; Elks et al., 2015; Pagan et al., 2015). Conversely, drastic increase of macrophage supply, evoked by overexpression of myeloid growth factors, can encourage resistance to mycobacterial infection, by preventing granuloma necroptosis (Pagan et al., 2015; Ramakrishnan, 2012). Taken together, recent findings from the zebrafish model are helping to critically dissect the highly debated dual role of macrophages in tuberculosis pathogenesis (Clay et al., 2007).

Human granulomas are amply vascularized, which suggested that, similarly to affecting tumor growth, curtailing vascularization might help to restrict granuloma formation. By injecting bacteria in the poorly vascularized zebrafish trunk tissue, the granuloma-driven promotion of angiogenesis could be mimicked in this model (Oehlers et al., 2015). Establishment of the intra-macrophage parasitosis, the production of RD1-encoded virulence factors, and the induction of local hypoxia is critical to mediate this response, which coincides with local induction of the angiogenic media-

tor *vegfaa*. In turn, depletion of Vegf signaling, which suppresses pathological angiogenesis, leads to contained granuloma expansion (Oehlers et al., 2015). Using the zebrafish-*Mm* model and genetic tools to control the function of Hif-1α/Hif-2α (the two main variants of hypoxia inducible factor alpha), it was found that hypoxia signaling not only controls angiogenesis, but also the production of nitric oxide (NO) by neutrophils, an important signaling mediator and antimicrobial factors (Elks et al., 2013). Interestingly, stabilization of Hif-1α stimulated activity of the nitric oxide synthase (Nos2a), while stabilization of the Hif-2α variant could antagonize NO production, with consequent opposing effects in inhibiting or promoting bacterial growth (Elks et al., 2013; Elks et al., 2015). These studies suggest angiogenic and hypoxia signaling pathways as possible targets for TB treatment. Several other host-directed therapeutic strategies have been proposed based on work in the zebrafish model and these are extensively covered in previous reviews. (Cronan & Tobin, 2014; Ramakrishnan, 2012; Torraca et al., 2014). In conclusion, the zebrafish *M. marinum* model has provided mechanistic insight into host factors that have been implicated either in protection against human TB or in the pathology of the disease, and provides a valuable anti-tubercular drug testing platform to develop novel therapeutic approaches.

Viral infections

Viral epidemics, with influenza and HIV/AIDS as prominent examples, have had devastating effects throughout human history and emerging viral diseases such as Dengue, Chikungunya and, most recently Zika, are a growing concern (Tilak et al., 2016) . While bacterial infections have been modeled in zebrafish for about 2 decades, the concept that the heterologous zebrafish model could be useful also to address viral infection with natural human pathogens, emerged relatively recently in the field (Goody et al., 2014; Levraud et al., 2014; Meijer & Spaink, 2011). In fact, while the zebrafish model proved immediately very useful to address economically relevant fish-specific viral infections, three main aspects represented a limitation into the use of zebrafish to model human viral disease. These include the tight and evolutionary rapid adaptation of viruses to their natural hosts, the large implication of a mature adaptive immunity during virus pathogenesis and the fact that the interferon-mediated signaling (the main pathway used by innate immune cells to counteract viral infections)

remains poorly characterized in (zebra)fish and diverges in some aspects from mammalian systems (Briolat et al., 2014; Levraud et al., 2014).

Despite these considerations, zebrafish models for several important human viral disease have now been established, including Chickungunya, Influenza and Herpes Simplex. (Antoine et al., 2014; Burgos et al., 2008; Gabor et al., 2014; Goody et al., 2014; Levraud et al., 2014; Palha et al., 2013).

Chikungunya Virus (CHIKV) is a mosquito-transmitted virus, causing serious and sometimes deadly illness in humans with acute fever, persistent rash, and debilitating muscle and joint pain. Infection of three-day-old zebrafish larvae with CHIKV showed that the pathogen can invade multiple host tissues such as muscles, liver, jaws and spinal cord cartilages, gills, fins, vascular endothelium and even eyes and brain (Palha et al., 2013). Thus, in some tissues, CHIKV infection in zebrafish mimics the pattern in humans. Interestingly, in zebrafish CHIKV infection persists persistent in the brain, while other tissues mostly clear the infection (Palha et al., 2013). Use of an *ifnφ1* fluorescent reporter line demonstrated that neutrophils are important to mediate an antiviral response to CHIKV infection via Ifn-signaling (Palha et al., 2013). The fact that CHIKV displays a remarkable brain tropism and persistence suggests that in humans too this pathogen might persist in this organ. The hypothesis of a brain reservoir in humans is in line with the fact that, in adults, some CHIKV symptoms can persist for years, even after the apparent eradication of the pathogen. Furthermore, CHIKV is known to cause encephalitis in newborns (Gerardin et al., 2016; Rajapakse et al., 2010). Therefore, further use of the zebrafish model could elucidate how CHIKV crosses the blood-brain barrier and persists in the central nervous system (CNS).

