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CellPress REVIEWS

Opinion

The Case for Modeling Human Infection in Zebrafish

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Zebrafish (*Danio rerio*) larvae are widely recognized for studying host–pathogen interactions *in vivo* because of their optical transparency, genetic manipulability, and translational potential. The development of the zebrafish immune system is well understood, thereby use of larvae enables investigation solely in the context of innate immunity. As a result, infection of zebrafish with natural fish pathogens including *Mycobacterium marinum* has significantly advanced our understanding of bacterial pathogenesis and vertebrate host defense. However, new work using a variety of human pathogens (bacterial, viral, and fungal) has illuminated the versatility of the zebrafish infection model, revealing unexpected and important concepts underlying infectious disease. We propose that this knowledge can inform studies in higher animal models and help to develop treatments to combat human infection.

Highlights

Mycobacterium marinum infection in zebrafish is an important system for studying natural host–pathogen interactions *in vivo*.

New work has shown that a wide variety of human pathogens can also be studied using a zebrafish infection model.

Zebrafish larvae are highly valuable in the discovery and dissection of fundamental concepts underlying human infection.

A better understanding of the biology of human pathogens *in vivo* will be necessary to develop new therapies to fight infection.

Why Use Zebrafish to Study Human Infection?

Zebrafish have been used for almost 30 years as a model to study developmental biology because larvae are optically accessible and develop rapidly [1]. Zebrafish are also genetically tractable, and enable investigation of innate immune responses in isolation from adaptive immunity – which does not develop fully until 4 weeks post-fertilization [2–4]. Despite anatomical differences between zebrafish and humans, zebrafish can be used to investigate human infection by injecting a corresponding site that best suits the research question (Figure 1A, Key Figure). As a result, zebrafish have become an important animal model to study host–pathogen interactions *in vivo*. *Mycobacterium marinum*, a natural pathogen of zebrafish that causes a tuberculosis-like disease, is a paradigm for investigating host–pathogen interactions *in vivo* and has significantly contributed to our understanding of human infection with *Mycobacterium tuberculosis* (reviewed in [5]). For example, *M. marinum* infection in zebrafish has shown that the virulence determinant RD1 (region of difference 1) induces the aggregation of infected macrophages to form granulomas characteristic of tuberculosis [6], that modulation of host tumor necrosis factor (TNF) levels by leukotriene A4 hydrolase (LTA4H) can have both protective and pathological roles [7,8] and that macrophage necrosis in granulomas depends on an inter-organelle signaling circuit induced by TNF [65].

Zebrafish share extensive genomic homology to humans, and >80% of human genes associated with diseases are present in zebrafish [4]. Importantly, counterparts of mammalian pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs), as well as downstream signaling components, have been demonstrated to play important roles in zebrafish host defense [9]. In this opinion article we examine recent literature showing how infection of zebrafish with human bacterial, viral, and fungal pathogens can be used to discover fundamental concepts underlying human infection (Figure 1).

Use of Zebrafish to Study Microbial Virulence Mechanisms *In Vivo*

Zebrafish larvae, unlike other vertebrate models, allow researchers to visualize the infection process *in vivo* from the single-cell to the whole-animal level, revealing unsuspected virulence mechanisms used by pathogens inside a human host. Bacteria belonging to the *Burkholderia cepacia* complex are important opportunistic pathogens of cystic fibrosis patients. Similarly to clinical cases in humans, zebrafish are highly susceptible to the *B. cepacia* complex, and different bacterial species can cause different infection outcomes ranging from persistent noninflammatory infection (nonfatal) to acute proinflammatory infection (fatal) [10,11]. By using zebrafish to study *Burkholderia cenocepacia* infection, it was observed for the first time that macrophages are crucial for promoting bacterial replication and acute proinflammatory infection [11]. The *B. cenocepacia* transcriptional regulator ShvR and its

