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Microreview

Real-time imaging and genetic dissection of host–microbe interactions in zebrafish

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Summary

Many aspects of host interactions with microbes can only be studied in the context of a whole organism. The zebrafish as a model organism has shown to be highly successful for studies of infection biology and the interactions of commensal microbiota with their hosts. Zebrafish are transparent during embryo and larval development and these early life stages are optimally suited for high-resolution imaging of host–microbe interactions in a vertebrate organism. This is facilitated by the development of a variety of fluorescent reporter lines that mark different immune cell types or subcellular compartments where pathogens reside. The zebrafish is an excellent vertebrate model for forward genetic screening and efficient tools for gene knock-down and targeted mutagenesis add further to the strength of this model organism. The use of zebrafish larvae for studying microbial infections has recently led to important new insights in host defence mechanisms, which are highlighted in this review focused on bacterial pathogens. Considering the highly conserved nature of the processes involved, including innate immune recognition, immunometabolism and autophagy, it is to be expected that these recent findings in zebrafish will have great translational value for biomedical applications.

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Introduction

Communication of multicellular organisms with the multitude of microbes surrounding them is vital for survival, not only to ensure effective defence against pathogens but also for mutualistic cohabitation purposes. Notwithstanding our current appreciation of the importance of the innate immune system for all aspects of this communication (O'Neill *et al.*, 2013), there is much that still remains to be learned about the mechanisms that can distinguish potentially threatening and non-threatening or beneficial microbes. Recently more insight has been gained into the downstream intracellular mechanisms that deal with bacteria that have penetrated inside tissues or have been phagocytosed, which among others led to the recognition of the importance of autophagy for defence against intracellular pathogens and for communication between immune cells (Deretic, 2011). However, many pathogens have potent virulence systems to defend themselves against autophagy or against other long known host defence mechanisms such as oxidative and nitrosative stress responses (Mostowy, 2013). Many aspects of these intricate interactions between pathogens and their hosts are poorly understood. An important reason why research in this area is still difficult is that cell culture systems that have been commonly used to identify molecular pathways involved in pathogen recognition are not very suitable to study factors that are only important in a whole organism setting. In the first place, the immune system of multicellular organisms not only responds to pathogen-associated molecular patterns (PAMPs), but also senses damage associated with invading microbes, and it is the combination of pathogen and danger signals that is crucial for appropriate activation of the immune response (O'Neill *et al.*, 2013). Second, the response of an organism to microbes is also linked to the actual physical location of the organism in particular tissues, for instance the gut responds differently to microbial communities than the skin and the response to microbial infection is probably different in many organs, as it is in the various immune cell types. Third, the virulence mechanisms of many pathogens require a tissue context, for example the formation of granulomatous aggregates of mycobacterium-infected and non-infected immune cells is central to the

pathology of tuberculosis (Ramakrishnan, 2012). These arguments give strong support to complement studies in cell culture systems with whole organism studies. Notwithstanding the importance of rodent models for infectious diseases, studies in non-mammalian hosts, such as the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and the zebrafish *Danio rerio*, are valuable to help understanding evolutionary conserved fundamental virulence mechanisms. Each of these models have their specific practical advantages and limitations, which are discussed in more detail in a recent review (O'Callaghan and Vergunst, 2010).

In the last decade the zebrafish has proven to be a versatile model for studying host–microbe interactions (Meeker and Trede, 2008; Kanther and Rawls, 2010; Meijer and Spaink, 2011; Berg and Ramakrishnan, 2012; Renshaw and Trede, 2012; van der Vaart *et al*., 2012). These studies benefit from the high similarity of the immune system of fish and mammals, enabling detailed comparative studies that are directly relevant for medical applications such as screening for novel medicines in disease models. In addition, the zebrafish offers several practical advantages over rodent studies such as a high fecundity and the availability of disease models that exploit the small size and optical transparency of zebrafish embryos and larvae. These transparent stages offer superior possibilities for high-resolution imaging of cellular migration processes in response to infections and for *in vivo* analysis of the interaction between microbes and host cells. To facilitate *in vivo* imaging, fluorescent reporter lines have been developed that mark different immune cell types of the zebrafish or subcellular compartments where pathogens reside (Mathias *et al*., 2006; Renshaw *et al*., 2006; Hall *et al*., 2009; He *et al*., 2009; Ellett and Lieschke, 2010; Gray *et al*., 2011; Wittamer *et al*., 2011; Li *et al*., 2012) (Fig. 1). These zebrafish reporter lines have also proved extremely useful for studying inflammatory diseases and the function of leukocytes in tumour microenvironments (Feng *et al*., 2010; Loynes *et al*., 2010; Walters *et al*., 2010; He *et al*., 2012). It can therefore be concluded that *in vivo* imaging is a major strength of the zebrafish model for studies of cellular interaction. Recently, this advantage has been greatly strengthened by the possibility to combine imaging with advanced tools for forward and reverse genetics and the full benefit of the recent availability of state of the art genomic tools such as RNA deep sequencing and whole-genome epigenetic analyses (Santoriello and Zon, 2012; Jiang *et al*., 2013; Kettleborough *et al*., 2013). The development of disease models was inspired by the important contribution of the zebrafish to developmental genetics, which firmly established this species as the preferred

