# Exploitation of host chemokine signalling by pathogenic mycobacteria

Torraca, V.

# Chapter 2

# Macrophage-pathogen interactions in infectious diseases: new therapeutic insights from the zebrafish host model

Vincenzo Torraca, Samrah Masud, Herman P. Spaink and Annemarie H. Meijer

*Institute of Biology, Leiden University, The Netherlands*

*Disease Models and Mechanisms, 2014 Jul;7(7):785-97*

# Chapter 2

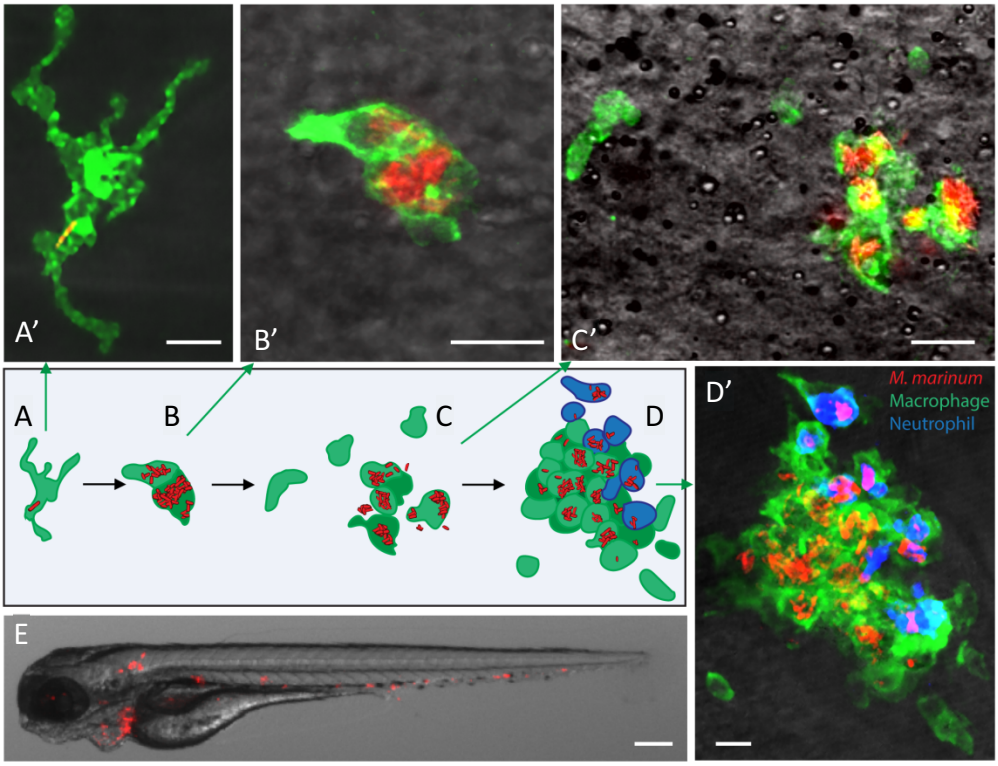
## Macrophage-pathogen interactions in infectious diseases: new therapeutic insights from the zebrafish host model

Vincenzo Torraca, Samrah Masud, Herman P. Spaink and Annemarie H. Meijer

*Institute of Biology, Leiden University, The Netherlands*

Studying macrophage biology in the context of a whole living organism provides unique possibilities to understand the contribution of this extremely dynamic cell subset in the reaction to infections, and has revealed the relevance of cellular and molecular processes that are fundamental to the cell-mediated innate immune response. In particular, various recently established zebrafish infectious disease models are contributing substantially to our understanding of the mechanisms by which different pathogens interact with macrophages and evade host innate immunity. Transgenic zebrafish lines with fluorescently labelled macrophages and other leukocyte populations enable non-invasive imaging at the optically transparent early life stages. Furthermore, there is a continuously expanding availability of vital reporters for subcellular compartments and for probing activation of immune defence mechanisms. These are powerful tools to visualise the activity of phagocytic cells in real time and shed light on the intriguing paradoxical roles of these cells in both limiting infection and supporting the dissemination of intracellular pathogens. This Review will discuss how several bacterial and fungal infection models in zebrafish embryos have led to new insights into the dynamic molecular and cellular mechanisms at play when pathogens encounter host macrophages. We also describe how these insights are inspiring novel therapeutic strategies for infectious disease treatment.

## GRAPHICAL ABSTRACT




The zebrafish-*M. marinum* model has become widely used to study human tuberculosis since the initial stages of granuloma formation recapitulate closely those initiated by *M. tuberculosis* in human lungs. The first step for granuloma formation is the establishment of an intra-macrophage infection niche (A-A'). Pathogenic mycobacteria (shown in red) resist in the host macrophage (shown in green) and replicate intracellularly until the host cell is unable to counteract the infection and activates mechanisms of programmed cell death (B-B'). Cell fragments and extracellular bacteria released by dying macrophages readily recruit new macrophages, which phagocytose the debris and take up the pathogen (C-C'). Repeated cycles of phagocytosis/bacterial expansion/macrophage death will determine the formation of the granuloma, an inflammatory lesion, predominantly consisting of macrophages that confine the infection. Neutrophils (shown in blue) associate poorly to the very initial stages of granuloma formation but become an important part of the lesion at later stages and can also contribute to bacterial clearance (D-D'). Lymphocytes are not essential to initiate granulomas but associate with these structures (both in adult zebrafish and in humans) in their more advanced stages. Lacking fully-developed adaptive immune cells, the embryonic and larval stages of the zebrafish infected with *M. marinum* (E) can serve as a platform to study the initiation of granulomas and the function of the innate immune cells in a context that anticipates the development of acquired immunity. Scale bars A-D: 10 µm. F: 200 µm.

## INTRODUCTION

The immune system has evolved through the constant interplay between microbes and their multicellular hosts. Selective forces acting on both sides have driven the evolution of a wide variety of virulence mechanisms in pathogens and alternative control mechanisms in their hosts. *In vivo* modelling of infectious disease is essential for understanding this complexity and translating it into novel therapeutic interventions. The immune system, innate and adaptive, is well conserved among vertebrates. The zebrafish (*Danio rerio*) offers an optically and genetically accessible vertebrate model to study host-pathogen interactions[1,2,3]. At the embryonic and early larval stages, zebrafish provide the opportunity of studying the relevance of innate immunity in a context where no adaptive response has yet been developed, given that early lymphocytes make their first appearance in 4-day-old larvae and a full adaptive immunity requires several weeks to be mounted[4,5].

Macrophages and neutrophils are the main phagocytic cell types of the innate immune system. Zebrafish models provide unique tools for studying the function of phagocytic cells, and these studies can effectively complement studies in other infectious disease models. Other recent reviews highlighted the use of zebrafish for understanding neutrophil biology[6,7]. Here, we will discuss six zebrafish models for important human pathogens (*Mycobacterium*, *Salmonella*, *Burkholderia*, *Staphylococcus*, *Shigella* and *Candida*), emphasising the novel insights that these models have recently provided into macrophage biology and highlighting how this could lead to the finding of new host-derived therapeutic strategies.

## ZEBRAFISH MACROPHAGE BIOLOGY AND TOOLS FOR INVESTIGATING MACROPHAGE FUNCTION

### Ontogeny and properties of early macrophages in zebrafish

The first macrophage precursors appear in the zebrafish embryo as early as 20 hours post fertilisation (hpf) from the anterior lateral plate mesoderm[8]. Following migration to the yolk sac, they differentiate and either invade the head mesenchyme, where they will later differentiate into microglial cells (the resident macrophages of the brain) or enter the blood circulation[8,9]. These cells, named primitive macrophages, retain proliferative capability and have been reported to exist in mammals too[8,10]. They can remove apoptotic cells, are able to sense and respond to invading microbes and can eradicate non-pathogenic infections. Primitive macrophages readily phagocytose microbes from the blood circulation or when present in tissues. In contrast, neutrophils (which develop slightly later) are less efficient in phagocytosing microbes in the blood but are potent scavengers of surface-associated bacteria[11].

Primitive macrophages are gradually replaced by different lineages of macrophages deriving from definitive haematopoiesis, the process that will produce specialised pluripotent cells with the ability to differentiate into all types of mature blood cells. The first wave of definitive haematopoiesis starts at 24 hpf in the posterior blood island or caudal haematopoietic tissue (CHT) with the differentiation of erythromyeloid progenitors[12]. By 48 hpf, these pluripotent progenitors are replaced with another subset of haematopoietic stem and progenitor cells (HSPCs), now able to also differentiate into the lymphoid lineage. These cells originate from the AGM (aorta, gonads and mesonephros), derived from the lateral posterior mesoderm. After leaving the AGM, they migrate to and

nest in the CHT and will provide the second wave of definitive haematopoiesis[12,13]. Development of HSPCs and their emergence from aortic endothelium is remarkably conserved between zebrafish and mammals[14,15,16].

Following the second wave of haematopoiesis, macrophage precursors are released into the circulation and will extravasate to seed tissues throughout the whole body, where they differentiate into tissue macrophages. Starting from 4 days post fertilisation (dpf), the kidney marrow, which is the main haematopoietic tissue of the adult fish, develops and will progressively replace the embryonic haematopoietic system. Another component of the mononuclear phagocyte system is represented by the dendritic cell (DC) population, which is also present in zebrafish larvae and can be detected from 8–12 dpf [17,18].

The infection studies discussed below, using zebrafish embryo and larval models, do not distinguish macrophages from circulating monocytes. Furthermore, possible functional differences between macrophages from primitive or definitive haematopoietic origins are generally not addressed. For more detailed and comparative descriptions of the processes of haematopoiesis in zebrafish and mammals we refer to other reviews[19,20].

**Macrophage defence mechanisms and subversion by intracellular pathogens**

Macrophages sense the presence of infection through microbial-specific molecules and host-derived inflammatory mediators. Their chemoattraction to the site of infection depends largely on the function of G-protein-coupled receptors[21,22]. Scavenger and complement receptors play a major role in phagocytosis[23] and Toll-like receptors (TLRs), in cooperation with other pattern-recognition receptors (PRRs), initiate the innate immune response[24]. TLRs, found on the cell surface and membranes of vesicular compartments, recognise pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively). Another main class of PRRs, the NOD-like receptors (NLRs), performs the same function in the cytosol[25,26]. Some NLRs participate in the assembly of the inflammasome, a multiprotein complex able to activate the caspase-1 cascade, which triggers processing of pro-inflammatory cytokines, such as IL1β (interleukin 1 beta) and full activation of the innate immune response[27].

When engulfed by macrophages, microorganisms are exposed to a number of defence mechanisms within the resulting phagosome and through its subsequent fusion with lysosomes. These include the production of reactive oxygen and nitrogen species (ROS and RNS, respectively)[28,29], exposure to antimicrobials, the activity of proteases and acidification[30,31,32]. Escape from the phagosome triggers septin caging and antibacterial autophagy as additional defence mechanisms[33,34].

Intracellular pathogens have evolved many strategies to counteract these defences. These counter-strategies are mediated by virulence factors, which are often secreted directly into the host cell via specialised secretion systems such as the T3SS (type III secretion system) of Gram-negative pathogens and the T7SS (type VII secretion system) of pathogenic mycobacteria[35,36]. Pathogens can also induce significant reprogramming of their host cells through manipulation of signalling pathways and chromatin remodelling; however, these mechanisms are still poorly understood[37,38]. Intracellular pathogens often block phagosome maturation and fusion with lysosomes or manipulate the vesicular system such that the phagosome is modified to resemble the endoplasmic reticulum or a Golgi-like

compartment[39]. Furthermore, several pathogens inject virulence factors that promote actin polymerisation to actively stimulate their uptake by both non-phagocytic and phagocytic cells[40]. Pathogens that are able to escape from the phagosome have mechanisms to evade autophagy and can spread from the initially infected cell to other cells by acquiring actin-based motility[40,41]. Other virulence mechanisms can induce inflammation and different cell-death programs to facilitate the dissemination of infection[42]. These different virulence strategies are schematically depicted in **Figure 1**.

**Macrophage markers and transgenic lines**

The development of transgenic zebrafish lines with fluorescently labelled leukocytes (**Supplementary Table 1**) has been key to the successful application of zebrafish for immunological studies. However, until recently, the lack of a specific reporter for the macrophage lineage limited the study of this myeloid subset. This has now been remedied with the development of the *csf1ra* and *mpeg1* reporter lines[43,44]. These genes are robust markers for macrophages at embryonic and larval stages, because they are co-expressed with the pan-leukocytic marker *lcp1* but not with the neutrophil markers *mpx* and *lyz*[45,46]. Despite the fact that *csf1ra* is macrophage-specific within the immune cell types, it is also expressed in neural crest cells and derivatives, such as the xanthophores. Nevertheless, the highly motile macrophages can be distinguished easily from the immobile xanthophores in time-course experiments[43]. Reporter lines using the *mpeg1* promoter label macrophages but not xanthophores (**Figure 2A**, **Supplementary Movie 1**) and, combined with a neutrophil marker, can show the different kinetics of macrophage and neutrophil responses to infection and wounding, as well as the dynamic interactions between the two cell types[44]. The *mpeg1* reporter also labels microglia and it has been suggested to label other antigen-presenting cells, such as the Langerhans dendritic cells, but these could not be detected before 8–9 dpf[18].

