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Torraca, V.

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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

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Chapter 2

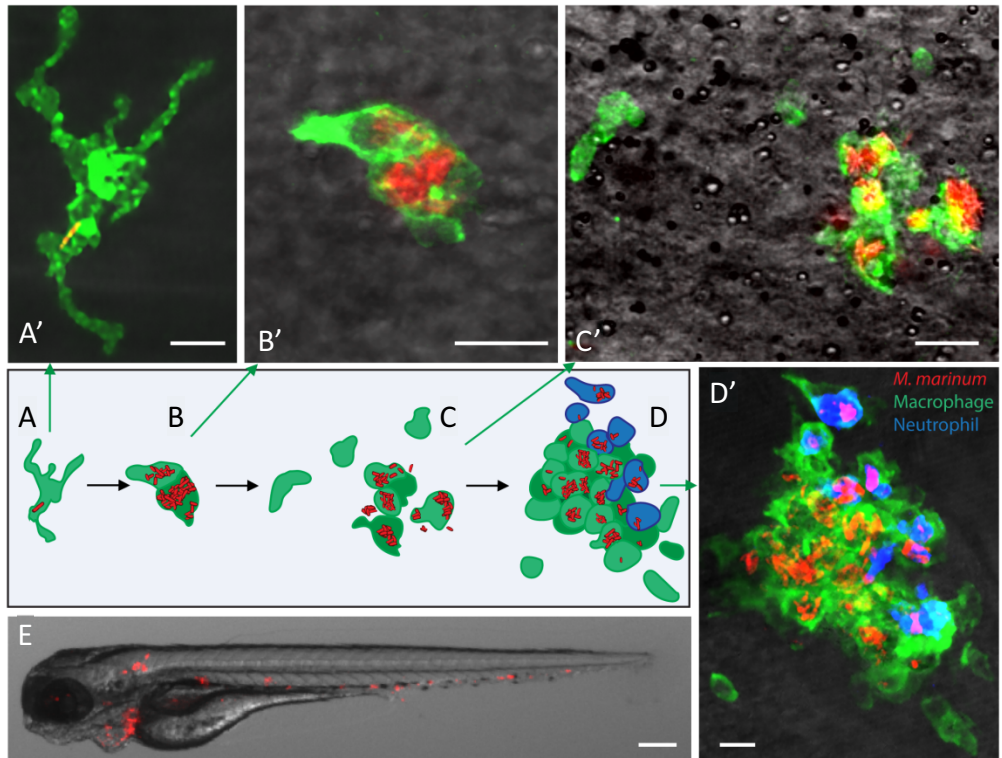
Macrophage-pathogen interactions in infectious diseases: new therapeutic insights from the zebrafish host model

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 μ m. F: 200 μ m.

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 mesoderm⁸. 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 bacteria¹¹.

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 progenitors¹². 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 phagocytosis²³ and Toll-like receptors (TLRs), in cooperation with other pattern-recognition receptors (PRRs), initiate the innate immune response²⁴. 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 IL1 β (interleukin 1 beta) and full activation of the innate immune response²⁷.

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

compartment³⁹. Furthermore, several pathogens inject virulence factors that promote actin polymerisation to actively stimulate their uptake by both non-phagocytic and phagocytic cells⁴⁰. 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 infection⁴². 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 *csflra* 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 *csflra* 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 experiments⁴³. 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 types⁴⁴. 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 dpf⁴⁸.

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, *spil/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 used⁴⁸. 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 differentiation⁴⁹.

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**).