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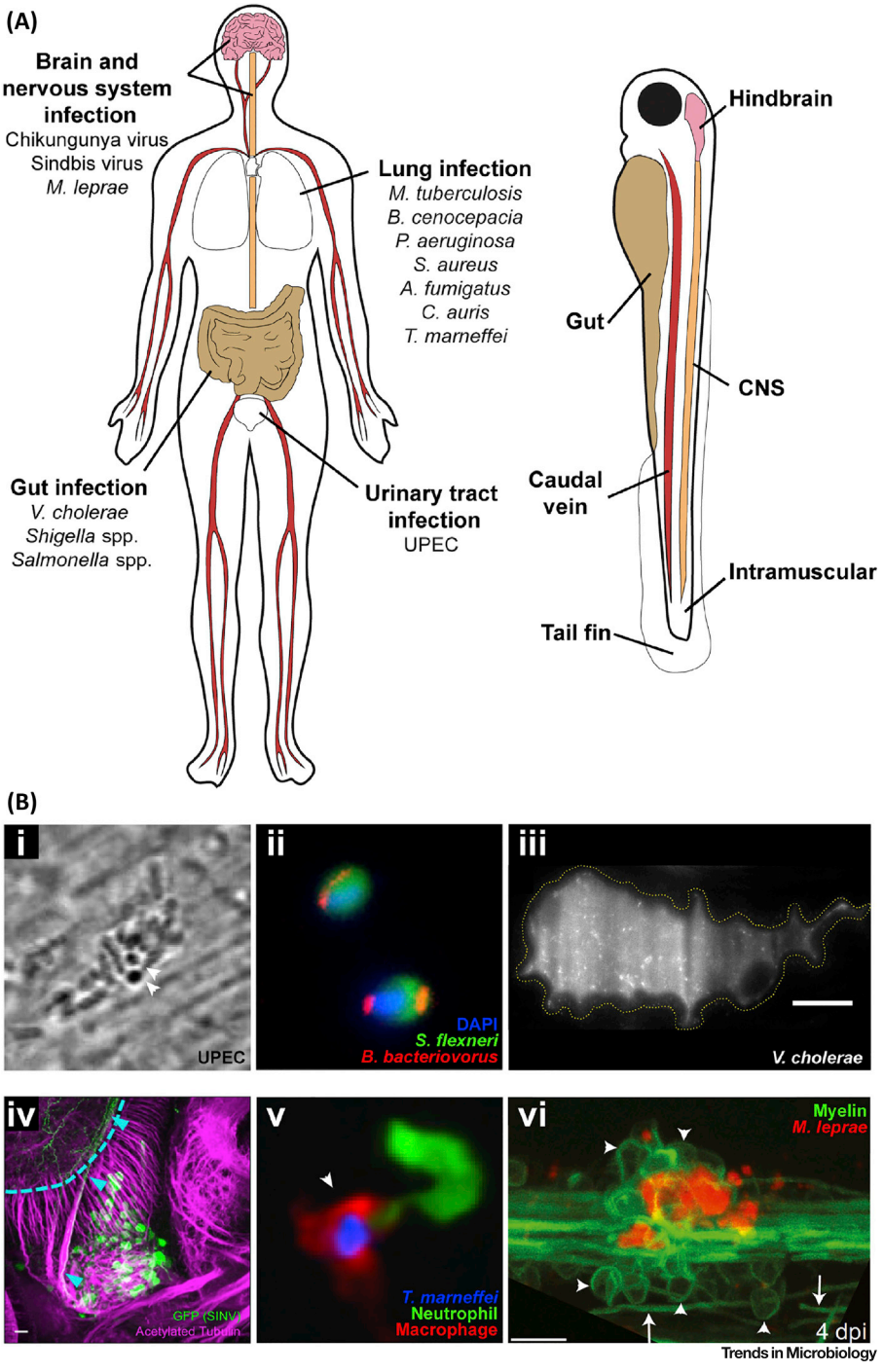
Key Figure

Human Infections Studied in Zebrafish



(See figure legend at the bottom of the next page.)