Expression of the Gal4 transcription factor under the control of macrophage or neutrophil promoters in combination with a UAS:nitroreductase-mCherry line allows for the specific ablation of one of the two phagocyte populations. This approach can be used to investigate their individual contributions to the immune response and infectious disease pathogenesis[43,47]. Alternatively, *spi1/pu.1* antisense morpholino knockdown can be used to block the development of either macrophages exclusively or of both macrophages and neutrophils, depending on the concentration used[48]. Similarly, *irf8* tools have also been used to deplete specific myeloid cell populations and to skew the development of their progenitors towards macrophages or neutrophils. Morpholino knockdown of *irf8* can completely deplete macrophage differentiation while stimulating an increased output of neutrophils and *irf8* overexpression can direct myeloid development towards macrophage differentiation[49].

Many other transgenic lines that label either the entire myeloid population, the early myeloid subset, microglia or all antigen-presenting cells are also very useful for the study of macrophage biology (**Supplementary Table 1**).

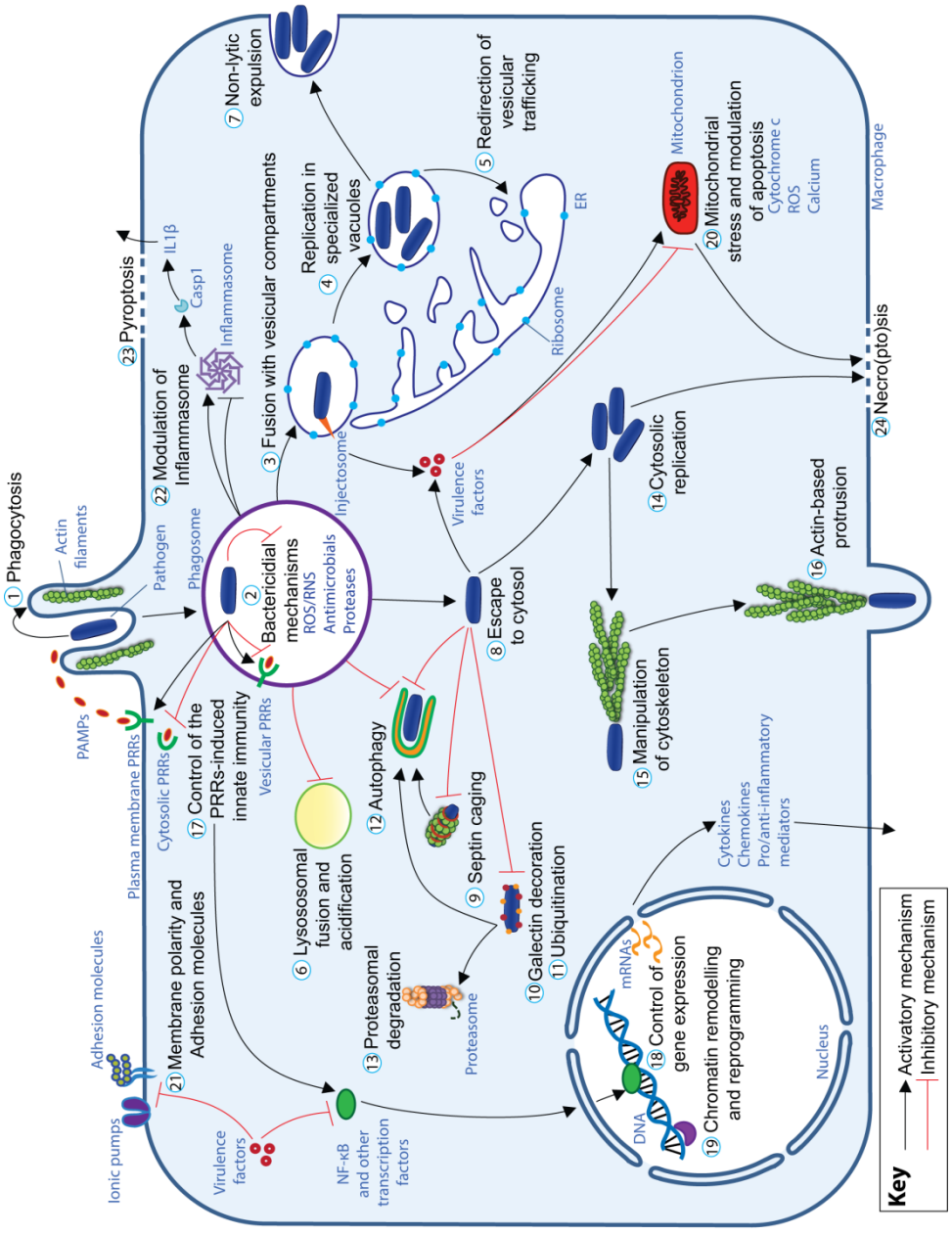



**Figure 1. Evasion of macrophage defence mechanisms by intracellular pathogens** *(Legend on the next page)*.

**Figure 1. Evasion of macrophage defence mechanisms by intracellular pathogens** *(Figure on the previous page)*. Upon phagocytosis (1), the pathogens generally reside within phagosomal compartments where a plethora of microbicidal components cooperate in a multidirectional assault to the microbes (2). By transferring virulence factors, often via secretion systems (injectosomes), some pathogens can avoid the classical maturation steps of these compartments, creating a favourable niche for their intracellular growth (3, 4, 5). Fusion of the phagosome with endosomes and/or lysosomes can be blocked (6) and fusion with Golgi- and reticulum-like vesicles can be promoted (3), resulting in the formation of specialised replicative vacuoles (4, 5), in some cases also directed to non-lytic expulsion (7). Several intracellular pathogens are able to escape directly into the cytosol (8). Here, septin cages (9), galectin decoration (10), ubiquitylation (11) and specific routes of antimicrobial autophagy (12) are activated to capture the escapers and redirect them to lytic compartments. Additionally, ubiquitylation of microbial proteins (11) labels these for proteasomal degradation (13). Several intracellular pathogens can efficiently counteract this second line of intracellular defence and replicate freely within the cytosol (14), frequently also manipulating the cell cytoskeleton (15) to sustain their extrusion and dissemination to other host cells (16). Intracellular infections have profound influences also on a wide spectrum of host functions. Cell signalling pathways can be manipulated to modulate the host inflammatory response (17) and control gene expression (18). Some pathogens are also known to induce epigenetic modification of their host cells, leading to reprogramming (19). Some virulence factors directly impact the homoeostatic mechanisms by interfering with normal mitochondrial functionality (20), membrane polarity and communication with the extracellular milieu (21). The ultimate possibility for the host to eradicate the infection is to initiate cell (pyroptotic, apoptotic or necroptotic) suicide programs (20, 22, 23, 24). However, the death mechanisms can also be modulated by pathogens, which can benefit from them by the induction of host damage, pathogen dissemination and the initiation of new replicative cycles.

## *In vivo* visualisation of macrophage function

Visualisation of live macrophage behaviour in zebrafish embryos can be achieved with great structural detail using digitally enhanced differential interference contrast (DIC) microscopy[8,50,51,52]. More recently, there has been tremendous progress in the use of transgenic marker lines (**Supplementary Table 1**) and labelled pathogens that facilitate live imaging in spatial and temporal dimensions (**Figure 2, Supplementary Movies 2-3**).

Photoconvertable fluorescent proteins such as Kaede and Dendra2 have been exploited to show that cells from the CHT can be recruited distally to infection foci and wounds[53] and that *Mycobacterium*-infected macrophages egress from primary granulomas to initiate secondary infection foci[52]. For imaging of phagocyte migration, pathogens or specific chemoattractants can be injected subcutaneously or into body cavities such as the otic vesicle and hindbrain ventricle, which can be reached without generating extensive tissue damage, thereby preventing wound-induced leukocyte mobilisation[11,54,55,56,57,58]. To visualise phagocytosis and the intracellular fate of bacteria, the pHrodo dye can be conjugated to bioparticles or to live or heat-killed bacteria (**Figure 2D**), providing constitutive fluorescence in one channel and additional fluorescence in another channel following exposure to an acidic environment[59]. Furthermore, the nature of the compartments where the pathogens reside can be investigated with combinations of different vital stains, most of which are permeable into zebrafish embryos when added to the water. Several pH-sensitive dyes (LysoSensor and LysoTracker) do not distinguish between lysosome-dependent or -independent phagosome acidification mechanisms, but they can be used simultaneously with methods for detection of the activity of lysosomal proteases (MagicRed-Cathepsin and DQ-BSA)[60].

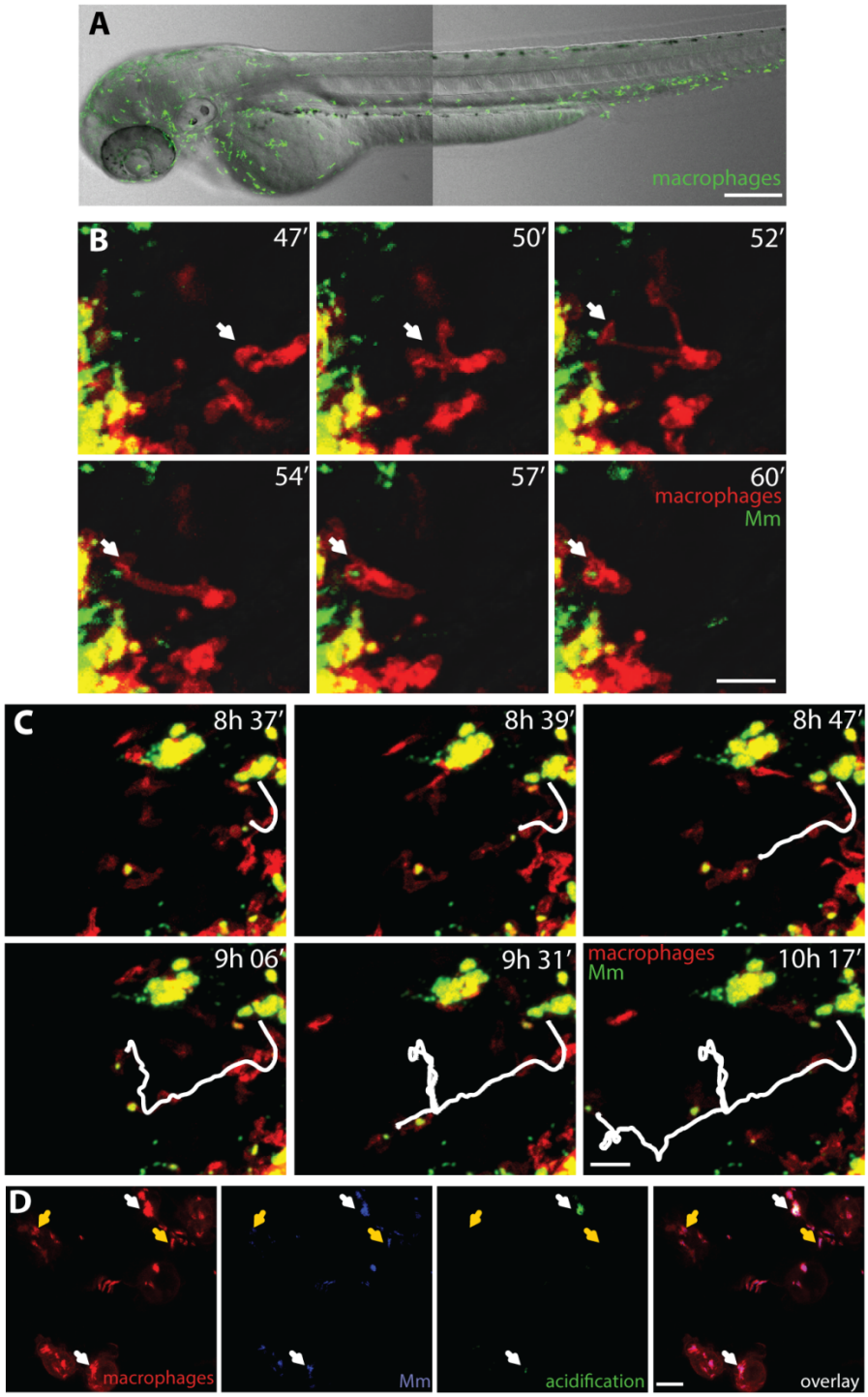



**Figure 2**. ***In vivo*** **imaging of macrophage responses to infection** *(Legend on the next page)*.

**Figure 2**. ***In vivo*** **imaging of macrophage responses to infection** *(Figure on the previous page)*. **A.** A 3-dpf *Tg(mpeg1:Gal4-VP16/UAS-E1b:Kaede)* zebrafish embryo showing the distribution pattern of macrophages (green). Random patrolling of macrophages is shown in Supplementary Movie 1. **B.** Phagocytosis of *M. marinum* (*Mm*; green) injected into the subcutaneous area overlying a somite in a 2-dpf *Tg(mpeg1:mCherry-F)* embryo. The arrow points at a macrophage (red) in the process of phagocytosis between 47 and 60 minutes post infection. The images are particulars and stills from **Supplementary Movie 2** (10 to 60 minutes post infection). **C.** Macrophage-mediated dissemination of *M. marinum* infection. The white track represents the path of an infected macrophage migrating away from the infection focus. The images are stills and particulars from **Supplementary Movie 3**, which was taken from the same embryo as in B at a more advanced stage of infection (~8 to ~10 hours post infection). **D.** Partial acidification of phagocytosed *M. marinum*. Bacteria double-labelled with constitutive mCrimson and pH-sensitive green pHrodo are contained within subcellular compartments of macrophages, which are intensely labelled by the membrane-bound mCherry of the *Tg(mpeg1:mCherry-F)* line. White arrows point at bacteria in acidified compartments, where the pHrodo dye is activated. Yellow arrows point at bacteria in non-acidified compartments. Note that most of the intracellular mycobacteria are not acidified, consistent with the ability of this pathogen to counteract phagosome maturation. Macrophages were imaged in the yolk sac circulation valley 5 hours after injection of bacteria into the caudal vein at 2 dpf. Images in A–C were acquired with the Zeiss Observer 6.5.32 laser-scanning confocal, with 10× (A) or 20× (B-C) objectives. Images in D were acquired with Leica TCS SPE confocal with a 20× objective. Figures and movies were processed with ImageJ. The zebrafish transgenic lines *Tg(mpeg1:Gal4-VP16/UAS-E1b:Kaede)* and *Tg(mpeg1:mCherry-F)* were previously described in other reports[44,61]. Scale bars: (A) 200 µm; (B-C) 25 µm; (D) 10 µm.