Influenza A virus (IAV) is the causative agent of annual epidemics of influenza. Similarly to CHIKV infection, IAV infection could be followed over time in zebrafish, using fluorescently-labelled viruses (Gabor et al., 2014). Strikingly, the viral kinetics and tissue tropisms in zebrafish recapitulate those observed in other models. Heart and skeletal muscles, blood endothelium and the mucosa-associated epithelium of the swim bladder accumulate the GFP-labelled virus, which is consistent with the fact that IAV preferentially infects human muscle, epithelial and endothelial cells *in*

vitro. The pathology evoked in zebrafish shows relevant parallels also at the molecular level, since the viremia coincides with upregulation of the antiviral transcripts of *ifnφ1* and Myxovirus influenza resistance a (*mxa*), the latter being a close fish ortholog of human MX1. The study also successfully proved that the zebrafish disease can be reverted by treatment with the known human anti-influenza drug Zanamivir, which indicates that zebrafish has a potential use as a screening platform for the discovery of novel antiviral compounds (Gabor et al., 2014).

Adult zebrafish have been used to study Herpes simplex virus type 1 (HSV-1) infection, a common cause of mucocutaneous orolabial, ocular and genital infections in humans (Antoine et al., 2014; Burgos et al., 2008). HSV-1 can also invade and damage the CNS, persist in nervous ganglia and lead to severe complications such as blindness and encephalitis. Following injection into the zebrafish abdominal cavity, the viral infection could spread to the midbody and ultimately reach the head, where it replicated abundantly in the CNS (Burgos et al., 2008). The current model of HSV-1 entry is that surface heparan sulfate derivatives mediate the initial viral adhesion, which in turn permits the fusion of the viral envelope with the host cell. These heparan sulfate moieties that act as viral receptors are remarkably conserved in zebrafish and are widely expressed in the CNS, like in mammals (Baldwin et al., 2013). The entry in the CNS causes the most severe HSV-1 complications and the penetration in nervous ganglia is a well-known mechanism by which this pathogen can establish latent infections. Therefore, the zebrafish model can be used to address the mechanisms responsible for HSV-1 CNS-invasion and provide new insight into how HSV-1 establishes latency and provokes repetitive episodes of disease reactivation.

Together, these studies have demonstrated that the possibility to longitudinally follow the infection course with fluorescently-labelled viruses in developing zebrafish embryos or adult fish is very attractive to model important aspects of human viral infections, such as the cellular and molecular bases of tissue and organ-specific viral tropisms. These successes indicate that it will be also be worth to explore the possibility of developing a zebrafish model for other problematic human viral infections, including Zika virus. Studying Zika infection in developing zebrafish embryos and larvae could be a valuable addition to mouse models that have only

recently been established and could provide new opportunities for studying the mechanistic basis of the association of this virus with microencephaly in newborns (Cugola et al., 2016; Li et al., 2016).

Fungal infections

A variety of fungi are present in the commensal flora of human mucosae and skin. Most of them represent opportunistic pathogens and can cause mycotic disease in immunocompromised individuals or when subjects are exposed to large doses. The increasing number of people following immunosuppressive regimens or that are HIV-positive have made fungal disease an important cause of illness, especially in hospitalized settings.