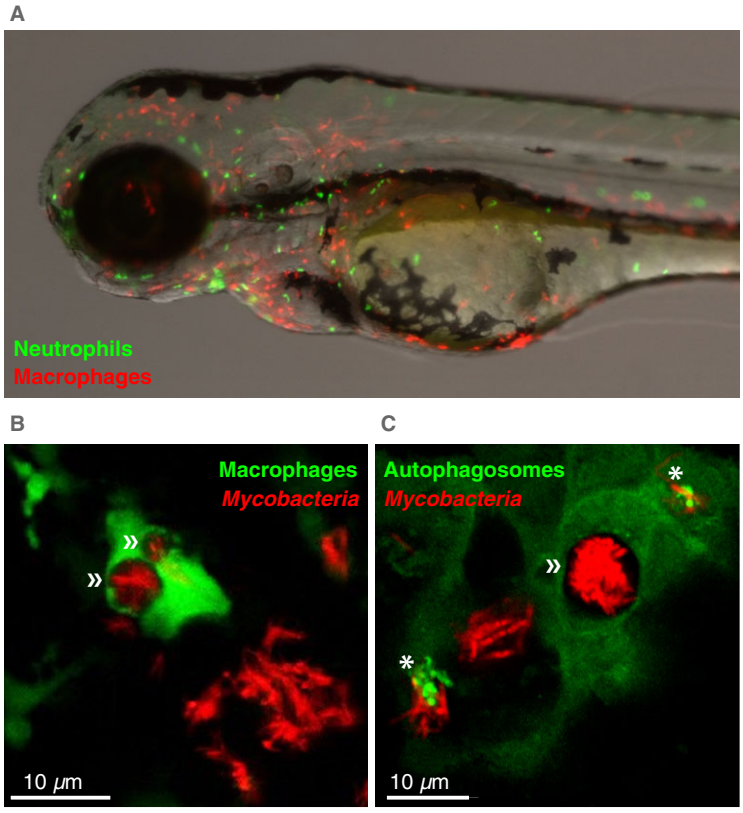

Fig. 1. Visualizing innate immune cell behaviour during host–pathogen interactions.
A. The use of transgenic zebrafish embryos with fluorescently labelled neutrophils [Tg(mpx:GFP)i114, Renshaw *et al*., 2006] and macrophages [Tg(mpeg1:mCherryF)ump2, G. Lutfalla, kind gift] allows the *in vivo* study of leukocyte behaviour during diverse processes such as the inflammatory response, tumorigenesis, and the interactions between immune cells and invading pathogens.
B. Here, we show a confocal micrograph of a macrophage [Tg(mpeg1:EGFP)gl22, Ellett *et al*., 2011] that has phagocytosed *Mycobacterium marinum* bacteria (mCherry labelled), which are now contained in intracellular vacuoles (as indicated by »).
C. The subcellular localization of pathogens can be specified by using transgenic lines that allow differentiation between the various compartments of the (auto-)phagolysosomal pathway. Here we show a confocal micrograph demonstrating the association between GFP-Lc3 decorated autophagosomes [Tg(CMV:EGFP-map1lc3b) line, He *et al*., 2009] and phagocytosed *M. marinum* (as indicated by *).

vertebrate model for forward genetic screens (Amsterdam and Hopkins, 2006; Santoriello and Zon, 2012). The most common strategy for forward genetic screening in zebrafish is random mutagenesis with ethylnitrosourea (ENU) (Amsterdam and Hopkins, 2006). This approach is extremely powerful for the discovery of new gene functions and disease associations, for instance in the field of infectious diseases demonstrated recently by the identification of a novel mycobacterial susceptibility locus (Tobin *et al*., 2010; Santoriello and Zon, 2012). In addition, antisense morpholino oligonucleotides are powerful tools for transient gene knock-down at embryo and larval stages (Bedell *et al*., 2011). The availability of the zebrafish reference genome sequence and efficient technologies for targeted mutagenesis, recently augmented with the TALEN and CRISPR/Cas systems, will speed up further research in this model (Clark *et al*., 2011; Blackburn *et al*., 2013; Howe *et al*., 2013; Kettleborough *et al*., 2013). The powerful combination of *in vivo* imaging with these tools for genetic dissection has been exploited in several disease models as highlighted by the recent studies of microbe–animal interactions that are discussed in this review.