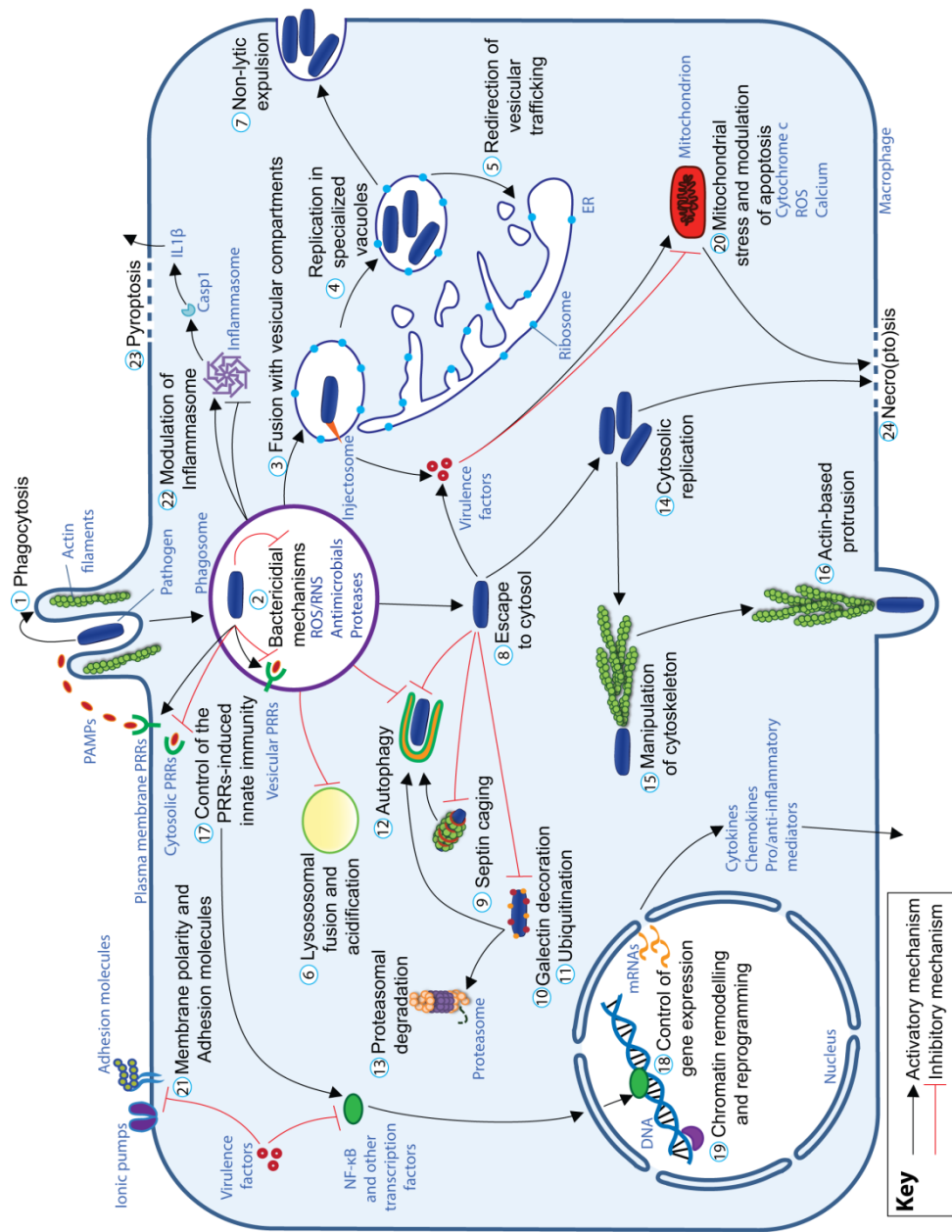


Figure 1. Evasion of macrophage defence mechanisms by intracellular pathogens (Legend on the next page).

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 homeostatic 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**).

Photoconvertible 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 wounds⁵³ and that *Mycobacterium*-infected macrophages egress from primary granulomas to initiate secondary infection foci⁵². 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 environment⁵⁹. 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.