Glossary

Antimicrobial resistance (AMR): resistance developed or acquired by microorganisms to antimicrobial compounds.
Autophagy: a cellular process for the degradation of cytoplasmic constituents.
Bdelloplast: a hallmark of predation by predatory bacteria in which the prey cell becomes rounded.
Bdellovibrio bacteriovorus: deltaproteobacteria that prey upon Gram-negative bacteria by invading their periplasm.
Biofilm: a community of microorganisms living in a self-produced extracellular matrix.
Clemastine: an antihistamine drug that enhances inflammasome activation.
Commensal: nonpathogenic bacteria that are involved in a symbiotic interaction between microbe and host.
Conidia (plural of conidium): asexual fungal spore.
Cyclic diguanosine monophosphate (cyclic-di-GMP): a second messenger molecule involved in different signaling pathways that coordinates the transition between motility and sessility.
Emergency granulopoiesis: increased *de novo* production of neutrophils by stem cells in response to infection.
Epigenetic reprogramming: changes in gene expression and cell physiology at the epigenome level by adding or removing covalent modifications on chromatin.
Immunological bottleneck: generated by a subset of immune cells that provide a niche for bacteria to evade clearance.
Inflammasome: a complex that assembles after pathogen recognition by host cells and that activates inflammatory responses.
Innate lymphoid cells (ILCs): immune cells derived from the lymphoid lineage that reside in peripheral tissues and do not express antigen-specific receptors.
LC3-associated phagocytosis (LAP): noncanonical autophagy pathway in which LC3 decorates single-membrane vesicles.
L-form: state in which bacteria do not have a cell wall.

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primary target (the antifungal cluster, *afc*) are both important virulence factors implicated in the transition from intracellular persistence to acute proinflammatory infection *in vivo* [12].

Pseudomonas aeruginosa can also cause serious infections in cystic fibrosis patients. Several groups have used zebrafish to investigate *P. aeruginosa* virulence factors and how the bacteria evade the immune system. Although studies have used different bacterial strains, the type III secretion system (T3SS) and quorum sensing have consistently been found to be necessary for virulence in zebrafish [13,14], in agreement with results from other animal models (reviewed in [15]). *P. aeruginosa* is widely capable of forming extracellular **biofilms** (see Glossary); however, these bacteria can also reside in host phagocytes *in vivo* [16]. Consistent with this, work using zebrafish has revealed that MgtC (a magnesium transport ATPase) is important for bacterial survival in macrophages and for progression to acute infection [17]. The transition from chronic to acute infection is modulated by bacterial levels of the second messenger molecule, **cyclic diguanosine monophosphate (cyclic-di-GMP)**. WarA, a methyltransferase involved in the biogenesis of lipopolysaccharide (LPS) O-antigen, interacts with SadC, a diguanylate cyclase that regulates cyclic-di-GMP levels, to allow bacteria to escape detection by zebrafish host immune cells [18].

Staphylococcus aureus is a **commensal** microbe; however, it can cause serious systemic infections through colonization of nares or indwelling medical devices. Zebrafish have helped to understand the mechanisms used by *S. aureus* to evade the host innate immune system. Both macrophages and neutrophils are important to contain the growth of *S. aureus* [19], but some macrophages and neutrophils can provide an **immunological bottleneck**, protecting a subset of bacteria that escape host cell killing and cause disseminated infection [20,21]. Moreover, zebrafish infection has shown how interactions with human skin commensals can promote colonization by *S. aureus* [22]. In this case, peptidoglycan from commensal bacteria can enhance *S. aureus* pathogenicity by reducing the ability of phagocytes to produce reactive oxygen species.

Neutropenia: dramatic reduction in the number of neutrophils at the whole-organism level.
Neutrophil extracellular traps (NETs): a host defense mechanism in which neutrophils release decondensed DNA, histones, and antimicrobial proteins in response to pathogenic microbes.
Peristaltic movements: rhythmic involuntary contraction and relaxation of muscles in the digestive tract.
Persister cells: a subset of dormant bacterial cells that are resistant to antibiotics.
Prostaglandins: bioactive lipids that have both pro- and anti-inflammatory roles.
Septins: a cytoskeletal component that interacts with membranes to form nonpolar filaments and rings.
Shuttling: a mechanism by which innate immune cells transfer microorganisms in the absence of death of the donor cell.
Trained innate immunity: innate immune memory generated by exposure to a microbial stimulus that primes myeloid lineage cells to respond more efficiently to a secondary stimulus.