A key study that demonstrated zebrafish embryos as a versatile model host for investigating host–microbe interactions showed that as early as 1 day post fertilization the embryos have developed primitive macrophages that are capable of phagocytosing bacteria injected into the blood circulation (Herbomel *et al*., 1999) (Supplementary Table S1). These primitive macrophages can effectively eliminate bacterial strains that are non-pathogenic and are also able to sense and migrate to localized infections. Subsequently it was shown that embryos have circulating neutrophils that produce myeloperoxidase (Mpx) enzyme by 2 days post fertilization (Lieschke *et al*., 2001). Soon after the description of the early development of these two major cell types of the innate immune system, it was shown that infection of zebrafish embryos with *Mycobacterium marinum*, a close relative of the human tuberculosis pathogen (*Mycobacterium tuberculosis*), leads to the formation of macrophage aggregates with pathological hallmarks of tuberculous granulomas (Davis *et al*., 2002). Since then, this has become the most widely used infection model in zebrafish and it has provided many new insights into the function of the innate immune system and granuloma formation in tuberculosis pathogenesis (Supplementary Table S1). The adult zebrafish has also been exploited for infection biology, especially for the discovery of virulence determinants of Streptococcal infections (Neely *et al*., 2002). These pioneering studies were followed by development of a wide variety of zebrafish models for studying infectious diseases or the commensal microbiota (Meeker and Trede, 2008; Kanther and Rawls, 2010; Meijer and Spaink, 2011). Infecting embryos is most commonly achieved by microinjection, which can be performed at different sites in order to study responses to systemic or localized infections (Benard *et al*., 2012). Such systemic studies can now also be performed at a very high throughput level (Carvalho *et al*., 2011; Spaink *et al*., 2013; Veneman *et al*., 2013). Methods have also been established to rear embryos under germ-free or gnotobiotic conditions in order to study host–microbe interactions in a controlled environment (Rawls *et al*., 2007; Pham *et al*., 2008). An important characteristic of zebrafish embryos and early larval stages is that *in vivo* functions of innate immune cells can be studied without contribution of the adaptive immune system, which becomes functional only after several weeks of development (Page *et al*., 2013). Haematopoiesis is remarkably similar between human and zebrafish and also the major signalling pathways involved in the activation and function of the immune system are highly conserved (Ellett and Lieschke, 2010; Stachura and Traver, 2011; van der Vaart *et al*., 2012). The best example of conserved infectious disease pathology is the formation of highly structured tuberculous granulomas during mycobacterial infection, allowing investigation of mechanisms underlying latency and reactivation (Swaim *et al*., 2006; Parikka *et al*., 2012). Microarray and RNA deep sequencing data sets have provided insights into the zebrafish transcriptome during infections and are powerful tools to provide leads for functional studies (van der Vaart *et al*., 2012). In addition to its usefulness for visualizing and genetically dissecting host–microbe interactions, the zebrafish also holds much promise as a high-throughput drug screening model using robotic technology (Spaink *et al*., 2013). The small size of the embryos and larvae makes them highly suited for screening chemical libraries, requiring often only minute quantities of chemical compounds that can be administered simply to the medium and are absorbed through the skin (Tamplin *et al*., 2012). The growing list of bacterial, viral and fungal pathogens that has been used for experimental infections in zebrafish has been detailed in several other reviews (Meeker and Trede, 2008; Kanther and Rawls, 2010; Meijer and Spaink, 2011). In this review we will focus on bacterial infections in zebrafish embryo and larval model systems. In addition to a comprehensive overview of the accomplishments in this field (Supplementary Table S1), we highlight recent studies that have advanced insights into defence mechanisms against pathogenic bacteria and that illustrate the powerful combination of imaging and genetics in the zebrafish model.

Regulation of the host innate immune response

Initiation of the innate immune defences is dependent on the recognition of pathogen-associated molecular