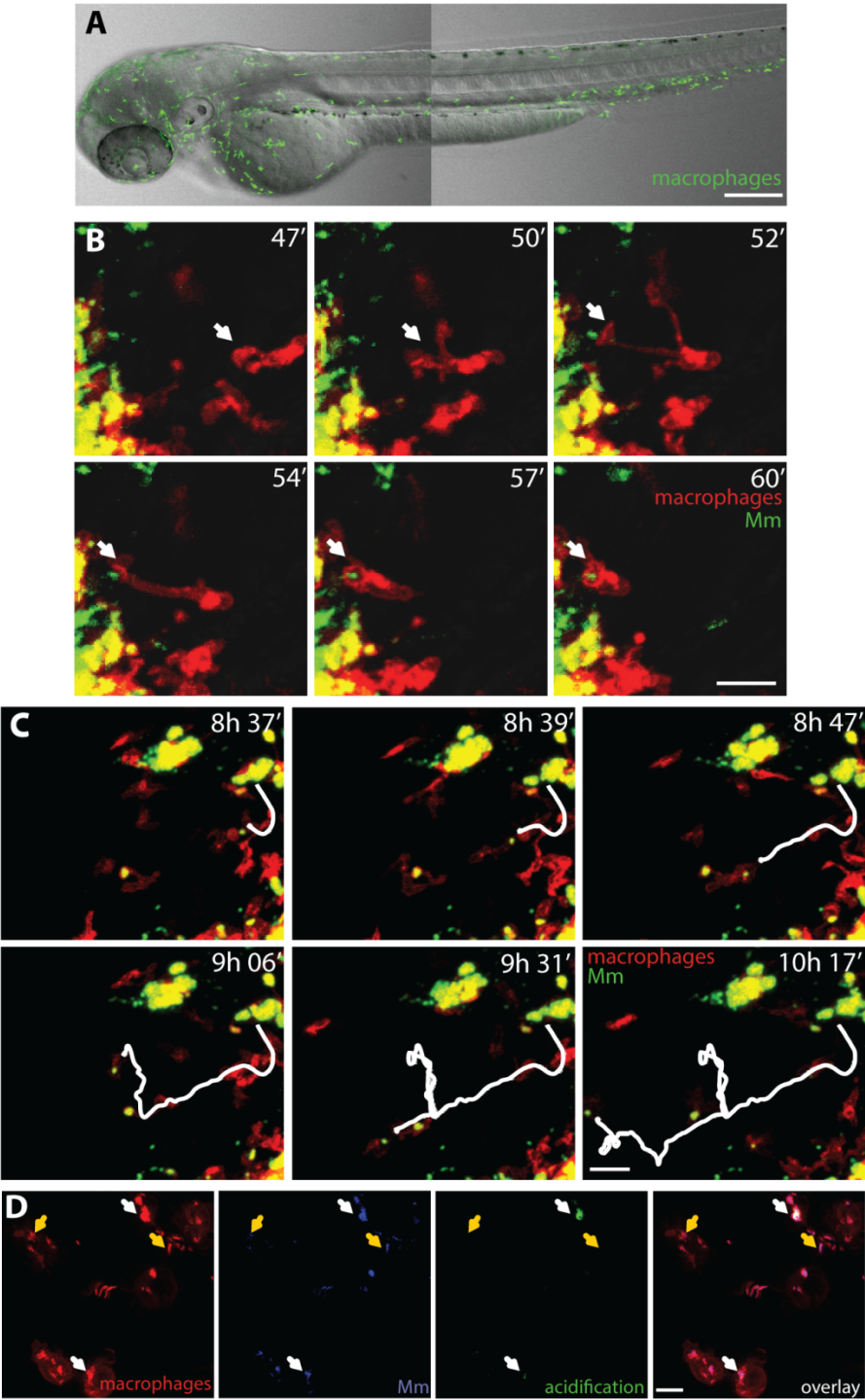


Figure 2. *In vivo* imaging of macrophage responses to infection (Legend on the next page).

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 tuberculosis⁷². 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**).