Different methods allow *in situ* detection of ROS and RNS responses during infection in zebrafish embryos[62,63,64,65]. Also, tools for visualising ATP, calcium effluxes and apoptosis have been efficiently used in zebrafish[60,66,67]. Furthermore, an increasing number of transgenic marker lines for vesicular compartments are becoming available that will help in elucidating the subcellular locations where pathogens reside *in vivo* (**Supplementary Table 2**).

## NEW INSIGHTS INTO MACROPHAGE-PATHOGEN INTERACTIONS

### *Mycobacterium marinum*

*M. marinum* is a natural pathogen of zebrafish that causes granulomatous necrotic lesion formation in host tissues. These lesions are histologically very similar to those generated by *Mycobacterium tuberculosis*, the aetiological agent of human tuberculosis[68,69]. In adult zebrafish, *M. marinum* can cause a latent infection and the bacteria can be reactivated from dormancy by immunosuppressive treatment, as is the case for *M. tuberculosis*, which is estimated to have infected one-third of the world population[70,71]. Tuberculosis therapy is limited by a number of problems, including the poor response of dormant mycobacteria to antibiotics, the increasing prevalence of multidrug-resistant strains and the lack of an effective vaccine against latent or reactivated tuberculosis[72]. The lack of a mouse model for tuberculosis that fully recapitulates the disease and the risk of working with the human pathogen owing to its airborne transmission emphasise the need for alternative models. The unique accessibility of the early stages of granuloma formation in zebrafish larvae has made the zebrafish-*M. marinum* host-pathogen pair one of the most productive models used to unravel the core pathogenic processes of mycobacterial infections[3,50,73] (**Figure 3A**). Notably, the use of this model has already provided several direct translational applications for human disease treatments (**Table 1**).

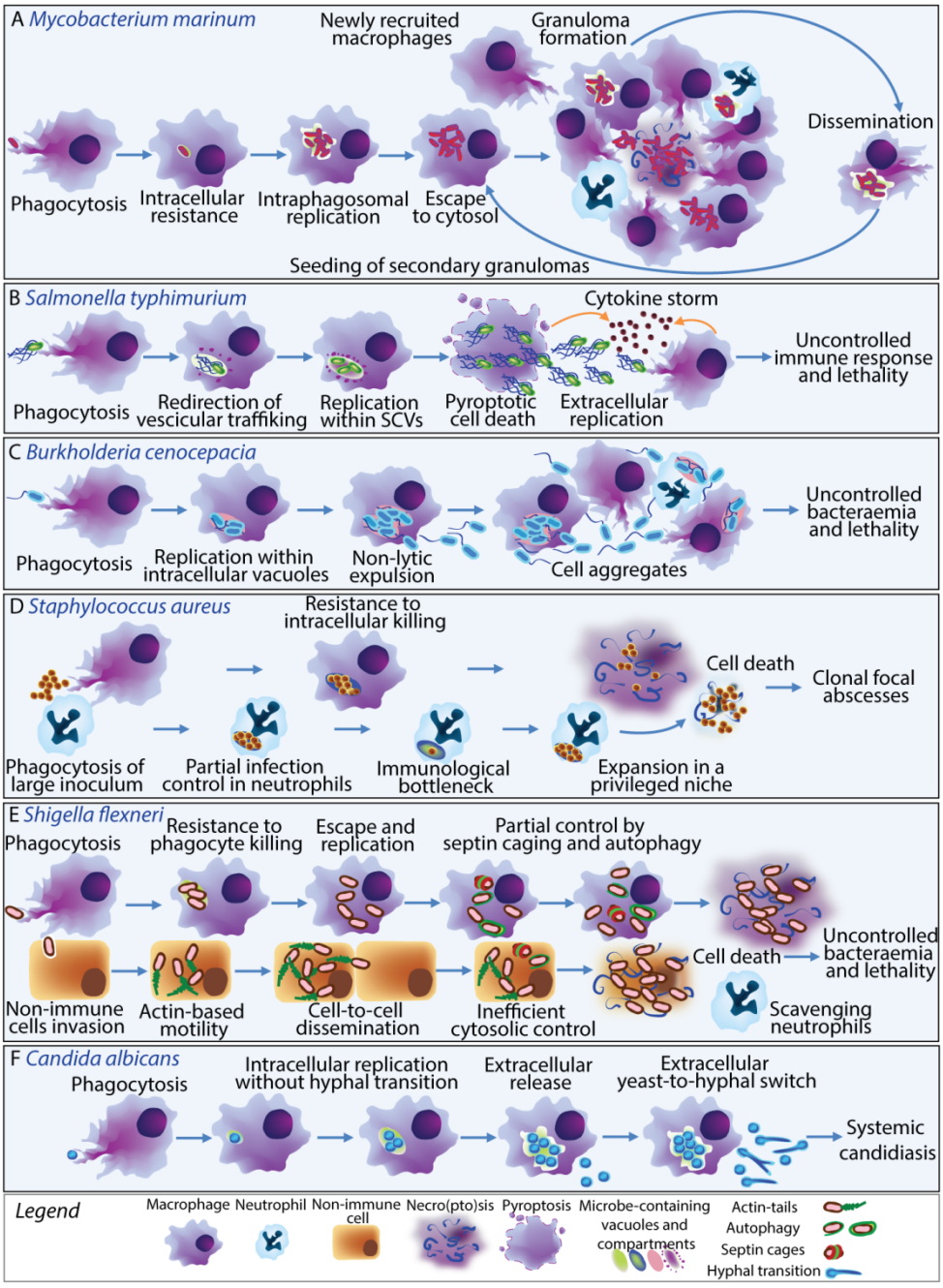



**Figure 3. Models of intracellular infections in zebrafish** *(Legend on the next page)*.

**Figure 3. Models of intracellular infections in zebrafish** *(Legend on the previous page)*. Schematic comparison of the infection phenotypes caused by different pathogens following intravenous injection in zebrafish embryos. **A.** *M. marinum* can replicate within phagosomes and also escape into the cytosol. Eventually, the host macrophages succumb to the infection and the pathogen spreads to new macrophages that have been recruited through bacterial virulence mechanisms. This leads to the formation of granulomatous lesions. Occasionally, infected macrophages can egress from the primary granuloma and seed secondary granulomas. **B.** *S. typhimurium* avoids the phagosomal defences by inducing the formation of non-lytic compartments [*Salmonella*-containing vacuoles (SCVs)]. Upon replication, the pathogen induces pyroptotic cell death. Extracellular *Salmonella* continues to replicate. Damage- and pathogen-associated signals contribute to uncontrolled inflammation ('cytokine storm'), which is rapidly fatal for the host. **C.** *B. cenocepacia* can also replicate within intracellular compartments. Additionally, it can be non-lytically expelled from the host macrophages. Within the extracellular environment, the pathogen stimulates leukocyte aggregation and continues replication. The resulting uncontrolled bacteraemia is the major cause of the fatal complications. **D.** *S. aureus* is largely resistant to intracellular killing when phagocytosed by macrophages and leads to their necrotic death. By contrast, when phagocytosed by neutrophils, most of the pathogen can be efficiently neutralised. However, resistant clones occasionally emerge and expand within these cells. This "intraphagocyte niche" is the reason of the monoclonality of focal staphylococcal abscesses. **E.** *S. flexneri* can invade non-immune cells. Within these cells, the pathogen escapes immediately into the cytosol and gains actin-based motility, by which it disseminates from cell to cell. Within macrophages, the pathogen can also escape from the phagosome, but here a more efficient cytosolic control partially combats the invader, delaying (although not avoiding) macrophage cell death. Neutrophils represent efficient scavengers for extracellular *Shigella* released from dying epithelial cells and macrophages but the infection is still rapidly lethal. **F.** Phagocytosis of *C. albicans* by macrophages leads to a standoff phase, where the host does not degrade the pathogen, but its virulence is contained as it remains locked into a yeast form. The fungus can still slowly replicate and eventually is released. Extracellularly, the yeast can germinate and the resulting fast-replicating hyphae will invade the whole organism, leading to systemic infection.

Infection of zebrafish embryos with *M. marinum* has demonstrated that macrophages are sufficient to initiate granuloma formation in the absence of adaptive immunity[50]. Subsequently, this model changed the widespread view of granulomas, historically regarded solely as host-protective structures, by showing that early granulomas promote mycobacterial dissemination (**Figure 3A**) and that their formation is driven by virulence determinants of the RD1 locus, encoding ESX-1, a secretion system conserved in all pathogenic mycobacteria[3,35,52,74]. Furthermore, the ESX-1-secreted protein ESAT-6 (Early secreted antigenic target 6) was found to induce matrix metalloproteinase Mmp9 production by epithelial cells surrounding the infection focus, which in turn facilitates macrophage infiltration. As a result, the application of Mmp9 antagonists has been suggested as a host-directed anti-tuberculosis therapy[75] (**Table 1**). The notion that granulomas are dynamic structures, even during latent infection, is supported by a study of *M. tuberculosis* infection in the macaque model[76].

Mycobacteria are well known for developing drug tolerance. The zebrafish embryo model has demonstrated that their intramacrophage localisation correlates with development of resistance and that granulomas promote dissemination of this resistant population. Upregulation of bacterial efflux pumps, which are required for intracellular growth, can mediate drug tolerance both in *M. marinum*-infected zebrafish and in *M. tuberculosis*-infected human macrophages. Efflux-pump inhibitors, already available on the market, can reduce this tolerance and their addition to standard anti-tuberculosis therapy might, therefore, shorten treatment duration[77,78] (**Table 1**).

| Target | Susceptible pathogens | Desired drug effect | Drugs tested | Ref. |
|---|---|---|---|---|
| **MMP9** Matrix metalloproteinase 9 | Mycobacteria | Reduction of ESAT6/Mmp9-dependent macrophage infiltration to mycobacterial infection | Not tested | 75 |
| **Efflux pumps** Multiple bacterial genes | Wide spectrum of bacteria | Prevention of drug tolerance development mediated by a macrophage-induced efflux mechanism | Verapamil* Reserpine* | 77,78 |
| **GR** Glucocorticoid receptor | Mycobacteria | Suppress detrimental inflammation in mycobacterial infection in patients with proinflammatory genotypes | Glucocorticoids* | 84 |
| **COXs** Cyclooxygenases | Mycobacteria | Suppress detrimental inflammation in mycobacterial infection in patients with proinflammatory genotypes | Acetylsalicylic acid (Aspirin)* | 84 |
| **BLT1** LTB4 receptor | Mycobacteria | Suppress detrimental inflammation in mycobacterial infection in patients with proinflammatory genotypes | U-75302*** | 84 |
| **CYPD** Cyclophilin D | Mycobacteria | Prevention of mitochondrial permeability transition, limiting ROS-dependent necroptosis | Alisporivir** | 83 |
| **ASMase** Acid sphingomyelinase | Wide spectrum of intracellular pathogens | Prevention of ceramide production, limiting ROS-dependent necroptosis | Desipramine* | 83 |
| **ROS** Reactive oxygen species | Wide spectrum of intracellular pathogens | Scavenging of ROS | N-acetylcysteine* Amifostine* Tempol* | 83 |
| **HIF-1α/HIF-2α** pathway Hypoxia-induced factor 1 and 2 α | Wide spectrum of pathogens | Stimulation of protective Hif- and iNOS-mediated RNS production in neutrophils | Not tested | 65 |
| **iNOS** Inducible nitric oxide synthase | Wide spectrum of pathogens | Stimulation of protective RNS production and beneficial emergency haematopoiesis | Not tested | 62,65 |
| **IRG1** pathway Immunoresponsive gene 1 | Wide spectrum of pathogens | Modulation of fatty acids catabolism and of mitochondrial ROS production | Not tested | 63 |
| **SQSTM1/p62** Sequestosome 1 | Wide spectrum of intracellular pathogens | Enhancement of infection control by selective autophagy mechanisms | Not tested | 86 |

**Table 1. Therapeutic strategies inspired by the zebrafish model to counteract intracellular infections** *(Legend on the next page)*.