patterns and danger signals by conserved families of pattern recognition receptors (PRRs), including the Toll-like receptors (TLRs), Nod-like receptors (NLRs) and RIG-I-like receptors (RLRs) (O'Neill *et al*., 2013). These receptors as well as most of the extracellularly associated recognition factors are conserved from fish to mammals (Stein *et al*., 2007; Pietretti and Wiegertjes, 2013). The first zebrafish mutant for a TLR signalling component was recently identified in an ENU mutagenesis screen (van der Vaart *et al*., 2013). This mutant contains a premature stop codon in the myeloid differentiation factor 88 gene (*myd88*), which encodes an adaptor molecule required for downstream signalling by all TLRs, except TLR3. Upon recruitment to the cytoplasmic face of TLRs, the MyD88 adaptor protein interacts with interleukin-1 receptor associated kinase 4 (IRAK4), which in turn recruits IRAK1 or IRAK2 to form the 'Myddosome' signalling complex (Gay *et al*., 2011). This complex is known to activate nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways, resulting in the induction of a wide range of downstream target genes involved in inflammation and antimicrobial mechanisms. The Myddosome also mediates signalling by the interleukin 1 (IL-1), IL-18 and IL-33 receptors and has been implicated in IFN-Υ receptor signalling. The zebrafish *myd88* mutant line (allele *myd88*^{hu3568}) has a truncated version of MyD88 that lacks the complete domain required for interaction with TLRs and part of the domain required for interaction with IRAK4 (van der Vaart *et al*., 2013). This mutation makes the fish severely immune-compromised, consistent with the effect of *MYD88* deficiency in human patients, who suffer from a primary immunodeficiency syndrome (von Bernuth *et al*., 2008). Under standard conditions for rearing zebrafish, survival of *myd88* mutant larvae was normal up to 8 days post fertilization (dpf), but mortality increased significantly during a critical period of 8–20 dpf. Survival of *myd88* mutants then stabilized during the late larval and juvenile stage, which might correlate with the onset of adaptive immunity. When challenged with bacterial pathogens, embryos of the *myd88* mutant line showed a strongly reduced expression of innate immune response genes and these embryos were more susceptible to infections with both acute (*Edwardsiella tarda* and *Salmonella typhimurium*) and chronic (*M. marinum* strains) bacterial pathogens. Challenge with an attenuated *S. typhimurium* strain [lipopolysaccharide (LPS) mutant Ra] also caused more severe infection levels under conditions of *myd88* deficiency, but *myd88* mutants were capable of limiting the growth of this strain by Myd88-independent signalling responses. Recognition of bacterial TLR ligands, flagellin and LPS, was compromised in *myd88* mutants. Flagellin is the ligand for TLR5, in zebrafish represented by two duplicated genes (*tlr5a* and *tlr5b*), whose combined morpholino knock-down also compromised flagellin recognition (Stockhammer *et al*., 2009). However, LPS, the ligand of mammalian TLR4, failed to stimulate two zebrafish TLR4 homologues (Tlr4aa and Tlr4bb) (Sepulcre *et al*., 2009; Sullivan *et al*., 2009). Therefore, it remains to be elucidated how recognition of LPS is linked to Myd88-dependent induction of pro-inflammatory gene expression (van der Vaart *et al*., 2013).

The myd88 mutation effect on responses to bacterial pathogens could be faithfully phenocopied by morpholino knock-down (van der Sar *et al*., 2006; Stockhammer *et al*., 2009; van der Vaart *et al*., 2013), and this knock-down approach has been used to study the function of Myd88 in the response of zebrafish larvae to intestinal commensal bacteria (Bates *et al*., 2007; Cheesman *et al*., 2011; Kanther *et al*., 2011; Oehlers *et al*., 2011b; Galindo-Villegas *et al*., 2012). Zebrafish embryos come into contact with commensal microbiota following hatching at 2–3 dpf. This contact was shown to induce transient expression of pro-inflammatory genes, such as *il1b*, in a MyD88-dependent manner (Galindo-Villegas *et al*., 2012). MyD88 was also shown to be required for expression of the acute phase response gene *serum amyloid alpha* (*saa*) in the gastrointestinal tract of zebrafish larvae (Kanther *et al*., 2011). These MyD88-dependent responses were undetectable in zebrafish larvae reared under germ free conditions (Kanther *et al*., 2011; Galindo-Villegas *et al*., 2012). A subset of innate immune response genes, including the neutrophil-specific markers *myeloperoxidase* (*mpx*) and *lysozyme C* (*lyz*) and the antimicrobial effector *defensin beta-like 1* (*defbl1*) did not require MyD88 for the response to commensal microbiota (Kanther *et al*., 2011; Galindo-Villegas *et al*., 2012). In agreement with these results, expression profiling of *myd88* deficient embryos challenged with bacterial pathogens also demonstrated that both Myd88-dependent and Myd88-independent signalling routes contribute to the induction of innate immune response genes (van der Vaart *et al*., 2012). In the presence of commensal microbes, zebrafish embryos were shown to be more resistant to viral infection, indicating that microbial priming after hatching is an essential trigger to shape the innate immune system (Galindo-Villegas *et al*., 2012). The hatching stage was also found to be associated with chromatin remodelling through histone modification of the promoters of *il1b* and antiviral genes, but not of antimicrobial genes like *lyz* and *defbl1* (Galindo-Villegas *et al*., 2012). These gene-specific chromatin modifications may function to protect zebrafish larvae against exposure to pathogens after hatching, while the transcriptionally inactive chromatin structure of these genes during earlier embryonic stages could be important to avoid excessive inflammation that might interfere with development. The importance of a tight control of pro-inflammatory gene expression was also evidenced by a morpholino knock-