Figure 1. (A) (Left) We highlight four major sites of human infection that are being studied using the zebrafish: the brain and nervous system, lungs, intestine, and bladder. For each site we list microbial pathogens that cause infection. (Right) We highlight common routes of injection in the zebrafish model: the hindbrain, gut, central nervous system (CNS), caudal vein, tail muscle (intramuscular), and tail fin. Human infection can be replicated in zebrafish using a corresponding infection site that best suits the research question. (B) Striking examples of host–pathogen interactions observed using zebrafish. (i) The tail fin is the thinnest part of zebrafish and is highly suited to high-resolution microscopy. Uropathogenic *Escherichia coli* (UPEC) injected in the tail fin can switch to the L-form state (white arrowheads) in the presence of antibiotics. *In vivo* observation of L-form switching may help to understand recurrent urinary tract infections (adapted from [25]). (ii) The hindbrain ventricle is a compartmentalized infection site that enables visualization of predator–prey interactions *in vivo*. In the hindbrain ventricle, *Bdellovibrio bacteriovorus* (red) preys on antibiotic-resistant *Shigella flexneri* [green, and DAPI (4′,6-diamidino-2-phenylindole), blue] to form bdelloplast structures, and works together with the host immune system to efficiently clear its bacterial prey (adapted from [27]). (iii) The zebrafish gut is organized similarly to the human gut, and enables researchers to dissect interactions between colonizing pathogens and microbiota. When fed to zebrafish, *Vibrio cholerae* (white) colonizes the gut (yellow dotted line) and expresses type VI secretion system (T6SS) effectors to modulate intestinal movements and enhance colonization efficiency (adapted from [28]). (iv) Caudal vein injections mimic systemic infections, and can be used to investigate which tissues and/or cell types are preferentially infected by microbial pathogens. When injected intravenously, Sindbis virus (green) infects the CNS by axonal transport. Once in the CNS, viruses infect cells in trigeminal ganglion and axons (magenta) in the optic tectum (blue arrowheads indicate infected axons; the dotted line indicates an axon-rich region) (adapted from [32]). (v) The tail muscle is a flat surface (ideal for microscopy) used to study immune cell recruitment. When injected intramuscularly, *Talaromyces marneffei* conidia (blue) is phagocytosed by neutrophils (green). Conidial β-glucan drives shuttling to macrophages (red) and promotes the dissemination of infection (the white arrowhead indicates the occurrence of intercellular transfer) (adapted from [38]). (vi) The zebrafish CNS can be used to analyze interactions of pathogens and/or immune cells with nerves. When injected into the CNS, macrophages infected with *Mycobacterium leprae* (red) cause dissociation of myelinated axons (green). The white arrows indicate intact myelin sheaths and the white arrowheads indicate myelin protrusions. This unexpected role for macrophages may be an important therapeutic target against leprosy in humans (adapted from [40]). Abbreviation: dpi, days post-infection.

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Collectively, infection of zebrafish with *B. cenocepacia*, *P. aeruginosa*, and *S. aureus* has helped to reveal virulence mechanisms, as well as the dichotomous (i.e., both pro- and antibacterial) role of innate immune cells that underlies host–pathogen interactions. Considering the striking parallels between zebrafish and human innate immune cells, it will be of interest to counteract these virulence mechanisms during human infection.

Use of Zebrafish to Study Bacterial Cell Biology *In Vivo*

The emergence of **antimicrobial-resistant (AMR)** bacteria has received great attention and has been identified by the World Health Organization as a top priority [23]. In addition to antibiotic resistance, bacteria have developed a multitude of strategies – such as **persister cells** and **L-form** switching – that also contribute to recurrent infections. L-form switching in the presence of antibiotics has been observed for a wide variety of bacteria *in vitro*, as well as in samples from cultivated macrophages, *Galleria mellonella*, and humans [24]. However, it is crucial to assess when switching occurs *in vivo*, for example, by uropathogenic *Escherichia coli* (UPEC) in patients suffering from recurrent urinary tract infections [25]. Benefiting from exciting advances in high-resolution microscopy and the optical accessibility of zebrafish larvae, researchers have followed bacterial cell biology *in vivo* during whole-animal infection. In this case, zebrafish were used to demonstrate that UPEC can rapidly switch to L-forms *in vivo* when fosfomycin is administered (Figure 1B). Strikingly, injected L-forms can survive as nonwalled cells *in vivo*, and return to walled cells when antibiotics are removed.