**Table 1. Therapeutic strategies inspired by the zebrafish model to counteract intracellular infections** *(Table on the previous page)***.** The zebrafish host model has contributed extensively to the investigation of novel therapeutic strategies, oriented on modulating host-derived responses. Metalloproteinase inhibitors can reduce the tissue inflammatory response guiding phagocytes towards mycobacterial infections, thus attenuating the expansion of primary granulomas and the seeding of secondary infectious foci. Efflux pumps, although not representing a host target, impact directly on the capability of the pathogens to adapt to the intracellular growth and their blockade can reduce drug tolerance. Several classes of established anti-inflammatory drugs are beneficial for subsets of tuberculosis and leprosy patients, dependent on their genotypically-determined inflammatory status (suppression of excessive inflammatory response). Levels of ROS and RNS work as a double-edged sword and their tight control can stimulate a positive outcome of the infectious process. Drugs scavenging, suppressing, or boosting ROS/RNS production are thereby valuable therapeutic tweezers to fine tune their balance. Finally, the possibility of stimulating pathogen-selective autophagy is a promising therapeutic approach. Specific drugs tested in the zebrafish model and supporting these approaches are listed: [*] Accepted drug; [**] Drug accepted for clinical trial; [***] Not accepted for clinical trial.

Another important insight into tuberculosis pathogenesis concerns the relevance of the inflammatory status. A zebrafish mutagenesis screen revealed Lta4h (Leukotriene A4 hydrolase) as a host factor that strongly correlates with *M. marinum* susceptibility[79]. This enzyme is required for producing LTB4 (Leukotriene B4), a powerful proinflammatory chemoattractant. Lta4h deficiency correlates with a less inflamed status, due to redirection of its substrates to anti-inflammatory lipoxins, resulting in reduced levels of proinflammatory cytokines such as tumour necrosis factor (TNF). The crucial role of TNF in controlling mycobacterial infection is exemplified by the increased risk of tuberculosis in patients with chronic inflammatory disorders treated with TNF antagonists[80]. However, excessive production of TNF is also associated with higher susceptibility to tuberculosis. This has been shown in zebrafish and other animal models as well as in tuberculosis meningitis patients, where a polymorphism at the *LTA4H* locus that causes increased TNF production has been linked with more progressive disease symptoms[79,81]. Hyper-inflamed and hypo-inflamed statuses have both been associated with necrotic death of infected macrophages and consequent extracellular release of the pathogen[82,83]. In conditions of low inflammation, macrophages passively undergo necrotic death because they are unable to control intracellular bacterial growth, whereas, in conditions of high inflammation, macrophages actively initiate two ROS-mediated necroptotic pathways, dependent on the activation of the mitochondrial permeability transition pore complex (mPTPC) and of the lysosomal acid sphingomyelinase (aSMase). Drugs targeting these pathways prevent activation of the necroptotic program in the zebrafish model[82]. The crucial role of the inflammatory status emphasises the importance of designing personalised patient therapies: patients with the proinflammatory *LTA4H* genotype might benefit from classical anti-inflammatory drugs [such as corticosteroids or non-steroidal anti-inflammatory drugs (NSAIDs)]; however, these drugs should be avoided in patients with the opposite genotype[79,84]. Drugs that directly block the ROS-linked necroptotic pathways will benefit the proinflammatory genotypes without generating detrimental effects on the other genotypes, because the necroptotic pathways are exclusively triggered in hyper-inflamed conditions[83] (**Table 1**).

The inflammatory response is initiated by TLR recognition of PAMPs. Myd88, a central adaptor in TLR signalling, was recently shown to be required for control of systemic *M. marinum* infection in zebrafish embryos[85]. In contrast, the initial recruitment of macrophages to a localised *M. marinum* infection in the hindbrain was found to be largely

Myd88 independent[58]. This effect was linked to the presence of PDIM (phthiocerol dimycocerosate) lipid on the surface of pathogenic mycobacteria, which masks the underlying PAMPs. Non-pathogenic mycobacteria, which lack PDIM, induce a robust immune response and are efficiently contained. Mutation of the PDIM transporter (*ΔmmpL7*) and of a factor involved in PDIM synthesis (*Δmas*) can restore Myd88-dependent macrophage recruitment and allow an efficient intracellular RNS response against invading bacteria. Interestingly, in the absence of a TLR response, macrophages can still be recruited. This Myd88-independent recruitment was found to be mediated by cell-surface phenolic glycolipids (PGLs), which induce macrophage recruitment through a pathway that is analogous to the mammalian CCL2-CCR2 (CC-motif chemokine ligand–receptor 2) axis. The macrophages recruited in this situation are suggested to be more permissive to intracellular bacterial growth, because they do not drive the strong intracellular RNS response. These observations might also explain why *M. tuberculosis* establishes infection in the lower rather than in the higher respiratory tracts, because the latter is exposed to resident and environmental microbes that can make macrophages more competent for intracellular killing via a continuous transduction of TLR-dependent immune signalling[58].

Although macrophages are the main cell type infected by *M. marinum* following intravenous injection, neutrophils are also important for early infection control in zebrafish. In the early granuloma, the protective role of neutrophils was found to depend on ROS production[56]. Prior to granuloma formation, neutrophils, both infected and non-infected, also produce RNS[65]. This RNS production is dependent on inducible nitric oxide synthase (iNOS) and can be modulated by genetic or pharmacological modulation of Hif-α (Hypoxia-inducible factor alpha) transcription factors. Increasing Hif-1α signalling or decreasing Hif-2α signalling primes neutrophils with higher levels of RNS prior to infection, thereby limiting susceptibility to mycobacterial infection. Increasing host RNS output by therapeutic targeting of the Hif-α pathway might shift the balance in favour of the host and can thereby be explored as a strategy to complement antibiotic interventions (**Table 1**).

In addition to the classical microbicidal mechanisms of macrophages, antibacterial autophagy has emerged as an important supplementary control mechanism in mycobacterial infections[34]. Using the zebrafish model, colocalisation of mycobacteria with the autophagic marker Lc3 has been demonstrated *in vivo*[2]. Moreover, actin tail formation and recruitment of septin cages have been visualised, the latter also being associated with Lc3 and autophagy[86]. The induction of dram1 (DNA damage-regulated autophagy modulator 1) during infection suggests an immunological function of this autophagy modulator and the zebrafish model could be further exploited to investigate therapeutic targeting of selective autophagy pathways[86,87].

Very recently, a zebrafish larval model has also been established to study a rapidly growing *Mycobacterium*, *M. abscessus*, an emerging pathogen that causes severe pulmonary infections in individuals with cystic fibrosis[61]. The study showed that the virulent rough morphotype of *M. abscessus* is transported to the central nervous system by macrophages, where bacteria released from dying cells form massive amounts of serpentine cords that grow too large to be phagocytosed, leading to acute and lethal infection. Furthermore, *M. tuberculosis* can also be disseminated by zebrafish larval macrophages and is sensitive to antibiotic treatment in this model[88]. It will be of interest to investigate novel therapeutic

strategies emerging from the study of *M. marinum* (**Table 1**) also in the zebrafish models for these human pathogens.

### *Salmonella enterica* serovar Typhimurium (*S. typhimurium*)

Like many other Gram-negative enterobacteria, *Salmonella* can infect a diverse range of hosts and cause zoonotic diseases[89]. The *S. enterica* serovar Typhimurium, often referred to as *S. typhimurium*, represents a common agent of enteric fever, gastroenteritis and bacteraemia, often linked to food poisoning. Although the bacterium is not a natural fish pathogen, zebrafish embryos are strongly susceptible to *S. typhimurium* in experimental settings. Pathogenesis in fish involves some of the acute symptoms seen in humans, including bacteraemia and a strong proinflammatory host response ('cytokine storm'), which are associated with early lethality of the zebrafish embryos[90,91]. The life cycle of *S. typhimurium* has been well characterised in infected cell cultures and in mammalian systems: the pathogen is able to alternate phases of intracellular replication within phagocytes and extracellular growth within the damaged tissue[92,93]. Similar observations have been made in the zebrafish embryo model. *S. typhimurium*, like *M. marinum*, can survive intracellularly in macrophages of zebrafish embryos (**Figure 3B**), but infected cells do not disseminate into tissues. Instead, following intravenous injection, the infection remains restricted to the vasculature, with bacteria multiplying both in macrophages and extracellularly at the epithelium of blood vessels[90]. Although infection with wildtype bacteria causes early lethality, bacteria with mutations in lipopolysaccharide (LPS) are attenuated in macrophages and are more sensitive to extracellular lysis, likely due to complement factors[90].

In sharp contrast with *M. marinum* infection, S. *typhimurium* infection leads to a cytokine storm within hours after intravenous injection[2,91,94]. At the transcriptional level, this response is similar to that observed in infection with the natural fish pathogen *Edwardsiella tarda*[85]. This proinflammatory transcriptional response provides a useful readout for characterising the consequences of mutation or knockdown of host genes involved in the immune response. Deficiencies in the TLR signalling components Myd88 and Traf6 strongly reduce expression of transcriptional regulators, signalling components and effectors of the immune response[85,95]. Conversely, these gene groups are hyper-induced following knockdown of the protein tyrosine phosphatase Shp1 (also known as Ptpn6)[96]. These observations are consistent with the function of these genes in mammalian animal models and human patients, where MYD88 and TRAF6 mutations are associated with immunodeficiencies and SHP1 mutations cause inflammatory phenotypes and autoimmune defects. Control of *S. typhimurium* and other infections in zebrafish is impaired both under conditions of a reduced or hyper-induced immune response, indicating the importance of highly balanced regulatory mechanisms[85,96]. Micro-RNAs (miRNAs), including members of the miR-146 family, have been implicated in fine-tuning of the mammalian innate immune response and, in zebrafish embryos, miR-146 is induced by *S. typhimurium* in a Myd88-Traf6-dependent manner. Although no major effects on known targets of the Myd88-Traf6 pathway were observed, apolipoprotein-mediated lipid transport emerged as a newly identified infection-inducible pathway under control of this miRNA family[97].

The signals involved in recruitment of phagocytes to local infection remain to be elucidated. Chemokines, such as Cxcl8 (Il8) and the orphan ligand Cxcl-c1c/Cxcl18b, are highly induced rapidly upon *S. typhimurium* infection[91]. Using other bacterial infection

models, the function of the CXCL8-CXCR2 signalling axis in neutrophil recruitment has been shown to be conserved in zebrafish[55,57]. Local *S. typhimurium* infection has also been shown to induce the recruitment of macrophages via the chemokine receptor Cxcr3.2, one of the zebrafish orthologues of human CXCR3[46]. The ligand association of Cxcr3.2 remains to be established.

In addition to phagocyte recruitment, localised infection also triggers emergency-driven haematopoiesis[62]. Early neutropaenia is a frequent outcome of *S. typhimurium* hindbrain infection in zebrafish embryos and this is compensated for by increased granulopoiesis. The activity of iNOS (and thus the production of the pleiotropic mediator nitric oxide), was found to be necessary to stimulate the expansion and proliferation of HSPCs in response to infection-dependent neutropaenia. The induction of iNOS is dependent on expression of the transcription factor C/ebpβ (CCAAT enhancer-binding protein β) in HSPCs. This is suggested to be an effect of elevated circulating levels of Gcsf (Granulocyte colony stimulating factor), produced by activated macrophages at the infection site[62].

The *S. typhimurium* infection model has recently led to new insight into the connection between infection control and host cell metabolic modulation[63]. Profound adaptations in glucose and lipid metabolism occur within infected immune cells. For example, in response to stimulation by *Salmonella* pathogenic factors, macrophages increase their uptake of lipids to fuel ROS production[63]. In line with this, *S. typhimurium* infection induces the expression of the mitochondria-associated enzyme Irg1 (Immunoresponsive gene 1) within infected zebrafish macrophages. This protein directs the catabolism of fatty acids to sustain mitochondrial oxidative phosphorylation and in turn leads to the production of mitochondrial ROS, contributing to intracellular degradation of phagocytosed bacteria. Irg1 holds promise as a new therapeutic target at the interface of inflammation and metabolism[62] (**Table 1**).

***Burkholderia cenocepacia***

The *B. cepacia* complex (*Bcc*) is represented by several closely related Gram-negative species that are able to survive freely in the environment or replicate within different hosts, including amoebae, invertebrates, vertebrates and plants[98]. In humans, opportunistic infection by *Bcc* frequently occurs in cystic fibrosis or immunocompromised individuals and represents a recurrent cause of fatal complications[99]. The capability to survive and infect a wide range of hosts suggests that *Bcc* species are highly adaptable to different niches and produce multiple virulence factors; however, the complex mechanisms of host-pathogen interactions underlying infections with *B. cenocepacia* and other *Bcc* strains remain largely unknown. In particular, it has been difficult to establish conclusively whether *B. cenocepacia* can survive intracellularly. Visualising infection in zebrafish embryos helped to answer this key question, by demonstrating the ability of this pathogen to survive within macrophages (**Figure 3C**). Following the creation of an intramacrophage replication niche, the bacterial infection disseminates by non-lytic expulsion from infected cells, induces immune cell aggregations and ultimately causes fatal systemic bacteraemia[100].

The zebrafish model system has also provided a valuable contribution to the investigation of the *in vivo* relevance of several *B. cenocepacia* virulence factors. A quorum-sensing-deficient cepR strain was shown to be strongly attenuated, indicated by a reduced ability to

replicate intracellularly and to disseminate efficiently from infected macrophages. Furthermore, differences in virulence were observed between strains from a panel of clinical isolates[100]. Loss of the third replicon, pC3 (a non-essential megaplasmid associated with several virulence determinants), results in highly attenuated infection in multiple hosts, including zebrafish embryos[101,102]. Mutants in pC3 are not able to grow significantly *in vivo* but are not eradicated, suggesting that the pC3-linked virulence factors are dispensable for intramacrophage survival[101]. A function in adaptation to a wide range of environments, rather than a direct role in modulating intracellular growth, might explain the prevalence of the pC3 replicon among *Bcc* isolates[102].