down study of the protein tyrosine phosphatase non-receptor type 6 (*ptpn6*) gene (Kanwal *et al*., 2013). Mammalian PTPN6 (SHP1) is a crucial factor for immune homeostasis that is thought to inhibit cytokine receptors as well as several kinases in TLR and cytokine signalling pathways (Abu-Dayyeh *et al*., 2008; An *et al*., 2008; Croker *et al*., 2008). When *ptpn6* deficient zebrafish embryos were challenged with *S. typhimurium* or *M. marinum*, they responded with an enhanced induction of pro-inflammatory genes compared with wild-type embryos. However, this increased innate immune response was contra productive as *ptpn6* knock-down embryos had a severely impaired ability to control infections. Consistent with the excessive inflammation observed under *ptpn6* knock-down conditions in zebrafish, mutations in human *PTPN6* have been associated with inflammatory and autoimmune diseases (Christophi *et al*., 2009; Eriksen *et al*., 2010; Zhu *et al*., 2010; Kanwal *et al*., 2013). Together, the zebrafish models for *myd88* and *ptpn6* deficiencies have demonstrated the translational value of zebrafish embryo and larval models for studying the regulation of immune responses, which ultimately can lead to better treatments for inflammatory disorders and autoimmune diseases.

Chemoattraction of leukocytes to infection foci

Transparent zebrafish larvae are particularly attractive as a model for studying leukocyte migration behaviour and elucidating the signals that guide these directional responses. The migration of neutrophils to sites of injury or sites of bacterial infection was found to rely on distinct signalling mechanisms (Deng *et al*., 2012). *In vivo* imaging in zebrafish larvae carrying a H_2O_2 sensor probe showed that a dynamic H_2O_2 gradient attracting neutrophils is generated around wounded tissue, but not in tissues infected with *Pseudomonas aeruginosa* (Niethammer *et al*., 2009; Deng *et al*., 2012). Neutrophil attraction to *P. aeruginosa* infection required phosphoinositide 3-kinase (PI3K) signalling, whereas bacterial *N*-formylmethionine peptides and host MyD88-dependent signalling were both dispensable for the initial recruitment of neutrophils to *P. aeruginosa* infection (Deng *et al*., 2012). Pharmacological inhibition and morpholino knock-down of the zebrafish chemokine receptor Cxcr2 demonstrated its requirement for neutrophil recruitment to localized *P. aeruginosa* infection (Deng *et al*., 2013). In agreement, knock-down of interleukin 8 (Cxcl8), the ligand of Cxcr2, inhibited neutrophil accumulation to localized *Escherichia coli* infection (Sarris *et al*., 2012). In addition to recruiting neutrophils from the immediate environment, the Cxcl8-Cxcr2 signalling axis mediated systemic activation and induced long-range mobilization of neutrophils from haematopoietic tissue to localized *P. aeruginosa* infection (Deng *et al*., 2013). Cxcl8-producing cells were transplanted into zebrafish larvae to generate a local source of chemokine, which enabled a detailed *in vivo* analysis of how Cxcl8 guides neutrophil migration (Sarris *et al*., 2012). Cxcl8 influenced neutrophil motility in two ways: first, by promoting neutrophil orthotaxis (i.e. increased speed of migration in the direction of the cellular implant) and, second, by restricting neutrophil motility in the immediate vicinity of the source of chemokine. Furthermore, Cxcl8 was found to be immobilized *in vivo* by heparin sulfate proteogylcans (HSPGs) in the extracellular matrix and this interaction turned out to be critical both for directing and for locally restricting neutrophil migration (Sarris *et al*., 2012). While local production of Cxcl8 was sufficient to instruct neutrophil migration, zebrafish has three additional homologues of human CXCL8 of which at least one (Cxclbb or Cxcl8-l2) also signals through Cxcr2 (Nomiyama *et al*., 2013; de Oliveira *et al*., 2013). Furthermore, like Cxcl8, a more distant Cxcl family member, Cxcl-c1c, is also locally induced at infection foci (Stockhammer *et al*., 2009). It remains to be elucidated which of these other members of the expanded zebrafish Cxcl family contribute to guiding neutrophil migration. The signals involved in recruitment of macrophages to infection also require further study. A homologue of human CXCR3 (Cxcr3.2) was implicated in recruitment of macrophages to localized *S. typhimurium* infection (Zakrzewska *et al*., 2010). Again, the family of putative ligands for this receptor is expanded in zebrafish and we are currently working to elucidate the receptor-ligand relationships. The CCR/CCL chemokine receptor/ligand families have also diverged between fish and mammals. Dissecting functional homologies with the mammalian chemokine receptor/ligand network and identifying the producer and target cells of zebrafish chemokines will require further extensive research efforts that are important for the use of zebrafish as a model for human disease.