Bacterial evolution, together with the overuse of antibiotics, has led to a gripping race between antibiotic resistance and drug development. However, bacteria cannot evolve resistance to ***Bdellovibrio bacteriovorus***, a nonpathogenic predatory bacterium that is being used as an alternative to antibiotics (reviewed in [26]). We have shown in zebrafish that *B. bacteriovorus* can prey on antibiotic-resistant *Shigella flexneri*, a Gram-negative enteropathogen, working in synergy with the host immune system to control infection [27]. High-resolution microscopy confirmed the *in vivo* formation of stable **bdelloplast** structures – rounded *S. flexneri* cells – following invasion by *B. bacteriovorus* (Figure 1B). Importantly, the use of *B. bacteriovorus* is safe for the host, even if the host is immunocompromised. Although further tests will be necessary to understand the full potential of *B. bacteriovorus* for human therapy, this study revealed a new approach to control infection by AMR Gram-negative bacteria.

Similarly to the investigation of predator–prey interactions, work using zebrafish has shown that bacterial competition can also be studied *in vivo*. A study using light-sheet fluorescence microscopy has demonstrated that *Vibrio cholerae*, a noninvasive intestinal pathogen, competes with other bacteria to colonize the gut in a type VI secretion system (T6SS)-dependent manner (Figure 1B) [28]. Interaction of *V. cholerae* with epithelial cells affects **peristaltic movements** of the zebrafish gut, displacing microbiota and promoting its own colonization. Similarly to the type VII secretion system (T7SS) in mycobacteria that plays a role in pathogenesis, *S. aureus* has a T7SS that secretes effectors important for bacterial competition [29]. From proteomic analysis of the T7SS secretome, a novel toxin named TspA (Type Seven-dependent Protein A) was discovered [30]. Although *in vitro* experiments failed to demonstrate a role for TspA, bacterial competition assays performed *in vivo* using zebrafish showed that TspA is important for intraspecies competition. Therefore, at least for *S. aureus*, zebrafish can be used to illuminate host factors that promote bacterial competition.

Use of Zebrafish to Study Emerging and Neglected Infectious Diseases

To understand emerging and neglected infectious diseases, researchers need well-characterized animal models that recapitulate key aspects of the infection process. Zebrafish can efficiently uncover so far unknown determinants that are central to infection by microbial pathogens. Infections with alphaviruses, such as Chikungunya and Sindbis virus, cause viral encephalopathies; however, the mechanisms by which these viruses enter the central nervous system (CNS) are poorly understood. Systemic viral infection in zebrafish has shown that Chikungunya and Sindbis viruses rapidly settle in the brain parenchyma, where they persist [31,32]. Whereas Chikungunya virus infects endothelial cells of the blood–brain barrier to reach the CNS, Sindbis virus infects peripheral nerve termini and propagates via axonal transport (Figure 1B) [32]. Together, these studies describe the entry pathways of different

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alphaviruses into the CNS that have been difficult to characterize using other animal models. It will be interesting to study Zika virus infection in zebrafish, and to unravel mechanisms underlying microencephaly linked to this re-emerging disease threat [33].

The wealth of zebrafish transgenic lines that express fluorescent proteins specifically in phagocytes has uniquely enabled researchers to study their dynamic interaction with invasive fungal pathogens (e.g., *Aspergillus*, *Candida*, and *Cryptococcus* spp.) (reviewed in [34]). In the case of *Aspergillus fumigatus*, an airborne opportunistic fungal pathogen, zebrafish have characterized different host responses against slow- and fast-germinating strains [35]. For slow-germinating strains, macrophages provide a protective niche and contribute to fungal persistence. By contrast, for fast-germinating strains, inflammatory activation of macrophages through MyD88-dependent signaling is triggered to clear **conidia**. Very recent work using zebrafish has focused on the emerging fungal pathogens *Candida auris* (a severe threat to hospital patients globally) and *Talaromyces marneffei* (an important opportunistic pathogen in HIV patients). In contrast to other *Candida* species, infection by *C. auris* does not cause **neutropenia** *in vivo* because **neutrophil extracellular traps (NETs)** are not produced to counteract infection [36]. During *T. marneffei* infection, conidia are phagocytosed by macrophages which provide a protective niche, whereas neutrophils have strong fungicidal activity dependent on myeloperoxidase [37]. Interestingly, β-glucan from *T. marneffei* cell walls can promote fungal dissemination by host cell **shuttling** (Figure 1B) [38]. Although shuttling involves the transfer of neutrophil phagosomes containing conidia to macrophages (i.e., from an acidic environment in neutrophils to a more acidic environment in macrophages), it does not involve killing the donor neutrophil.