Together, these studies demonstrate the usefulness of the zebrafish model for analysis of *B. cenocepacia* mechanisms of intracellular survival and virulence.

***Staphylococcus aureus***

*S. aureus* is the causative agent of a wide range of infectious pathologies such as sty, pneumonia, endocarditis, osteomyelitis and septicaemia[103], which remain important causes of morbidity and of complications in hospitalised patients. Although not a natural pathogen of zebrafish, both embryos[104] and adults[105] exhibit clear acute symptoms when infected with this Gram-positive pathogen, providing a useful model for bacteraemia (**Figure 3D**).

*S. aureus* has long been considered an extracellular pathogen, but there is accumulating evidence that it can also survive and replicate in phagocytes[106]. The zebrafish embryo model has contributed significantly to our understanding of the nature and relevance of the intracellular phase in the life cycle of this pathogen[47,104]. Live imaging showed that, upon intravenous infection, *S. aureus* is completely phagocytosed by macrophages and neutrophils[104,107]. Although some embryos clear the infection in a phagocyte-dependent manner, other embryos develop overwhelming infection, indicating that the bacteria can subvert the phagocyte-killing mechanisms[47].

When larvae are co-infected with two isogenic, but differently labelled, *S. aureus* clones, the infection evolves by forming focal lesions that are predominantly monoclonal and, during the course of overwhelming infection, the ratio between the original strains is often skewed towards one predominating strain[47]. This phenomenon is fully dependent on phagocyte activity. These data suggest that most of the phagocytes are able to clear the infection but a population of phagocytes provides an intracellular protective niche in which some bacteria gain the ability to replicate and resist, to ultimately be released and disseminate. Consistent with this, co-infection in a murine sepsis model resulted in kidney abscesses that contained predominantly one strain of *S. aureus* and thus were likely founded by single bacteria[108]. The relevance of this work for clinical treatments is underscored by a recent study showing that the use of sub-curative antibiotic doses can support the preferential expansion of antibiotic-resistant bacteria during a mixed infection[109]. Selective ablation of macrophages or neutrophils in the zebrafish model has revealed that neutrophils are most likely to form the privileged niche responsible for disseminated infection of *S. aureus*[104]. Interesting remaining questions include elucidation of the mechanisms by which some bacteria from the initial inoculum are able to avoid being killed by neutrophils and determination of whether *S. aureus* can also resist macrophages *in vivo*, as suggested by human cell culture studies[110].

### *Shigella flexneri*

*S. flexneri*, a human-adapted *Escherichia coli* species, is a causative agent of diarrhoea and dysentery in humans, generally deriving from orofaecal contaminations. Like other enterobacteria, it mostly affects the digestive tract; however, in advanced infectious stages, it can lead to bacteraemia and systemic sepsis. In the early phase of infection, the pathogen can interact with membranes of host cells, inject virulence determinants and induce ruffling and internalisation[40]. In this actively induced ingestion mechanism resides its capability to establish intracellular infection in non-phagocytic cells, such as epithelial cells associated with the digestive tract. Once it is internalised, it can escape from phagosomes and survive freely in the cytosol. Subsequently, the pathogen can spread through intestinal epithelial cells by actin-based motility (**Figure 3E**). Microfold cells allow *Shigella* to transverse the intestinal epithelium, where they encounter macrophages. Death of infected macrophages and subsequent destabilisation of the epithelium due to inflammation allows more *Shigella* to infiltrate the tissue and invade epithelial cells through the basal membrane. Survival and replication in macrophages, eventually followed by macrophage pyroptosis, is fundamental to allowing dissemination and extensive colonisation of the intestinal epithelium[111].

Recently, a zebrafish model for *S. flexneri* was established and this has been used to show that intravenously administrated bacteria can survive and replicate both in macrophages and in non-immune cells[86]. The pathogenicity of *Shigella* is highly dependent on the presence of T3SS virulence factors and avirulent strains can be successfully combated by the zebrafish innate immune system and are not able to induce phagocytosis in non-phagocytic cells. Live imaging shows that replication of *S. flexneri* in macrophages ultimately induces cell death, whereas bacteria are more efficiently contained and degraded within neutrophils. Neutrophils also act as scavengers, eliminating infected dead cells. Macrophages are not able to retain the infection within vacuoles and bacteria spread into the cytosol, where they can colocalise with actin and septin cages (**Figure 3E**).

In mammalian cultured cells, cytosolic *S. flexneri* can be targeted for autophagy via both ubiquitin-dependent and -independent pathways, and, as a counteractive mechanism, the bacteria can secrete virulence factors to escape autophagy[41,112]. Colocalisation with the autophagy marker Lc3 followed by electron microscopic analysis in the zebrafish model confirmed that autophagy targeting is associated with entrapment of *S. flexneri* in septin cage-like structures[33,86]. Reduction of autophagy, via knockdown of the autophagy-related receptor p62, increases the infection burden of zebrafish larvae and this effect is specific only for the T3SS-positive strain that is able to escape into the cytosol[86]. These data support the hypothesis that antibacterial protection provided by efficient autophagic machinery is essential to properly counteract *S. flexneri* infection. The ability to monitor *S. flexneri* infection in a transparent zebrafish host provides new possibilities to assess the relevance of autophagy *in vivo* in immune and non-immune cells, and to develop new strategies for anti-bacterial therapies targeting this process (**Table 1**).

### *Candida albicans*

*C. albicans* is an opportunistic dimorphic fungus that grows in yeast and hyphal forms[113]. Most of the human population carries *C. albicans* as a harmless constituent of the epidermal, mucosal and intestinal flora. However, uncontrolled systemic candidiasis and fungal growth on the mucosal surfaces can cause severe and life-threatening infectious complications, particularly in immunocompromised individuals. In zebrafish embryos, as

in humans, *C. albicans* can be phagocytosed by both neutrophils and macrophages[114,115]. Live observations reveal that, in a non-compromised zebrafish host, this intracellular localisation leads to a transitory standoff phase in which the yeast form survives and replicates, but does not germinate or lyse the host cell (**Figure 3F**). Subsequently, the fungi switch to the more virulent hyphal form and proliferate exuberantly in individuals that fail to contain the infection, whereas they revert to the yeast form in most surviving embryos[115]. Intracellular yeast forms are unable to undergo the yeast-to-hyphal transition, even under conditions of impaired oxidative-stress response, in contrast with previous *in vitro* data in which filamentous growth was observed within cultured macrophages[116]. Therefore, macrophages apparently have an enhanced ability to control infection in the *in vivo* environment.

Although germination was shown to be independent of the phagocyte-specific NADPH oxidase (PHOX), this enzyme was found to be essential to produce an efficient oxidative-stress response against *C. albicans* and to control filamentous growth[115,116]. Previously, the limitation of fungal growth was ascribed mainly to direct fungal destruction by ROS; however, imaging in zebrafish revealed a non-canonical role for PHOX and for the epithelial dual NADPH oxidase (DUOX) in recruitment of phagocytes to *C. albicans* infection sites. Therefore, impaired phagocyte recruitment to invading *Candida* under conditions of NADPH oxidase deficiency seems to be the cause of the overall reduction in containment of the infection and, consequently, of massive extracellular hyphal growth. Although localised infection with wildtype *C. albicans* is unable to induce chemoattraction under conditions of NADPH oxidase deficiency, infection with a yeast-locked mutant strain (*edt1Δ/Δ*) can be efficiently counteracted by phagocyte recruitment and internalisation, even in pan-NADPH-oxidase-depleted conditions. This suggests that the hyphal transition (or another edt1-associated program) is also able to attenuate ROS-independent phagocyte recruitment, thus explaining the relevance of host ROS-driven chemoattraction mechanisms to counteract *C. albicans* infection[116].

## CONCLUDING REMARKS

The study of intracellular pathogens in zebrafish macrophages has led to new mechanistic insights that are inspiring novel host-directed therapeutic strategies (**Table 1**). The real-time imaging possibilities in zebrafish will also be very useful for elucidating the mechanisms underlying macrophage migration processes, as has already been demonstrated by the study of neutrophils in the larval system[6,7,55]. A question that is very relevant both for infectious diseases and for cancer biology concerns the presence of different pro- and anti-inflammatory macrophage subtypes in zebrafish. Classically activated (M1) and alternatively activated (M2) macrophages, resembling the phenotypes of mammalian macrophages, have been identified in different fish species[117]. That different macrophage subtypes might already be present in early zebrafish larvae has been suggested, but this remains to be further investigated[118]. The early larval stages, which are optimally suited for imaging and for genetic and pharmacological interventions, can give much information on the intracellular survival mechanisms of pathogens, as demonstrated by the studies discussed herein. The early larval stages are also very useful for studying the response of microglia to brain injuries or infection, contributing to a deeper understanding of the role of these specialised macrophages in neurodegenerative diseases[67,119]. Studying the antigen-presentation function of macrophages and DCs at later developmental stages is becoming increasingly feasible owing to advances in technologies for generating stable

mutant lines[120,121,122]. Dynamic interactions between macrophages and neutrophils that are emerging from recent studies in zebrafish are of considerable interest for further study[44,56,65]. The use of the zebrafish model has already provided insights into the *in vivo* relevance of intracellular defence mechanisms such as ROS and RNS production and autophagy. We expect that further use of this powerful model will continue to make important contributions towards the understanding of innate immunity and of the virulence strategies that pathogens use to subvert innate host defences.

**ACKNOWLEDGMENTS**

The authors thank Georges Lutfalla (University of Montpellier II) and Graham J. Lieschke (Monash University) for the kind gift of the *Tg(mpeg1:mCherry-F)* and *Tg(mpeg1:Gal4-VP16/UAS-E1b:Kaede)* lines, respectively. These lines were used to make **Figure 2** and **Supplementary Movies 1-2**.

V.T. is a Marie Curie fellow in the Initial Training Network FishForPharma (PITN-GA-2011-289209) funded by the 7th Framework Programme of the European Commission. S.M. is supported by a fellowship from the Higher Education Commission of Pakistan. Work that led to this Review was additionally supported by the European 7th framework project ZF-Health (FP7-Health-2009-242048), by the Smart Mix Program (NWOA_6QY9BM) of The Netherlands Ministry of Economic Affairs and The Netherlands Ministry of Education, Culture and Science, and by the Leiden University Fund (LUF).

**FOOTNOTES**

This article is part of a Special Issue, Spotlight on Zebrafish: Translational Impact.

The format and spelling of the contents in this chapter have been adapted from the published article to conform it to the thesis outline.

| Marker | Transgenic line | Specificity | Promoter sequence | Ref. |
|---|---|---|---|---|
| ***apoeb*** apolipoprotein Eb | *Tg(apoeb:lyn-eGFP)* | Microglial cells | Obtained from a BAC clone containing the *apoeb* locus, by BAC recombineering at the translation start | 60 |
| ***coro1a*** coronin, actin binding protein, 1A | *Tg(coro1a:eGFP)* | Macrophages, neutrophils and thymocytes[(a)] | -7.03 kb of *coro1a* upstream the translation start | 123 |
| ***csf1ra/fms*** colony stimulating factor 1 receptor, a | *TgBAC(csf1ra:Gal4-VP16 / UAS-E1b:Eco.NfsB-mCherry)* | Macrophages (highly motile), and xantophore cells (immobile) | Obtained from a BAC clone containing the *csf1ra* locus, by BAC recombineering at the translation start | 43 |
| ***fli1a*** friend leukaemia integration 1a | *Tg(fli1a:eGFP)* | Primitive macrophages (dull), endothelial cells (bright) and subsets of erythrocytes (dull) | 5'UTR of *fli* was obtained from a PAC library | 124 |
| ***lyz/lysC*** lysozyme | *Tg(lyz:eGFP)* | Neutrophils[(b)] | -11 kb of *lyz* upstream the translation start | 125 |
| | *Tg(lyz:DsRed2)* | Neutrophils[(b)] | -6.35 kb of *lyz* upstream the translation start | 125 |
| | *Tg(-4.1lyz:eGFP)* | Neutrophils[(b)] | -4.1 kb of *lyz* upstream the translation start | 126 |
| | *Tg(-2.4lyz:eGFP)* | Neutrophils and primitive macrophages[(c)] | -2.4 kb of *lyz* upstream the translation start | 127 |
| | *Tg(lyz:Gal4-VP16)* | Neutrophils[(b)] | -11 kb of *lyz* construct as in ref.[125] | 128 |
| ***mhc2dab*** major histocompatibility complex class II DAB gene | *Tg(mhc2dab:eGFP)* *Tg(mhc2dab:mCherry)* | Antigen presenting cells (APCs)[(d)] | -3.8 kb of *mhc2dab* upstream the transcription start | 17 |
| ***mpeg1*** macrophage expressed 1 | *Tg(mpeg1:eGFP)* *Tg(mpeg1:mCherry)* *Tg(mpeg1:Gal4-VP16)* | Macrophages[(e)] | -1.86 kb of *mpeg1* upstream the translation start | 44 |
| | *Tg(mpeg1:mCherry-F)* | Macrophages | -1.86 kb of *mpeg1* upstream the translation start | 61 |
| | *Tg(mpeg1:Dendra2)* | Macrophages[(e)] | As in ref.[44] | 129 |
| | *Tg(mpeg1:YFP)* | Macrophages[(e)] | As in ref.[44] | 83 |
| ***mpx/mpo*** myeloid-specific peroxidase | *TgBAC(mpx:eGFP)* | Neutrophils[(f)] | Obtained from a BAC clone containing the *mpx* locus, by BAC recombineering at the translation start | 130 |
| | *Tg(mpx:GFP)* | Neutrophils (bright) and a subset of macrophages (dull) | -8 kb of *mpx* upstream the translation start | 131 |
| | *Tg(-8mpx:mCherry)* *Tg(-8mpx:DsRed-F)* *Tg(-8 mpx:eGFP-F)* | Neutrophils (bright) and a subset of macrophages (dull) | As in ref.[131] | 132 |
| | *Tg(-8mpx:Dendra2)* | Neutrophils (bright) and a subset of macrophages (dull) | As in ref.[131] | 133 |