The zebrafish model has been used to study several fungal pathogens of global health interest, which include *Candida albicans*, *Aspergillus fumigatus*, *Mucor circinelloides* and *Cryptococcus neoformans*. All these studies have shown that an appropriate competency of the innate immunity is important to curtail fungal infections (Chao et al., 2010; Knox et al., 2014; Tenor et al., 2015; Voelz et al., 2015). However, the involvement and relevance of macrophage and neutrophils in the response to each of these pathogens (or at least to the particular strains used in these studies) shows interesting specificity. During *Mucor* and *Candida* infection, both macrophages and neutrophils are highly recruited to the infection site and both phagocytose the fungal spores (Chao et al., 2010; Voelz et al., 2015). In sharp contrast, it is observed that *Aspergillus* conidia (asexual fungal spores) and *Cryptococcus* cells are essentially engulfed by macrophages, with neutrophils playing only a marginal function in counteracting these pathogens (Knox et al., 2014; Tenor et al., 2015). *Aspergillus fumigatus* is a dimorphic fungus that grows in yeast and hyphal forms. Infected zebrafish showed that neutrophils did not engulf the fungal spores (conidia), but can tightly associate with the hyphal form of the fungus (Knox et al., 2014). This suggests differential specificity of macrophage and neutrophil responses to the vegetative and reproductive fungal forms (Knox et al., 2014).

Similarly to *A. fumigatus*, *Candida albicans* is an opportunistic dimorphic fungus and most of humans are healthy carries of this commensal. The most frequent *Candida* infections are those that remain localized to the

mucosal tissues, but life-threatening conditions can derive from systemic dissemination, especially in immunocompromised individuals (Brothers et al., 2013). Interestingly, when *Candida albicans* is injected locally in the zebrafish hindbrain, it readily causes disseminated infection and high mortality, which is associated to its germination from yeast to hypha. Both zebrafish macrophages and neutrophils can phagocytose *Candida* (Brothers et al., 2013). Uptake of the yeast form is important to contain the transition to the hyphal and more invasive form, indicating that immune cells are also crucial to counteract the yeast-to-hyphal transition of dimorphic fungi. While this model mimics human systemic candidiasis, injection of *Candida albicans* into the swimbladder of zebrafish larvae can be used to model mucosal Candida colonization and to study the distinctive immune mechanisms at play on the mucosal surfaces (Gratacap & Wheeler, 2014).

Recent use of the zebrafish model has been critical to better characterize the mechanism of virulence of *Cryptococcus neoformans*, which represents an emerging and often fatal human pathogen (Bojarczuk et al., 2016; Tenor et al., 2015). Cryptococcal infection in humans generally initiates in the lung. However, the pathogen displays a remarkable tropism for the CNS, which is the main life-threatening complication of this fungal disease. Live imaging in zebrafish demonstrated that the predisposition of this pathogen to colonize the brain is maintained in this host and that the capability of the pathogen to cross the blood brain barrier depends on the virulence gene FNX1 and on a known cryptococcal invasion-promoting pathway previously identified in a murine model (Tenor et al., 2015). Additionally, longitudinal studies in zebrafish showed that macrophages are important to counteract the acute infection with this pathogen (Bojarczuk et al., 2016). However, it was observed that cryptococci can still largely proliferate intracellularly in macrophages, and, within 24 hours, they can counteract macrophage phagocytosis by progressively increasing their capsule size until this reaches an extent that severely limits further phagocytosis. This study suggests that the early proinflammatory activation of macrophages can control cryptococcal infection in healthy individuals, while intracellular survival and modification of the cryptococcal capsule will lead to uncontrolled progression of infection in immunocompromised patients (Bojarczuk et al., 2016).

Concluding remarks

Modeling of infectious diseases using the early life stages of zebrafish is continuing to demonstrate striking similarities in the mechanism of action of the innate immune system across vertebrates, which not only is evolutionary relevant, but also adds a high biomedical value to the use of the zebrafish model. Notably, in many cases the zebrafish platform has served as a valuable springboard to more extended studies in mammals. In other cases, the zebrafish has worked well as a surrogate system to model certain disease features that have otherwise been difficult to reproduce or study in mammalian models. Considering the expanding genetic toolbox for zebrafish research and the advanced use for non-invasive intravital imaging, it is to be expected that the zebrafish model will attract an increasingly larger scientific audience and continue to enforce its position in translational research. With state-of-art genome editing techniques now being successfully applied in zebrafish, it will be possible to generate a collection of key immune gene knockouts that will help to better understand the core mechanisms of immune recognition and pathogen virulence and to generate knowledge that can be exploited for developing novel therapeutic strategies to combat infectious and inflammatory diseases.

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