Reactive oxygen production as an innate immune defence mechanism

The production of reactive oxygen and nitrogen species (ROS and RNS) is one of the most effective mechanisms of host defence against intracellular pathogens. ROS are generated by the NADPH oxidase (NOX) and dual oxidase (DUOX) families of NADPH oxidases, which in human includes seven members in total (NOX1–5 and DUOX1–2) (Bedard and Krause, 2007). The zebrafish has a single homologue of DUOX, which is expressed in epidermal and intestinal epithelial cells in a manner dependent on the Nod1 and Nod2 receptors (Flores *et al*., 2010; Oehlers *et al*., 2011a). Morpholino knock-down of *duox*, *nod1* or *nod2* impaired the ability of larvae to control

S. typhimurium infection (Flores *et al*., 2010; Oehlers *et al*., 2011a).

Oxidative killing is also important for control of mycobacterial infection, but ROS production can act as a double edged sword (Yang *et al*., 2012; Roca and Ramakrishnan, 2013). *M. marinum* infection in zebrafish larvae starts from infected macrophages that aggregate into granuloma-like structures, which also attract neutrophils. These neutrophils can phagocytose dying macrophages and have a protective role in the early granuloma, which was shown to depend on NADPH oxidase (Nox2) by knock-down analysis of its cytochrome b-245 alpha and beta subunits (cyba/p22phox and cybb/gp91phox) (Yang *et al*., 2012). ROS can also increase the mycobactericidal activity of macrophages, but when produced in excess it induces macrophage cell death and release of mycobacteria into the growth permissive extracellular milieu (Roca and Ramakrishnan, 2013). This damaging effect of ROS is the explanation for increased tuberculosis susceptibility of zebrafish larvae that produce high levels of tumour necrosis factor (TNF) and pro-inflammatory lipids due to overexpression of leukotriene A4 hydrolase (*lta4h*) (Tobin *et al*., 2010; Roca and Ramakrishnan, 2013). The high TNF state of these larvae triggered mitochondrial ROS production and programmed necrosis (necroptosis) of infected macrophages via receptor-interacting serine-threonine kinase 1 and 3 (Rip1 and Rip3) signalling, and this was independent of Nox2 (Roca and Ramakrishnan, 2013). Subsequent experiments revealed two cooperative mechanisms behind TNF-mediated necroptosis. The first involves regulation of the mitochondrial permeability transition pore complex (mPTPC) by cyclophilin D (CypD), while the second occurs through activation of lysosomal acid sphingomyelinase (aSMase) that produces ceramide, an inducer of apoptosis and RIP1-dependent necroptosis. Combination treatment with CypD and aSMase inhibiting drugs (alisporivir and desipramine) could reverse the tuberculosis susceptibility of larvae in the high TNF state. These drugs are already in phase III clinical trial (alisporivir) or clinical use (desipramine) for other applications and the zebrafish work has now identified them as promising candidates for supporting antibiotic therapy of tuberculosis meningitis patients with pro-inflammatory *LTA4H* genotypes, which have a poor prognosis under conventional treatment regimens (Tobin *et al*., 2012; Roca and Ramakrishnan, 2013).