**Supplementary Table 1. Zebrafish fluorescent reporter lines for immune cells** *(continued on the next page)***.**

| | | | | |
|---|---|---|---|---|
| ***myd88*** myeloid differentiation primary response gene (88) | *Tg(myd88:eGFP)* *Tg(myd88:DsRed2)* | Subsets of myeloid leukocytes, distal pronephric ducts and cloaca | -3.7 kb of *myd88* upstream the translation start | 59 |
| ***ptprc/cd45*** protein tyrosine phosphatase, receptor type, C | *Tg(ptprc:DsRed)* | Macrophages, granulocytes and T lymphocytes | -7.6 kb of *ptprc* upstream the transcription start | 14 |
| ***spi1b/pu.1*** spleen focus forming virus (SFFV) proviral integration oncogene spi1b | *Tg(-5.3spi1b:eGFP)* | Early myeloid cells[(g)] | -5.3 kb of *spi1b* upstream the translation start | 134 |
| | *Tg(-9.0spi1b:eGFP)* | Early myeloid cells (bright) and muscles (dull)[(g)] | -9 kb of *spi1b* upstream the translation start | 135 |
| | *Tg(-4spi1b:Gal4 / UAS:eGFP)* | Early myeloid cells[(g)] | -4 kb of *spi1b* upstream the translation start | 60 |
| | *Tg(-4spi1b:lyn-eGFP)* | Early myeloid cells[(g)] | As in ref.[60] | 46 |

**Supplementary Table 1. Zebrafish fluorescent reporter lines for immune cells** *(continued from the previous page)*. Notes: [(a)] Expression in macrophages, neutrophils and thymocytes was shown for embryonic and young larval stages. Expression in myelomonocyte progenitors in head-kidneys of adults was also documented; [(b)] Discrepancies about expression of the construct in primitive macrophages reported; [(c)] Observations limited to embryonic and early larval stage. Labelling of primitive macrophages was determined based on the presence of fluorescent cells over the yolk at 26 hpf. Expression in macrophages from the second wave of primitive haematopoiesis and definitive haematopoiesis not demonstrated. [(d)] APCs are here indicated as macrophages, dendritic cells, B lymphocytes and eosinophils. Fluorescence visible from 5dpf and abundantly labelling APCs only from 12dpf; expression in keratinocytes reported in adults and juveniles; [(e)] Loss of expression after 6 dpf and in the adults reported; labelling of Langerhans dendritic cells in larvae suggested; [(f)] Existence of a set of $GFP^{low}$ macrophage-like cells distinguishable by confocal microscopy reported by some laboratories; [(g)] Expression documented from 12 to 30 hpf; Expression in early lymphoid cells reported for adult *Tg(-9.0spi1b:eGFP).*

| Marker | Reporter construct | Specificity | Notes | Ref. |
|---|---|---|---|---|
| **rab5c** RAB5c, member RAS oncogene family | *Tg(h2afx:eGFP-rab5c)*[(a)] | Early endosomes | - | 136 |
| | *Tg(UAS:mCherry-rab5c_S36N)*[(b)] | Early endosomes | S36N substitution, dominant negative | 136 |
| | *Tg(UAS:mCherry-rab5c_Q81L)*[(b)] | Early endosomes | Q81L substitution, constitutively active | 136 |
| ***rab7a*** RAB7, member RAS oncogene family a | *Tg(h2afx:eGFP-rab7)*[(a)] | Late endosomes | - | 136 |
| | *Tg(UAS:mCherry-rab7_T22N)*[(b)] | Late endosomes | T22N substitution, dominant negative | 136 |
| | *Tg(UAS:mCherry-rab7_Q67L)*[(b)] | Late endosomes | Q67L substitution, constitutively active | 136 |
| ***rab7b*** RAB7, member RAS oncogene family b | *Plasmid(UAS:GFP-rab7b_T22N)*[(b)] | Late endosomes | T22N substitution, dominant negative; the reporter consists of a UAS-expression plasmid | 137 |
| ***rab11a*** RAB11a, member RAS oncogene family | *Tg(h2afx:eGFP-rab11a)*[(a)] | Recycling endosomes | - | 136 |
| | *Tg(UAS:mCherry-rab11a_S25N)*[(b)] | Recycling endosomes | S25N substitution, dominant negative | 136 |
| | *Tg(UAS:mCherry-rab11a_Q70L)*[(b)] | Recycling endosomes | Q70L substitution, constitutively active | 136 |
| ***lamp1*** lysosomal-associated membrane protein 1 | *Tg(hsp70l:lamp1-RFP)*[(c)] | Lysosomes | - | 137 |
| ***lamp2*** lysosomal membrane glycoprotein 2 | *Tg(hsp70l:lamp2-eGFP)*[(c)] | Lysosomes | - | 137 |
| ***cd63/lamp3*** Cd63 antigen | *Plasmid(CMV/SP6:GFP-cd63)*[(d)] | Lysosomes | The reporter consists of an expression plasmid for CMV-expression or mRNA preparation | 60 |
| ***map1lc3b*** microtubule-associated protein 1 light chain 3 beta | *Tg(CMV:eGFP-map1lc3b)*[(d)] | Autophagosomes | - | 138 |
| | *Tg(hsp70l:RFP-Rno.Map1lc3b)*[(c)] | Autophagosomes | Contains the Map1lc3b sequence from the rat (*Rattus norvegicus*) | 137 |
| ***gabarapa*** GABA(A) receptor-associated protein a | *Tg(CMV:eGFP-gabarapa)*[(d)] | Autophagosomes | - | 138 |
| ***rab32a*** RAB32a, member RAS oncogene family | *Tg(4xUAS:eGFP-rab32a,myl7:eGFP)*[(b)] | Vesicular trafficking | - | 137 |
| | *Plasmid(UAS:GFP-rab32a_T27N)*[(b)] | Vesicular trafficking | T27N substitution, dominant negative; the reporter consists of a UAS-expression plasmid | 137 |
| ***rab38b*** | *Tg(UAS:GFP-rab38b-T23N, cmlc2:GFP)*[(b)] | Vesicular trafficking | T23N substitution, dominant negative | 137 |

**Supplementary Table 2. Zebrafish reporters of subcellular compartments.** Notes: [(a)] *h2afx*: H2A histone family, member X constitutive promoter; [(b)] *UAS*: Upstream Activating Sequence for Gal4-dependent expression; [(c)] *hsp70l*: heat shock cognate 70-kd protein, like inducible promoter; [(d)] *CMV*: Cytomegalovirus constitutive promoter.

## HYPERLINK TO SUPPLEMENTARY MOVIES

Supplementary Movies are available online at the following URL:
**https://drive.google.com/open?id=0B_188Sfgn4xoUkRVNjNKdlNhSEE**

## REFERENCES

1 Renshaw SA, Trede NS. A model 450 million years in the making: zebrafish and vertebrate immunity. Dis Model Mech. 2012 Jan;5(1):38-47.
2 van der Vaart M, Spaink HP, Meijer AH. Pathogen recognition and activation of the innate immune response in zebrafish. Adv Hematol. 2012;2012:159807.
3 Ramakrishnan L. Looking within the zebrafish to understand the tuberculous granuloma. Adv Exp Med Biol. 2013;783:251-66.
4 Lam SH, Chua HL, Gong Z, Lam TJ, Sin YM. Development and maturation of the immune system in zebrafish, Danio rerio: a gene expression profiling, in situ hybridization and immunological study. Dev Comp Immunol. 2004 Jan;28(1):9-28.
5 Page DM, Wittamer V, Bertrand JY, Lewis KL, Pratt DN, Delgado N, Schale SE, McGue C, Jacobsen BH, Doty A, Pao Y, Yang H, Chi NC, Magor BG, Traver D. An evolutionarily conserved program of B-cell development and activation in zebrafish. Blood. 2013 Aug 22;122(8):e1-11.
6 Henry KM, Loynes CA, Whyte MK, Renshaw SA. Zebrafish as a model for the study of neutrophil biology. J Leukoc Biol. 2013 Oct;94(4):633-42.
7 Shelef MA, Tauzin S, Huttenlocher A. Neutrophil migration: moving from zebrafish models to human autoimmunity. Immunol Rev. 2013 Nov;256(1):269-81.
8 Herbomel P, Thisse B, Thisse C. Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development. 1999 Sep;126(17):3735-45.
9 Herbomel P, Thisse B, Thisse C. Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina, and epidermis through a M-CSF receptor-dependent invasive process. Dev Biol. 2001 Oct 15;238(2):274-88.
10 Takahashi K, Naito M, Takeya M. Development and heterogeneity of macrophages and their related cells through their differentiation pathways. Pathol Int. 1996 Jul;46(7):473-85.
11 Colucci-Guyon E, Tinevez JY, Renshaw SA, Herbomel P. Strategies of professional phagocytes in vivo: unlike macrophages, neutrophils engulf only surface-associated microbes. J Cell Sci. 2011 Sep 15;124(Pt 18):3053-9.
12 Bertrand JY, Kim AD, Violette EP, Stachura DL, Cisson JL, Traver D. Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo. Development. 2007 Dec;134(23):4147-56.
13 Murayama E, Kissa K, Zapata A, Mordelet E, Briolat V, Lin HF, Handin RI,Herbomel P. Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. Immunity. 2006 Dec;25(6):963-75.
14 Bertrand JY, Chi NC, Santoso B, Teng S, Stainier DY, Traver D. Haematopoietic stem cells derive directly from aortic endothelium during development. Nature. 2010 Mar 4;464(7285):108-11.
15 Kissa K, Herbomel P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. Nature. 2010 Mar 4;464(7285):112-5.
16 Boisset JC, van Cappellen W, Andrieu-Soler C, Galjart N, Dzierzak E, Robin C. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature. 2010 Mar 4;464(7285):116-20.
17 Wittamer V, Bertrand JY, Gutschow PW, Traver D. Characterization of the mononuclear phagocyte system in zebrafish. Blood. 2011 Jun 30;117(26):7126-35.
18 Svahn AJ, Graeber MB, Ellett F, Lieschke GJ, Rinkwitz S, Bennett MR, Becker TS. Development of ramified microglia from early macrophages in the zebrafish optic tectum. Dev Neurobiol. 2013 Jan;73(1):60-71.
19 Stachura DL, Traver D. Cellular dissection of zebrafish hematopoiesis. Methods Cell Biol. 2011;101:75-110.
20 Jagannathan-Bogdan M, Zon LI. Hematopoiesis. Development. 2013 Jun;140(12):2463-7.
21 Xu LL, Warren MK, Rose WL, Gong W, Wang JM. Human recombinant monocyte chemotactic protein and other C-C chemokines bind and induce directional migration of dendritic cells in vitro. J Leukoc Biol. 1996 Sep;60(3):365-71.
22 Cotton M, Claing A. G protein-coupled receptors stimulation and the control of cell migration. Cell Signal. 2009 Jul;21(7):1045-53.
23 Elomaa O, Kangas M, Sahlberg C, Tuukkanen J, Sormunen R, Liakka A, Thesleff I, Kraal G, Tryggvason K. Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages. Cell. 1995 Feb 24;80(4):603-9.