A variety of mycobacteria have been studied using zebrafish, including *Mycobacterium leprae* (the causative agent of leprosy in humans) for which the only *in vivo* models previously used were the mouse foot pad and armadillo. Although working with *M. leprae* in zebrafish has some limitations (e.g., the long doubling time of *M. leprae*, which therefore cannot be studied within the timeframe of larval development [39]), it is possible to visualize macrophage–pathogen interactions and study the early stages of demyelination and axonal damage characteristic of leprosy in humans [40]. Work has shown that the macrophage response to *M. leprae* phenolic glycolipid-1, and not the direct interaction of bacteria with myelinating glia, induces demyelination of oligodendrocytes in the CNS (Figure 1B) [40,41]. During human infection, *M. leprae* is known to invade Schwann cells which are responsible for myelination of nerves in the peripheral nervous system, suggesting that the precise neuropathy of human leprosy is not fully replicated in zebrafish larvae [42]. However, this newly found role for macrophages during *M. leprae* infection, as discovered using zebrafish, may be an important therapeutic target to counteract pathology underlying human disease.

Use of Zebrafish to Discover Innovative Concepts in Host Defense

Since the pioneering work of Philippe Herbomel and colleagues, who described the development of macrophages and neutrophils, as well as their ability to phagocytose pathogens in the zebrafish embryo [43,44], these cells have been the subject of intense investigation during bacterial infection of zebrafish. However, the breadth of immune cells available for zebrafish host defense is not yet fully known. A study using single-cell RNA sequencing in adult zebrafish, and immune challenge using bacterial (*Vibrio anguillarum*) or parasitic (*Anisakis simplex*) stimuli, discovered cells that resemble **innate lymphoid cells (ILCs)** which previously had only been studied in mammals [45]. In zebrafish it was found that only ~10% of these ILC-like cells express cytokine receptors, making them different from mouse and human ILCs (where 100% of cells express cytokine receptors). These results indicate that zebrafish may be useful for discovery of therapies using ILCs, and highlight there is much still to discover about the zebrafish immune system.

Autophagy is an intracellular degradation process that is crucial for cellular homeostasis and host defense. The zebrafish was among the first animal models employed to study bacterial autophagy *in vivo*, and showed that p62 (a selective autophagy receptor that interacts with ubiquitin) controls *S. flexneri* and *M. marinum in vivo* [46–48]. For *Salmonella* Typhimurium infection, bacterial association with LC3 (a key autophagy marker) is observed in phagosomes and other intracellular vesicles

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[49]. Furthermore, association with LC3 is independent of ATG13, but is dependent on Rubicon and NADPH, which suggests that *S.* Typhimurium replication in macrophages is restricted by **LC3-associated phagocytosis (LAP)** [49]. For *S. aureus* infection, p62-mediated autophagy and LAP have opposing roles in neutrophils [50,51] because p62-selective autophagy promotes bacterial clearance, whereas LAP helps bacteria to establish an intracellular niche.

The **inflammasome** is a multiprotein complex that assembles after pathogen recognition by host cells. Although mice have been widely used to study inflammasome biology, zebrafish can offer an evolutionary perspective on components and concepts that are highly conserved from fish to human. In the *Shigella*-zebrafish infection model, it was discovered that **septins**, a poorly understood component of the cytoskeleton, control inflammation and caspase-1 activity [52]. However, the precise role of the septin cytoskeleton in this process awaits investigation. Work using *S.* Typhimurium revealed that bacteria are eliminated in neutrophils through guanylate-binding protein 4 (GBP4) inflammasome-dependent production of **prostaglandins** [53]. In macrophages, an evolutionarily conserved protein named Caiap (CARD- and ANK-containing inflammasome adaptor protein) is necessary to activate caspase-1 and control *S.* Typhimurium infection [54]. Gram-negative bacteria can also induce noncanonical inflammasome activation, where oligomerization of NLRP3 (NOD-, LRR-, and pyrin domain-containing 3) is initiated following the activation of caspase-11 instead of caspase-1 (reviewed in [55]). In zebrafish, Caspb mediates noncanonical inflammasome activation activated through a pyrin-like domain that recognizes Gram-negative LPS [56]. During *M. marinum* infection, activation of the inflammasome occurs through Caspa and Caspb pathways: whereas activation of the Caspa pathway enhances bacterial dissemination and drives granuloma expansion, activation of the Caspb pathway enhances host defense [57]. In view of these studies, as well as the use of **clemastine** as an antimycobacterial agent in zebrafish [58], the inflammasome is clearly an important target for host-directed therapies to combat infection.