A recent study in zebrafish showed that the production of mitochondrial ROS (mROS) is fuelled by the utilization of fatty acids and revealed a novel role for immunoresponsive gene 1 (*irg1*) in this process connecting cellular metabolism with infection control (Hall *et al*., 2013). Irg1 is a homologue of bacterial 2-methylcitrate dehydratase involved in metabolism of propionate short-chain fatty acids. Expression of *irg1* in zebrafish is upregulated in response to *S. typhimurium* infection in a MyD88-independent manner (Hall *et al*., 2013; van der Vaart *et al*., 2013). A related gene, irg1-like (*irg1l*), is induced by exposure to *E. tarda* (van Soest *et al*., 2011). The primary response transcription factor Cebpβ was found to induce the specific expression of *irg1* in macrophages during infection, and in turn *cebpβ* expression was controlled cooperatively by glucocorticoid receptor signalling and by the JAK/STAT pathway (Hall *et al*., 2013). When larvae were infected with *S. typhimurium* under *irg1* knock-down conditions, macrophages developed significantly increased bacterial loads. Live imaging with probes for mitochondria (MitoTracker) and for mitochondrial superoxide (MitoSox) showed that infected macrophages produce mROS through an Irg1-dependent mechanism. Treatment with etomoxir, an inhibitor of a rate-limiting enzyme in fatty acid β-oxidation, blocked the mROS production induced by infection or LPS stimulation, while glycolysis inhibitor treatment had no significant effect. Further live imaging studies showed that uptake of a fluorescent fatty acid analogue (BODIPY FL C_{16}) was enhanced in the mitochondria of macrophages in LPS-stimulated larvae. Co-injection of fatty acids with *S. typhimurium* enhanced mROS production in larval macrophages in an Irg1-dependent manner and increased their ability to control infection. Consistent with the studies in zebrafish larvae, mROS production and bactericidal activity of murine macrophages was also shown to depend on fatty acid β-oxidation. Besides its effect on mROS production, Irg1 can also augment macrophage bactericidal activity by catalysing the production of itaconic acid, a compound that inhibits isocitrate lyase, an enzyme in the glyoxylate cycle that bacteria use to convert fatty acids into glucose. Irg1-dependent production of itaconic acid was shown to have antimicrobial activity against *S. typhimurium* and *M. tuberculosis* in murine macrophages (Michelucci *et al*., 2013). The discovery that Irg1 functions at the interface of inflammation and metabolism makes this protein an interesting therapeutic target (Hall *et al*., 2013). This illustrates that immunometabolism is an emerging research field that may open new avenues for disease treatment.

Function of nitric oxide in immune defence

Another link between immunity and metabolism is the conversion of L-arginine into the antimicrobial compound nitric oxide (NO) by inducible nitric oxidase synthase (iNos or Nos2). The antimicrobial effects of NO can be enhanced by reaction between NO and oxygen radicals, resulting in the formation of potent reactive nitrogen species (RNS) such as peroxynitrite. Activity of the iNos pathway can be visualized *in situ* in zebrafish larvae by

immunodetection of iNos itself, by using DAF-FM as a probe for direct measurement of NO levels, or by using an anti-nitrotyrosine antibody to measure nitrosylation of tyrosines, which provides a read-out for historical NO production (Lepiller *et al*., 2007; Clay *et al*., 2008; Hall *et al*., 2012; Elks *et al*., 2013). During *M. marinum* infection of zebrafish embryos, the iNos pathway is activated in a subset of infected macrophages, but a more prominent response was detected in bystander neutrophils (Clay *et al*., 2008; Elks *et al*., 2013). In uninfected embryos, neutrophils display basal levels of tyrosine nitrosylation due to the expression of myeloperoxidase, which is able to nitrosylate tyrosine residues in the absence of peroxynitrite. Within 1 day following *M. marinum* infection, neutrophil nitrosylation levels were shown to be significantly enhanced (Elks *et al*., 2013). A similar increase in neutrophil nitrosylation levels could be achieved in the absence of infection by overexpression of a dominant active form of the transcriptional regulator Hypoxia inducible factor alpha (Hif-1α). When embryos were subsequently infected with *M. marinum* they developed lower bacterial burdens than in the absence of stabilized Hif-1α. In agreement, pharmacological stabilization of Hif-1α by treating embryos with dimethyloxaloylglycine (DMOG) prior to infection also increased resistance to *M. marinum*. Genetic depletion of *inos* (*nos2a*) and the use of pharmacological inhibitors (L-NAME and L-NIL) blocked the protective effect of stabilized Hif-1α, showing that this transcription factor acts through an iNos-dependent mechanism. Neutrophil nitrosylation levels could also be enhanced by decreasing Hif-2α signalling (by overexpressing a dominant negative form) and this also increased resistance to *M. marinum* infection. These results demonstrate that Hif-1α and Hif-2α have opposing effects on host susceptibility to mycobacterial infection. Furthermore, these data indicate that modulating the Hif pathway primes neutrophils to bacterial challenge and that increased levels of RNS in neutrophils before infection aids the host in combatting *M. marinum*. An important remaining question is how neutrophils with activated iNos interact with macrophages, the main cell type containing internalized *M. marinum* (Elks *et al*., 2013). In addition to its direct antimicrobial function, iNos has also been implicated in the emergency haematopoiesis pathway that functions to expand the neutrophil population during infection of zebrafish larvae (Hall *et al*., 2012).