24 O'Neill LA, Golenbock D, Bowie AG. The history of Toll-like receptors - redefining innate immunity. Nat Rev Immunol. 2013 Jun;13(6):453-60.
25 Bertin J, Nir WJ, Fischer CM, Tayber OV, Errada PR, Grant JR, Keilty JJ, Gosselin ML, Robison KE, Wong GH, Glucksmann MA, DiStefano PS. Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB. J Biol Chem. 1999 May 7;274(19):12955-8.
26 Inohara N, Koseki T, del Peso L, Hu Y, Yee C, Chen S, Carrio R, Merino J, Liu D, Ni J, Núñez G. Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. J Biol Chem. 1999 May 21;274(21):14560-7.
27 Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell. 2002 Aug;10(2):417-26.
28 Minakami R, Sumimotoa H. Phagocytosis-coupled activation of the superoxide-producing phagocyte oxidase, a member of the NADPH oxidase (nox) family. Int J Hematol. 2006 Oct;84(3):193-8.
29 El-Gayar S, Thüring-Nahler H, Pfeilschifter J, Röllinghoff M, Bogdan C. Translational control of inducible nitric oxide synthase by IL-13 and arginine availability in inflammatory macrophages. J Immunol. 2003 Nov 1;171(9):4561-8.
30 Schmidtchen A, Frick IM, Andersson E, Tapper H, Björck L. Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. Mol Microbiol. 2002 Oct;46(1):157-68.
31 Park YK, Bearson B, Bang SH, Bang IS, Foster JW. Internal pH crisis, lysine decarboxylase and the acid tolerance response of Salmonella typhimurium. Mol Microbiol. 1996 May;20(3):605-11.
32 Vandal OH, Pierini LM, Schnappinger D, Nathan CF, Ehrt S. A membrane protein preserves intrabacterial pH in intraphagosomal Mycobacterium tuberculosis. Nat Med. 2008 Aug;14(8):849-54.
33 Mostowy S, Bonazzi M, Hamon MA, Tham TN, Mallet A, Lelek M, Gouin E, Demangel C, Brosch R, Zimmer C, Sartori A, Kinoshita M, Lecuit M, Cossart P. Entrapment of intracytosolic bacteria by septin cage-like structures. Cell Host Microbe. 2010 Nov 18;8(5):433-44.
34 Deretic V, Saitoh T, Akira S. Autophagy in infection, inflammation and immunity. Nat Rev Immunol. 2013 Oct;13(10):722-37.
35 Abdallah AM, Gey van Pittius NC, Champion PA, Cox J, Luirink J, Vandenbroucke-Grauls CM, Appelmelk BJ, Bitter W. Type VII secretion—mycobacteria show the way. Nat Rev Microbiol. 2007 Nov;5(11):883-91.
36 Baxt LA, Garza-Mayers AC, Goldberg MB. Bacterial subversion of host innate immune pathways. Science. 2013 May 10;340(6133):697-701.
37 Masaki T, Qu J, Cholewa-Waclaw J, Burr K, Raaum R, Rambukkana A. Reprogramming adult Schwann cells to stem cell-like cells by leprosy bacilli promotes dissemination of infection. Cell. 2013 Jan 17;152(1-2):51-67.
38 Wang Y, Curry HM, Zwilling BS, Lafuse WP. Mycobacteria inhibition of IFN-gamma induced HLA-DR gene expression by up-regulating histone deacetylation at the promoter region in human THP-1 monocytic cells. J Immunol. 2005 May 1;174(9):5687-94.
39 Duclos S, Desjardins M. Subversion of a young phagosome: the survival strategies of intracellular pathogens. Cell Microbiol. 2000 Oct;2(5):365-77.
40 Ogawa M, Handa Y, Ashida H, Suzuki M, Sasakawa C. The versatility of Shigella effectors. Nat Rev Microbiol. 2008 Jan;6(1):11-6.
41 Ogawa M, Yoshimori T, Suzuki T, Sagara H, Mizushima N, Sasakawa C. Escape of intracellular Shigella from autophagy. Science. 2005 Feb 4;307(5710):727-31.
42 Hilbi H, Moss JE, Hersh D, Chen Y, Arondel J, Banerjee S, Flavell RA, Yuan J, Sansonetti PJ, Zychlinsky A. Shigella-induced apoptosis is dependent on caspase-1 which binds to IpaB. J Biol Chem. 1998 Dec 4;273(49):32895-900.
43 Gray C, Loynes CA, Whyte MK, Crossman DC, Renshaw SA, Chico TJ. Simultaneous intravital imaging of macrophage and neutrophil behaviour during inflammation using a novel transgenic zebrafish. Thromb Haemost. 2011 May;105(5):811-9.
44 Ellett F, Pase L, Hayman JW, Andrianopoulos A, Lieschke GJ. mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. Blood. 2011 Jan 27;117(4):e49-56.
45 Meijer AH, van der Sar AM, Cunha C, Lamers GE, Laplante MA, Kikuta H, Bitter W, Becker TS, Spaink HP. Identification and real-time imaging of a myc-expressing neutrophil population involved in inflammation and mycobacterial granuloma formation in zebrafish. Dev Comp Immunol. 2008;32(1):36-49.
46 Zakrzewska A, Cui C, Stockhammer OW, Benard EL, Spaink HP, Meijer AH. Macrophage-specific gene functions in Spi1-directed innate immunity. Blood. 2010 Jul 22;116(3):e1-11.
47 Prajsnar TK, Hamilton R, Garcia-Lara J, McVicker G, Williams A, Boots M, Foster SJ, Renshaw SA. A privileged intraphagocyte niche is responsible for disseminated infection of Staphylococcus aureus in a zebrafish model. Cell Microbiol. 2012 Oct;14(10):1600-19.
48 Su F, Juarez MA, Cooke CL, Lapointe L, Shavit JA, Yamaoka JS, Lyons SE. Differential regulation of primitive myelopoiesis in the zebrafish by Spi-1/Pu.1 and C/ebp1. Zebrafish. 2007 Fall;4(3):187-99.
49 Li L, Jin H, Xu J, Shi Y, Wen Z. Irf8 regulates macrophage versus neutrophil fate during zebrafish primitive myelopoiesis. Blood. 2011 Jan 27;117(4):1359-69.

50 Davis JM, Clay H, Lewis JL, Ghori N, Herbomel P, Ramakrishnan L. Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity. 2002 Dec;17(6):693-702.
51 Herbomel P, Levraud JP. Imaging early macrophage differentiation, migration, and behaviors in live zebrafish embryos. Methods Mol Med. 2005;105:199-214.
52 Davis JM, Ramakrishnan L. The role of the granuloma in expansion and dissemination of early tuberculous infection. Cell. 2009 Jan 9;136(1):37-49.
53 Yoo SK, Starnes TW, Deng Q, Huttenlocher A. Lyn is a redox sensor that mediates leukocyte wound attraction in vivo. Nature. 2011 Nov 20;480(7375):109-12.
54 Benard EL, van der Sar AM, Ellett F, Lieschke GJ, Spaink HP, Meijer AH. Infection of zebrafish embryos with intracellular bacterial pathogens. J Vis Exp. 2012 Mar 15;(61). pii: 3781.
55 Sarris M, Masson JB, Maurin D, Van der Aa LM, Boudinot P, Lortat-Jacob H, Herbomel P. Inflammatory chemokines direct and restrict leukocyte migration within live tissues as glycan-bound gradients. Curr Biol. 2012 Dec 18;22(24):2375-82.
56 Yang CT, Cambier CJ, Davis JM, Hall CJ, Crosier PS, Ramakrishnan L. Neutrophils exert protection in the early tuberculous granuloma by oxidative killing of mycobacteria phagocytosed from infected macrophages. Cell Host Microbe. 2012 Sep 13;12(3):301-12.
57 Deng Q, Sarris M, Bennin DA, Green JM, Herbomel P, Huttenlocher A. Localized bacterial infection induces systemic activation of neutrophils through Cxcr2 signaling in zebrafish. J Leukoc Biol. 2013 May;93(5):761-9.
58 Cambier CJ, Takaki KK, Larson RP, Hernandez RE, Tobin DM, Urdahl KB, Cosma CL, Ramakrishnan L. Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids. Nature. 2014 Jan 9;505(7482):218-22.
59 Hall C, Flores MV, Chien A, Davidson A, Crosier K, Crosier P. Transgenic zebrafish reporter lines reveal conserved Toll-like receptor signaling potential in embryonic myeloid leukocytes and adult immune cell lineages. J Leukoc Biol. 2009 May;85(5):751-65.
60 Peri F, Nüsslein-Volhard C. Live imaging of neuronal degradation by microglia reveals a role for v0-ATPase a1 in phagosomal fusion in vivo. Cell. 2008 May 30;133(5):916-27.
61 Bernut A, Herrmann JL, Kissa K, Dubremetz JF, Gaillard JL, Lutfalla G, Kremer L. Mycobacterium abscessus cording prevents phagocytosis and promotes abscess formation. Proc Natl Acad Sci U S A. 2014 Mar 11;111(10):E943-52.
62 Hall CJ, Flores MV, Oehlers SH, Sanderson LE, Lam EY, Crosier KE, Crosier PS. Infection-responsive expansion of the hematopoietic stem and progenitor cell compartment in zebrafish is dependent upon inducible nitric oxide. Cell Stem Cell. 2012 Feb 3;10(2):198-209.
63 Hall CJ, Boyle RH, Astin JW, Flores MV, Oehlers SH, Sanderson LE, Ellett F, Lieschke GJ, Crosier KE, Crosier PS. Immunoresponsive gene 1 augments bactericidal activity of macrophage-lineage cells by regulating β-oxidation-dependent mitochondrial ROS production. Cell Metab. 2013 Aug 6;18(2):265-78.
64 Bilan DS, Pase L, Joosen L, Gorokhovatsky AY, Ermakova YG, Gadella TW, Grabher C, Schultz C, Lukyanov S, Belousov VV. HyPer-3: a genetically encoded H(2)O(2) probe with improved performance for ratiometric and fluorescence lifetime imaging. ACS Chem Biol. 2013 Mar 15;8(3):535-42.
65 Elks PM, Brizee S, van der Vaart M, Walmsley SR, van Eeden FJ, Renshaw SA, Meijer AH. Hypoxia inducible factor signaling modulates susceptibility to mycobacterial infection via a nitric oxide dependent mechanism. PLoS Pathog. 2013;9(12):e1003789.
66 Cheung CY, Webb SE, Love DR, Miller AL. Visualization, characterization and modulation of calcium signaling during the development of slow muscle cells in intact zebrafish embryos. Int J Dev Biol. 2011;55(2):153-74.
67 Sieger D, Moritz C, Ziegenhals T, Prykhozhij S, Peri F. Long-range Ca2+ waves transmit brain-damage signals to microglia. Dev Cell. 2012 Jun 12;22(6):1138-48.
68 Prouty MG, Correa NE, Barker LP, Jagadeeswaran P, Klose KE. Zebrafish-Mycobacterium marinum model for mycobacterial pathogenesis. FEMS Microbiol Lett. 2003 Aug 29;225(2):177-82.
69 Swaim LE, Connolly LE, Volkman HE, Humbert O, Born DE, Ramakrishnan L. Mycobacterium marinum infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. Infect Immun. 2006 Nov;74(11):6108-17.
70 Parikka M, Hammarén MM, Harjula SK, Halfpenny NJ, Oksanen KE, Lahtinen MJ, Pajula ET, Iivanainen A, Pesu M, Rämet M. Mycobacterium marinum causes a latent infection that can be reactivated by gamma irradiation in adult zebrafish. PLoS Pathog. 2012 Sep;8(9):e1002944.
71 Gengenbacher M, Kaufmann SH. Mycobacterium tuberculosis: success through dormancy. FEMS Microbiol Rev. 2012 May;36(3):514-32.
72 Ottenhoff TH, Kaufmann SH. Vaccines against tuberculosis: where are we and where do we need to go? PLoS Pathog. 2012;8(5):e1002607.

73 Cronan MR, Tobin DM. Fit for consumption: zebrafish as a model for tuberculosis. Dis Model Mech. 2014 Jul;7(7):777-84.
74 Volkman HE, Clay H, Beery D, Chang JC, Sherman DR, Ramakrishnan L. Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. PLoS Biol. 2004 Nov;2(11):e367.
75 Volkman HE, Pozos TC, Zheng J, Davis JM, Rawls JF, Ramakrishnan L. Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium. Science. 2010 Jan 22;327(5964):466-9.
76 Lin PL, Rodgers M, Smith L, Bigbee M, Myers A, Bigbee C, Chiosea I, Capuano SV, Fuhrman C, Klein E, Flynn JL. Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model. Infect Immun. 2009 Oct;77(10):4631-42.
77 Adams KN, Takaki K, Connolly LE, Wiedenhoft H, Winglee K, Humbert O, Edelstein PH, Cosma CL, Ramakrishnan L. Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. Cell. 2011 Apr 1;145(1):39-53.
78 Adams KN, Szumowski JD, Ramakrishnan L. Verapamil, and its metabolite norverapamil, inhibit macrophage-induced, bacterial efflux pump-mediated tolerance to multiple anti-tubercular drugs. J Infect Dis. 2014 Aug 1;210(3):456-66.
79 Tobin DM, Vary JC Jr, Ray JP, Walsh GS, Dunstan SJ, Bang ND, Hagge DA, Khadge S, King MC, Hawn TR, Moens CB, Ramakrishnan L. The lta4h locus modulates susceptibility to mycobacterial infection in zebrafish and humans. Cell. 2010 Mar 5;140(5):717-30.
80 Wallis RS. Tumour necrosis factor antagonists: structure, function, and tuberculosis risks. Lancet Infect Dis. 2008 Oct;8(10):601-11.
81 Tsenova L, Bergtold A, Freedman VH, Young RA, Kaplan G. Tumor necrosis factor alpha is a determinant of pathogenesis and disease progression in mycobacterial infection in the central nervous system. Proc Natl Acad Sci U S A. 1999 May 11;96(10):5657-62.
82 Clay H, Volkman HE, Ramakrishnan L. Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death. Immunity. 2008 Aug 15;29(2):283-94.
83 Roca FJ, Ramakrishnan L. TNF dually mediates resistance and susceptibility to mycobacteria via mitochondrial reactive oxygen species. Cell. 2013 Apr 25;153(3):521-34.
84 Tobin DM, Roca FJ, Oh SF, McFarland R, Vickery TW, Ray JP, Ko DC, Zou Y, Bang ND, Chau TT, Vary JC, Hawn TR, Dunstan SJ, Farrar JJ, Thwaites GE, King MC, Serhan CN, Ramakrishnan L. Host genotype-specific therapies can optimize the inflammatory response to mycobacterial infections. Cell. 2012 Feb 3;148(3):434-46.
85 van der Vaart M, van Soest JJ, Spaink HP, Meijer AH. Functional analysis of a zebrafish myd88 mutant identifies key transcriptional components of the innate immune system. Dis Model Mech. 2013 May;6(3):841-54.
86 Mostowy S, Boucontet L, Mazon Moya MJ, Sirianni A, Boudinot P, Hollinshead M, Cossart P, Herbomel P, Levraud JP, Colucci-Guyon E. The zebrafish as a new model for the in vivo study of Shigella flexneri interaction with phagocytes and bacterial autophagy. PLoS Pathog. 2013;9(9):e1003588.
87 Meijer AH, van der Vaart M, Spaink HP. Real-time imaging and genetic dissection of host-microbe interactions in zebrafish. Cell Microbiol. 2014 Jan;16(1):39-49.
88 Carvalho R, de Sonneville J, Stockhammer OW, Savage ND, Veneman WJ, Ottenhoff TH, Dirks RP, Meijer AH, Spaink HP. A high-throughput screen for tuberculosis progression. PLoS One. 2011 Feb 16;6(2):e16779.
89 Fàbrega A, Vila J. Salmonella enterica serovar Typhimurium skills to succeed in the host: virulence and regulation. Clin Microbiol Rev. 2013 Apr;26(2):308-41.
90 van der Sar AM, Musters RJ, van Eeden FJ, Appelmelk BJ, Vandenbroucke-Grauls CM, Bitter W. Zebrafish embryos as a model host for the real time analysis of Salmonella typhimurium infections. Cell Microbiol. 2003 Sep;5(9):601-11.
91 Stockhammer OW, Zakrzewska A, Hegedûs Z, Spaink HP, Meijer AH. Transcriptome profiling and functional analyses of the zebrafish embryonic innate immune response to Salmonella infection. J Immunol. 2009 May 1;182(9):5641-53.
92 Dunlap NE, Benjamin WH Jr, Berry AK, Eldridge JH, Briles DE. A 'safe-site' for Salmonella typhimurium is within splenic polymorphonuclear cells. Microb Pathog. 1992 Sep;13(3):181-90.
93 Salcedo SP, Noursadeghi M, Cohen J, Holden DW. Intracellular replication of Salmonella typhimurium strains in specific subsets of splenic macrophages in vivo. Cell Microbiol. 2001 Sep;3(9):587-97.
94 Ordas A, Hegedus Z, Henkel CV, Stockhammer OW, Butler D, Jansen HJ, Racz P, Mink M, Spaink HP, Meijer AH. Deep sequencing of the innate immune transcriptomic response of zebrafish embryos to Salmonella infection. Fish Shellfish Immunol. 2011 Nov;31(5):716-24.
95 Stockhammer OW, Rauwerda H, Wittink FR, Breit TM, Meijer AH, Spaink HP. Transcriptome analysis of Traf6 function in the innate immune response of zebrafish embryos. Mol Immunol. 2010 Nov-Dec;48(1-3):179-90.
96 Kanwal Z, Zakrzewska A, den Hertog J, Spaink HP, Schaaf MJ, Meijer AH. Deficiency in hematopoietic phosphatase ptpn6/Shp1 hyperactivates the innate immune system and impairs control of bacterial infections in zebrafish embryos. J Immunol. 2013 Feb 15;190(4):1631-45.