The concept of **trained innate immunity** is based on the development by innate immune cells of 'memory' for pathogens after infection via **epigenetic reprogramming**. Work in mice has shown that infection with *Candida albicans*, or vaccination with bacillus Calmette–Guérin (BCG), can protect against secondary infections in a macrophage-dependent and T cell-independent manner (reviewed in [59]). BCG invasion of bone marrow induces expansion of the hematopoietic stem cell niche, and these cells differentiate into trained macrophages that are highly efficient at controlling *M. tuberculosis* [60]. In mammalian models, innate immunity can be studied in isolation by repressing adaptive immunity; however, in zebrafish larvae there is no need to do so. The first studies to suggest that trained innate immunity can be studied using zebrafish larvae showed that, upon infection with *S.* Typhimurium or *S. flexneri*, the hematopoietic stem cell niche expands and **emergency granulopoiesis** is induced [61,62]. Neutrophil production after primary infection with *S. flexneri* protects against secondary infection with *S. flexneri* [62]. By contrast, primary infection with Sindbis virus renders larvae highly susceptible to a secondary infection with *S. flexneri* [63]. It is thus of great interest to use zebrafish to illuminate epigenetic changes underlying host defense, and to direct the investigation of epigenetic changes underlying trained innate immunity in humans.

Concluding Remarks and Future Perspectives

In this opinion article we highlight the innovative use of zebrafish infection to enhance our understanding of human infectious disease (Table S1 in the supplemental information online). The future is promising for microbiologists studying zebrafish infection. Cutting-edge microscopy techniques, including fluorescent probe technology and super-resolution microscopy, are highly suitable for application in zebrafish. Moreover, embryos can be obtained in large numbers, thereby enabling high-throughput investigation. As a result, zebrafish larvae have been at the forefront of cell biology *in vivo*, elegantly exemplified by a recent study using automated high-throughput investigation combined with lattice light-sheet microscopy to show organelle (endoplasmic reticulum, mitochondria) dynamics at the single-cell level [64]. Although datasets obtained using zebrafish and novel microscopy techniques will be large and complex, efficient data-handling systems are concurrently being developed. Together with advances in RNA sequencing and other 'omic' approaches, we predict that

Outstanding Questions

How crucial is it to precisely mimic 'natural' host–pathogen conditions during zebrafish infection? Can zebrafish help to define what it means to be a human pathogen?

What are the barriers that may prevent a human pathogen from infecting a zebrafish? Can zebrafish illuminate the breadth of microbial virulence factors and host response pathways triggered during human infection?

Epigenetic modifications underlie different mechanisms of human disease and offer new avenues for medical treatment. Can epigenetic modifications in humans be predicted from investigation using the zebrafish infection model?

How can zebrafish infection studies be used to efficiently inform future experiments using higher vertebrate animal models? Can data obtained from zebrafish infection be directly translated to human therapies?

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microbiologists employing these novel approaches and large datasets to study zebrafish infection can provide profound insights into human infection.

In closing, the full potential of the zebrafish model to study infectious disease has yet to be realized (see Outstanding Questions). Zebrafish are not intended to replace other vertebrate models such as mice, but instead can reveal fundamental concepts of microbial pathogenesis and host defense, and in this way help to develop innovative therapies to combat human infections.

SUPPLEMENTAL INFORMATION

Supplemental information associated with this article can be found online at https://doi.org/10.1016/j.tim.2019.08.005.

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