The emerging role of autophagy in innate immune defence

Immunity and metabolism are further connected through autophagy, a fundamental catabolic mechanism used to degrade unnecessary or dysfunctional cellular components. The recent recognition that autophagy also has diverse functions in the immune system created a paradigm shift in the immunology field (Deretic, 2011). The hallmark of autophagy is the decoration of double membrane autophagosome vesicles with the lipidated form of LC3, encoded by *MAP1LC31B*, the vertebrate orthologue of yeast *ATG8*. GFP-Lc3 transgenic zebrafish enable live imaging of autophagy (He *et al*., 2009) and were used to demonstrate the *in vivo* association between GFP-Lc3 and phagocytosed *M. marinum* and *Shigella flexneri* bacteria (Fig. 1C; van der Vaart *et al*., 2012; Mostowy *et al*., 2013). These studies indicate that defence mechanisms based on autophagy can be highly complex and in addition to autophagosomes with double membranes involves rapid shuttling of LC3 containing vesicles that target LC3 also to single membrane phagosomal compartments. The specific induction of an autophagy modulator (DNA damage-regulated autophagy modulator 1, *dram1*) during infection in zebrafish, downstream of TLR and TNF signalling, suggests an immunological function which we are currently investigating in our laboratory (Stockhammer *et al*., 2010; van der Vaart *et al*., 2013). Intracellular *S. flexneri* bacteria, which escape into the cytosol and spread by actin-based motility, are targeted to the autophagolysosomal pathway by cage-like structures of GTP-binding septin proteins, a process recently demonstrated *in vivo* using the zebrafish model (Mostowy *et al*., 2013). *M. marinum*, which can also form actin tails, could recruit septin cages and GFP-Lc3 similar to *S. flexneri* (Mostowy *et al*., 2013). Septin caging is known to require the function of the selective autophagy receptor p62 (sequestome 1, SQSTM1) (Mostowy *et al*., 2011). This receptor belongs to a new class of pattern recognition receptors named SLRs (sequestosome 1/p62-like receptors) by analogy with the TLRs, NLRs and RLRs (Deretic, 2011). SLRs link innate immunity to autophagy by specifically targeting ubiquitinated bacteria and microbicidal peptides to autophagosomes. Knock-down of p62 in zebrafish larvae significantly reduced septin caging and the ability of macrophages to control infection, indicating that p62-mediated autophagy plays an important role in host defence against *S. flexneri in vivo* (Mostowy *et al*., 2013). Stimulating this selective autophagy pathway represents a potential therapeutic strategy for infectious diseases caused by intracellular pathogens.

Conclusion

The use of zebrafish larvae for studying microbial infection has led to important new insights in host defence mechanisms that in several cases appear to be common for all vertebrates. In many instances, however, the results have still to be extended to studies in mammalian systems in order to show the translational value for biomedical applications. Nonetheless, considering the

conserved nature of the processes under study, such as the innate immune recognition processes, myeloid defence mechanisms, immunometabolism and the autophagic destruction machinery, we can predict that most findings described in this review will appear to be relevant for biomedical research. In this respect, also the knowledge obtained from defence mechanisms in non-vertebrate models, such as *Drosophila*, *C. elegans*, and the slime mould *Dictyostelium*, will be of great importance for comparative studies. The rapid increase of available high-throughput technologies in the zebrafish tool box (Lessman, 2011; Spaink *et al*., 2013) will enable us to apply new insights resulting from functional studies for knowledge-based drug screens and could lead to new approaches for rational drug design. In addition, new reporter lines that provide readouts for activation of the immune system are highly useful tools for *in vivo* visualization of immune responses to pathogens (Kanther and Rawls, 2010; Palha *et al*., 2013). Other needs to further advance the use of the zebrafish model are the availability of specific antibodies for distinguishing immune cell types and technologies for generating cell-specific and conditional knockout mutants.

Bottlenecks that still remain in the understanding of infectious diseases include the lack of knowledge on the first recognition steps of microbes by the host cells and the initial signalling events of communication between various cell types of tissues infected by microbes, including non-immune cells. That such communication is important is illustrated by work of the group of Ramakrishnan and co-workers, showing the requirement of *mmp9* activation in epithelial cells for recruitment of macrophages and granuloma formation during mycobacterial infection in zebrafish (Volkman *et al*., 2010). The observation that activation of *mmp9* in host epithelium is driven by a mycobacterial secreted protein is a good example of how microbial virulence factors can exploit interactions between host cell types to their own advantage (Volkman *et al*., 2010). It can be expected that a multitude of other factors important for host defence and targeted by pathogens still remain to be identified. Elucidating the functions of these factors in host–pathogen interactions will be facilitated by the unique possibilities of the zebrafish model for high-resolution *in vivo* imaging combined with forward and reverse genetics. Identification of the cellular producers and targets of the chemokines that control host responses to infection and elucidation of the mechanisms that pathogens can use to manipulate these and other signalling pathways of the innate immune system are important challenges where the zebrafish model can contribute. Furthermore, real-time imaging in zebrafish provides great opportunities to help understanding how macrophages and neutrophils interact and cooperate *in vivo* to combat pathogens.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Brief overview of mechanistic insights gained from studying bacterial pathogenesis and host defence mechanisms in zebrafish embryo and larval infection models.