97 Ordas A, Kanwal Z, Lindenberg V, Rougeot J, Mink M, Spaink HP, Meijer AH. MicroRNA-146 function in the innate immune transcriptome response of zebrafish embryos to Salmonella typhimurium infection. BMC Genomics. 2013 Oct 10;14:696.
98 Mahenthiralingam E, Baldwin A, Dowson CG. Burkholderia cepacia complex bacteria: opportunistic pathogens with important natural biology. J Appl Microbiol. 2008 Jun;104(6):1539-51.
99 Saiman L, Siegel J. Infection control in cystic fibrosis. Clin Microbiol Rev. 2004 Jan;17(1):57-71.
100 Vergunst AC, Meijer AH, Renshaw SA, O'Callaghan D. Burkholderia cenocepacia creates an intramacrophage replication niche in zebrafish embryos, followed by bacterial dissemination and establishment of systemic infection. Infect Immun. 2010 Apr;78(4):1495-508.
101 Agnoli K, Schwager S, Uehlinger S, Vergunst A, Viteri DF, Nguyen DT, Sokol PA, Carlier A, Eberl L. Exposing the third chromosome of Burkholderia cepacia complex strains as a virulence plasmid. Mol Microbiol. 2012 Jan;83(2):362-78.
102 Agnoli K, Frauenknecht C, Freitag R, Schwager S, Jenul C, Vergunst A, Carlier A, Eberl L. The third replicon of members of the Burkholderia cepacia Complex, plasmid pC3, plays a role in stress tolerance. Appl Environ Microbiol. 2014 Feb;80(4):1340-8.
103 Lowy FD. Staphylococcus aureus infections. N Engl J Med. 1998 Aug 20;339(8):520-32.
104 Prajsnar TK, Cunliffe VT, Foster SJ, Renshaw SA. A novel vertebrate model of Staphylococcus aureus infection reveals phagocyte-dependent resistance of zebrafish to non-host specialized pathogens. Cell Microbiol. 2008 Nov;10(11):2312-25.
105 Lin B, Chen S, Cao Z, Lin Y, Mo D, Zhang H, Gu J, Dong M, Liu Z, Xu A. Acute phase response in zebrafish upon Aeromonas salmonicida and Staphylococcus aureus infection: striking similarities and obvious differences with mammals. Mol Immunol. 2007 Jan;44(4):295-301.
106 Rigby KM, DeLeo FR. Neutrophils in innate host defense against Staphylococcus aureus infections. Semin Immunopathol. 2012 Mar;34(2):237-59.
107 Li YJ, Hu B. Establishment of multi-site infection model in zebrafish larvae for studying Staphylococcus aureus infectious disease. J Genet Genomics. 2012 Sep 20;39(9):521-34.
108 Holtfreter S, Radcliff FJ, Grumann D, Read H, Johnson S, Monecke S, Ritchie S, Clow F, Goerke C, Bröker BM, Fraser JD, Wiles S. Characterization of a mouse-adapted Staphylococcus aureus strain. PLoS One. 2013 Sep 2;8(9):e71142.
109 McVicker G, Prajsnar TK, Williams A, Wagner NL, Boots M, Renshaw SA, Foster SJ. Clonal expansion during Staphylococcus aureus infection dynamics reveals the effect of antibiotic intervention. PLoS Pathog. 2014 Feb 27;10(2):e1003959.
110 Kubica M, Guzik K, Koziel J, Zarebski M, Richter W, Gajkowska B, Golda A, Maciag-Gudowska A, Brix K, Shaw L, Foster T, Potempa J. A potential new pathway for Staphylococcus aureus dissemination: the silent survival of S. aureus phagocytosed by human monocyte-derived macrophages. PLoS One. 2008 Jan 9;3(1):e1409.
111 Ashida H, Ogawa M, Kim M, Suzuki S, Sanada T, Punginelli C, Mimuro H, Sasakawa C. Shigella deploy multiple countermeasures against host innate immune responses. Curr Opin Microbiol. 2011 Feb;14(1):16-23.
112 Mostowy S, Sancho-Shimizu V, Hamon MA, Simeone R, Brosch R, Johansen T, Cossart P. p62 and NDP52 proteins target intracytosolic Shigella and Listeria to different autophagy pathways. J Biol Chem. 2011 Jul 29;286(30):26987-95.
113 Gow NA, van de Veerdonk FL, Brown AJ, Netea MG. Candida albicans morphogenesis and host defence: discriminating invasion from colonization. Nat Rev Microbiol. 2011 Dec 12;10(2):112-22.
114 Chao CC, Hsu PC, Jen CF, Chen IH, Wang CH, Chan HC, Tsai PW, Tung KC, Wang CH, Lan CY, Chuang YJ. Zebrafish as a model host for Candida albicans infection. Infect Immun. 2010 Jun;78(6):2512-21.
115 Brothers KM, Newman ZR, Wheeler RT. Live imaging of disseminated candidiasis in zebrafish reveals role of phagocyte oxidase in limiting filamentous growth. Eukaryot Cell. 2011 Jul;10(7):932-44.
116 Brothers KM, Gratacap RL, Barker SE, Newman ZR, Norum A, Wheeler RT. NADPH oxidase-driven phagocyte recruitment controls Candida albicans filamentous growth and prevents mortality. PLoS Pathog. 2013;9(10):e1003634.
117 Forlenza M, Fink IR, Raes G, Wiegertjes GF. Heterogeneity of macrophage activation in fish. Dev Comp Immunol. 2011 Dec;35(12):1246-55.
118 Feng Y, Santoriello C, Mione M, Hurlstone A, Martin P. Live imaging of innate immune cell sensing of transformed cells in zebrafish larvae: parallels between tumor initiation and wound inflammation. PLoS Biol. 2010 Dec 14;8(12):e1000562.
119 Sieger D, Peri F. Animal models for studying microglia: the first, the popular, and the new. Glia. 2013 Jan;61(1):3-9.
120 Clark KJ, Voytas DF, Ekker SC. A TALE of two nucleases: gene targeting for the masses? Zebrafish. 2011 Sep;8(3):147-9.
121 Blackburn PR, Campbell JM, Clark KJ, Ekker SC. The CRISPR system—keeping zebrafish gene targeting fresh. Zebrafish. 2013 Mar;10(1):116-8.

122 Kettleborough RN, Busch-Nentwich EM, Harvey SA, Dooley CM, de Bruijn E, van Eeden F, Sealy I, White RJ, Herd C, Nijman IJ, Fényes F, Mehroke S, Scahill C, Gibbons R, Wali N, Carruthers S, Hall A, Yen J, Cuppen E, Stemple DL. A systematic genome-wide analysis of zebrafish protein-coding gene function. Nature. 2013 Apr 25;496(7446):494-7.
123 Li L, Yan B, Shi YQ, Zhang WQ, Wen ZL. Live imaging reveals differing roles of macrophages and neutrophils during zebrafish tail fin regeneration. J Biol Chem. 2012 Jul 20;287(30):25353-60.
124 Lawson ND, Weinstein BM. In vivo imaging of embryonic vascular development using transgenic zebrafish. Dev Biol. 2002 Aug 15;248(2):307-18.
125 Hall C, Flores MV, Storm T, Crosier K, Crosier P. The zebrafish lysozyme C promoter drives myeloid-specific expression in transgenic fish. BMC Dev Biol. 2007 May 4;7:42.
126 Zhang Y, Bai XT, Zhu KY, Jin Y, Deng M, Le HY, Fu YF, Chen Y, Zhu J, Look AT, Kanki J, Chen Z, Chen SJ, Liu TX. In vivo interstitial migration of primitive macrophages mediated by JNK-matrix metalloproteinase 13 signaling in response to acute injury. J Immunol. 2008 Aug 1;181(3):2155-64.
127 Kitaguchi T, Kawakami K, Kawahara A. Transcriptional regulation of a myeloid-lineage specific gene lysozyme C during zebrafish myelopoiesis. Mech Dev. 2009 May-Jun;126(5-6):314-23.
128 Elks PM, van Eeden FJ, Dixon G, Wang X, Reyes-Aldasoro CC, Ingham PW, Whyte MK, Walmsley SR, Renshaw SA. Activation of hypoxia-inducible factor-1α (Hif-1α) delays inflammation resolution by reducing neutrophil apoptosis and reverse migration in a zebrafish inflammation model. Blood. 2011 Jul 21;118(3):712-22.
129 Harvie EA, Green JM, Neely MN, Huttenlocher A. Innate immune response to Streptococcus iniae infection in zebrafish larvae. Infect Immun. 2013 Jan;81(1):110-21.
130 Renshaw SA, Loynes CA, Trushell DM, Elworthy S, Ingham PW, Whyte MK. A transgenic zebrafish model of neutrophilic inflammation. Blood. 2006 Dec 15;108(13):3976-8.
131 Mathias JR, Perrin BJ, Liu TX, Kanki J, Look AT, Huttenlocher A. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish. J Leukoc Biol. 2006 Dec;80(6):1281-8.
132 Yoo SK, Deng Q, Cavnar PJ, Wu YI, Hahn KM, Huttenlocher A. Differential regulation of protrusion and polarity by PI3K during neutrophil motility in live zebrafish. Dev Cell. 2010 Feb 16;18(2):226-36.
133 Yoo SK, Huttenlocher A. Spatiotemporal photolabeling of neutrophil trafficking during inflammation in live zebrafish. J Leukoc Biol. 2011 May;89(5):661-7.
134 Ward AC, McPhee DO, Condron MM, Varma S, Cody SH, Onnebo SM, Paw BH, Zon LI, Lieschke GJ. The zebrafish spi1 promoter drives myeloid-specific expression in stable transgenic fish. Blood. 2003 Nov 1;102(9):3238-40.
135 Hsu K, Traver D, Kutok JL, Hagen A, Liu TX, Paw BH, Rhodes J, Berman JN, Zon LI, Kanki JP, Look AT. The pu.1 promoter drives myeloid gene expression in zebrafish. Blood. 2004 Sep 1;104(5):1291-7.
136 Clark BS, Winter M, Cohen AR, Link BA. Generation of Rab-based transgenic lines for in vivo studies of endosome biology in zebrafish. Dev Dyn. 2011 Nov;240(11):2452-65.
137 Ellis K, Bagwell J, Bagnat M. Notochord vacuoles are lysosome-related organelles that function in axis and spine morphogenesis. J Cell Biol. 2013 Mar 4;200(5):667-79.
138 He C, Bartholomew CR, Zhou W, Klionsky DJ. Assaying autophagic activity in transgenic GFP-Lc3 and GFP-Gabarap zebrafish embryos. Autophagy. 2009 May;5(4):520-6.