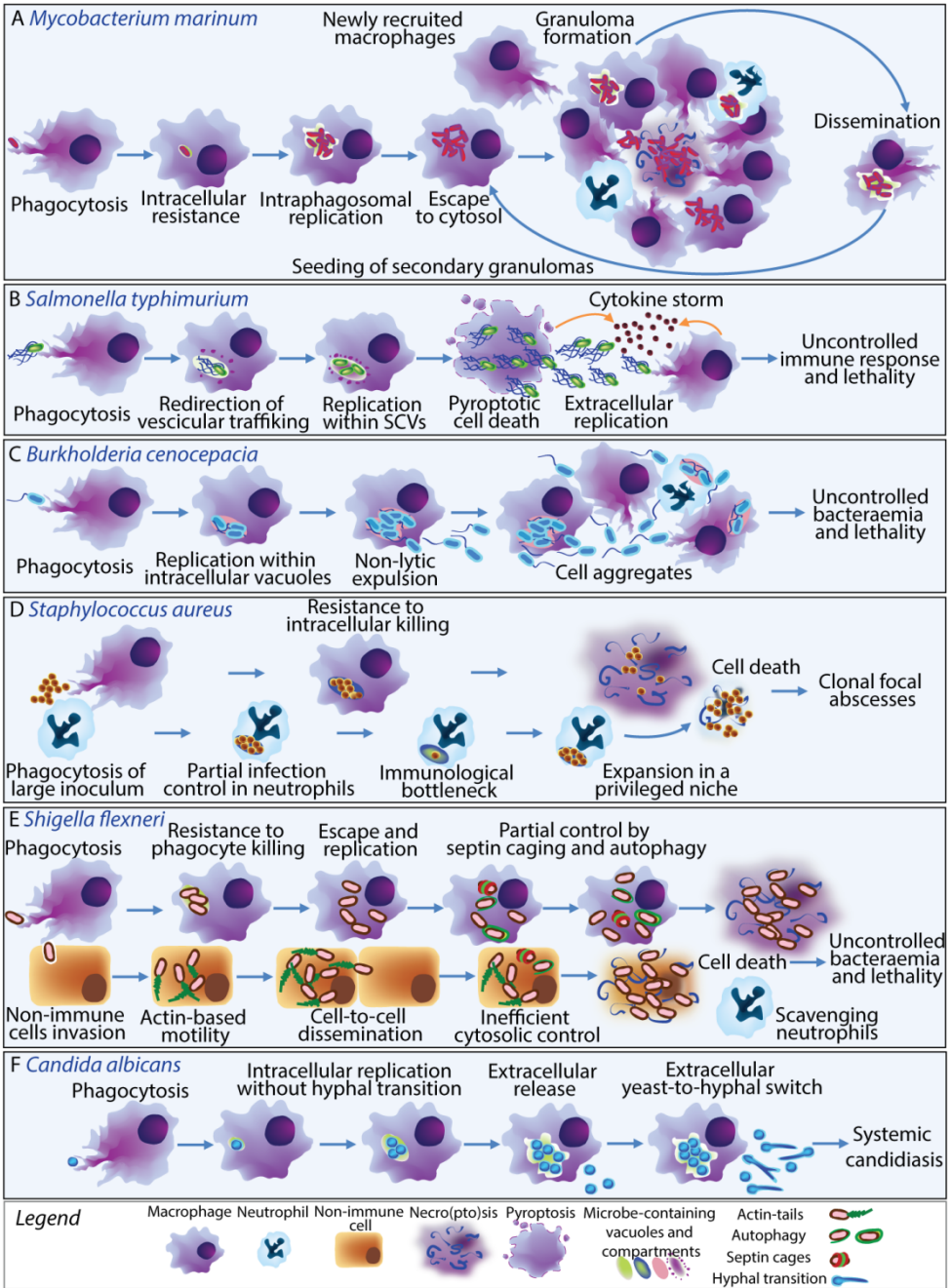


Figure 3. Models of intracellular infections in zebrafish (Legend on the next page).

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 "intraphaagocyte 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 immunity⁵⁰. 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 therapy⁷⁵ (**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 model⁷⁶.

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 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* susceptibility⁷⁹. 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 antagonists⁸⁰. 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 model⁸². 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 conditions⁸³ (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 embryos⁸⁵. In contrast, the initial recruitment of macrophages to a localised *M. marinum* infection in the hindbrain was found to be largely

Myd88 independent⁵⁸. 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 signalling⁵⁸.

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 production⁵⁶. Prior to granuloma formation, neutrophils, both infected and non-infected, also produce RNS⁶⁵. 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-1 α signalling or decreasing Hif-2 α 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 infections³⁴. 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 autophagy⁸⁶. The induction of draml (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 fibrosis⁶¹. 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 model⁸⁸. 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 diseases⁸⁹. 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 vessels⁹⁰. 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 factors⁹⁰.

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 family⁹⁷.

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* infection⁹¹. 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 CXCR3⁴⁶. The ligand association of Cxcr3.2 remains to be established.

In addition to phagocyte recruitment, localised infection also triggers emergency-driven haematopoiesis⁶². 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/ebp β (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 site⁶².

The *S. typhimurium* infection model has recently led to new insight into the connection between infection control and host cell metabolic modulation⁶³. 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 production⁶³. 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 metabolism⁶² (**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 plants⁹⁸. In humans, opportunistic infection by *Bcc* frequently occurs in cystic fibrosis or immunocompromised individuals and represents a recurrent cause of fatal complications⁹⁹. 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 bacteraemia¹⁰⁰.

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 isolates¹⁰⁰. 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 survival¹⁰¹. 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* isolates¹⁰².

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 septicemia¹⁰³, which remain important causes of morbidity and of complications in hospitalised patients. Although not a natural pathogen of zebrafish, both embryos¹⁰⁴ and adults¹⁰⁵ 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 phagocytes¹⁰⁶. 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 mechanisms⁴⁷.

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 strain⁴⁷. 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 bacteria¹⁰⁸. 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 infection¹⁰⁹. 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 studies¹¹⁰.

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 internalisation⁴⁰. 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 epithelium¹¹¹.

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 cells⁸⁶. 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 cytosol⁸⁶. 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 forms¹¹³. 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 embryos¹¹⁵. 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 macrophages¹¹⁶. 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* infection¹¹⁶.

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 species¹¹⁷. That different macrophage subtypes might already be present in early zebrafish larvae has been suggested, but this remains to be further investigated¹¹⁸. 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.

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

Modelling macrophage-pathogen interactions in zebrafish

myd88 myeloid differentiation primary response gene (88)	<i>Tg(myd88:eGFP)</i> <i>Tg(myd88:DsRed2)</i>	Subsets of myeloid leukocytes, distal pronephric ducts and cloaca	-3.7 kb of <i>myd88</i> upstream the translation start	59
ptprc/cd45 protein tyrosine phosphatase, receptor type, C	<i>Tg(ptprc:DsRed)</i>	Macrophages, granulocytes and T lymphocytes	-7.6 kb of <i>ptprc</i> upstream the transcription start	14
spi1b/pu.1 spleen focus forming virus (SFFV) proviral integration oncogene spi1b	<i>Tg(-5.3spi1b:eGFP)</i>	Early myeloid cells ^(a)	-5.3 kb of <i>spi1b</i> upstream the translation start	134
	<i>Tg(-9.0spi1b:eGFP)</i>	Early myeloid cells (bright) and muscles (dull) ^(a)	-9 kb of <i>spi1b</i> upstream the translation start	135
	<i>Tg(-4spi1b:Gal4 / UAS:eGFP)</i>	Early myeloid cells ^(a)	-4 kb of <i>spi1b</i> upstream the translation start	60
	<i>Tg(-4spi1b:lyn-eGFP)</i>	Early myeloid cells ^(a)	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	<i>Tg(h2afx:eGFP-rab5c)^(a)</i>	Early endosomes	-	136
	<i>Tg(UAS:mCherry-rab5c_S36N)^(b)</i>	Early endosomes	S36N substitution, dominant negative	136
	<i>Tg(UAS:mCherry-rab5c_Q81L)^(b)</i>	Early endosomes	Q81L substitution, constitutively active	136
rab7a RAB7, member RAS oncogene family a	<i>Tg(h2afx:eGFP-rab7)^(a)</i>	Late endosomes	-	136
	<i>Tg(UAS:mCherry-rab7_T22N)^(b)</i>	Late endosomes	T22N substitution, dominant negative	136
	<i>Tg(UAS:mCherry-rab7_Q67L)^(b)</i>	Late endosomes	Q67L substitution, constitutively active	136
rab7b RAB7, member RAS oncogene family b	<i>Plasmid(UAS:GFP-rab7b_T22N)^(b)</i>	Late endosomes	T22N substitution, dominant negative; the reporter consists of a UAS-expression plasmid	137
rab11a RAB11a, member RAS oncogene family	<i>Tg(h2afx:eGFP-rab11a)^(a)</i>	Recycling endosomes	-	136
	<i>Tg(UAS:mCherry-rab11a_S25N)^(b)</i>	Recycling endosomes	S25N substitution, dominant negative	136
	<i>Tg(UAS:mCherry-rab11a_Q70L)^(b)</i>	Recycling endosomes	Q70L substitution, constitutively active	136
lamp1 lysosomal-associated membrane protein 1	<i>Tg(hsp70l:lamp1-RFP)^(c)</i>	Lysosomes	-	137
lamp2 lysosomal membrane glycoprotein 2	<i>Tg(hsp70l:lamp2-eGFP)^(c)</i>	Lysosomes	-	137
cd63/lamp3 Cd63 antigen	<i>Plasmid(CMV/SP6:GFP-cd63)^(d)</i>	Lysosomes	The reporter consists of an expression plasmid for CMV-expression or mRNA preparation	60
map1lc3b microtubule-associated protein 1 light chain 3 beta	<i>Tg(CMV:eGFP-map1lc3b)^(d)</i>	Autophagosomes	-	138
	<i>Tg(hsp70l:RFP-Rno.Map1lc3b)^(c)</i>	Autophagosomes	Contains the Map1lc3b sequence from the rat (<i>Rattus norvegicus</i>)	137
gabarapa GABA(A) receptor- associated protein a	<i>Tg(CMV:eGFP-gabarapa)^(d)</i>	Autophagosomes	-	138
rab32a RAB32a, member RAS oncogene family	<i>Tg(4xUAS:eGFP-rab32a,myl7:eGFP)^(b)</i>	Vesicular trafficking	-	137
	<i>Plasmid(UAS:GFP-rab32a_T27N)^(b)</i>	Vesicular trafficking	T27N substitution, dominant negative; the reporter consists of a UAS-expression plasmid	137
rab38b	<i>Tg(UAS:GFP-rab38b-T23N, cmlc2:GFP)^(b)</i>	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_188Sfgn4xoUkRVNjNKdINhSEE

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