Review Paper

Evolution of the Zebrafish Model: From Development to Immunity and Infectious Disease

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ABSTRACT

The successful zebrafish developmental model has now expanded to being used as a model for the analysis of host–pathogen interactions during infectious disease. Numerous pathogens have been demonstrated to infect zebrafish and new mechanisms of virulence, as well as host defense have been uncovered using this new model. In this review we summarize the literature on how the zebrafish infectious disease model is being used to decipher virulence mechanisms used by various pathogens and the host defense mechanisms initiated to combat infection.

INTRODUCTION

The best strategies to stop the progression of infectious disease will come from having a complete understanding of the virulence mechanisms used by pathogens during an infection. However, a microbial infection is made up of a complicated series of dynamic interactions between the pathogen and its host, with each interaction influencing the progression and ultimate outcome of the disease process. As the host reacts to the presence of the pathogen by initiating defense mechanisms, the microbe in turn must respond to survive the attack. A fully responsive host is necessary to analyze the changing host environment, as well as accurately study how a pathogen responds to that changing environment. Therefore, in vivo identification of virulence regulation and specific host–pathogen interactions is fundamental to the study of pathogenesis and one of the major challenges for microbial pathogenesis research.

One of the most informative methods used to study host–pathogen interactions is through animal model systems in which the responses of both the host and the pathogen can be analyzed. The availability of complete or partial genomes of numerous organisms has highlighted the evolutionary conservation of defense mechanisms used against microbial predators. New nonvertebrate model systems developed to study human disease have proven quite informative. Recently developed model host systems have been successful using plants (Arabidopsis thaliana) and nematodes (Caenorhabditis elegans) for the study of pathogenesis. Arabidopsis and C. elegans have both been used to identify numerous virulence genes in the human pathogen Pseudomonas aeruginosa.1–4 When these mutants were subsequently analyzed in a murine model of infection, a high percentage were avirulent or reduced in virulence, supporting the utility of using a nonvertebrate model for the study of human infectious disease.5,6 These nonverte-
brate and plant models were a distinct advantage over larger mammalian systems because of the ability to easily genetically manipulate the host.1–3,7 Another model using the soil ameba Dictyostelium discoideum has been developed to study the intracellular survival mechanisms of Legionella species8 as well as the virulence mechanisms of the opportunistic human pathogen P. aeruginosa.9 Additionally, the nonvertebrate host model, Drosophila melanogaster, was used to identify the Toll family of receptors, one of the origins of host defense that is shared across a diverse range of species.10–12 The wealth of information available on Drosophila has been exploited to analyze infectious disease caused by both Mycobacterium marinum13 and Listeria monocytogenes.14

**ZEBRAFISH AS AN INFECTION MODEL**

Recently, a new vertebrate animal model for the study of host–pathogen interactions during infection was developed using the zebrafish (Danio rerio).15,16 Successful infection of zebrafish has been demonstrated using a variety of pathogens including the zoonotic fish pathogens Mycobacterium marinum,15,17–20 Edwardsiella tarda,21,22 Salmonella arizonae,15 Vibrio anguillarum,23 and Streptococcus iniae (Fig. 1).16,24 Furthermore, infection of zebrafish has been successfully demonstrated with pathogens that are not known to infect fish such as Salmonella typhimurium,25 Bacillus subtilis,26 Escherichia coli,26 multiple species of Listeria,27 Streptococcus agalactiae (M.N. Neely and D.R. Runft, unpublished data) and Streptococcus pyogenes (Fig. 1).16,24,28 Snakehead rhabdovirus, a virus that infects warm-water fish, was also used for zebrafish infection to analyze upregulation of multiple host proteins involved in the immune response.21,29 Two additional viruses, infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV), were analyzed in the zebrafish for potential use as vectors for gene transfer experiments in hematopoietic cells.30 Also adding to our knowledge of infection processes in zebrafish, a novel truncated form of fibronectin (FN2) expressed on zebrafish cells was identified by Zhao et al.,31 and was subsequently found to cause cells to be more susceptible to IHNV infection, possibly by mediating virus attachment.32

Even though several other animal models are already established, there are multiple advantages to using the zebrafish as an infectious disease model, some of which include low cost, easy maintenance, requirement of minimal laboratory space, and easy handling. The adult zebrafish size is particularly useful for monitoring disease progression because a single longitudinal section of the whole animal can be mounted on one slide for histologic analysis, instead of only a small tissue sample, as is the case with most mammalian models. For the study of vertebrate development, the growth of zebrafish embryos ex vivo has allowed analysis of mutations that would be fatal during fetal development and therefore not easily analyzed in the mouse model system. As an infectious disease model this attribute is also advantageous, because infections can be done in a step-wise fashion as immune defenses arise in the developing embryo to answer specific questions about the development and function of defense mechanisms. Another significant advantage of using the zebrafish embryos is their transparency for the first 3 weeks of life. This allows the observation of fluorescently labeled bacteria in real time inside the developing embryo. This scenario was used quite successfully to examine early host responses to infection with M. marinum.15,20 Using this technique, Davis et al.15 was able to observe two previously unrecognized macrophage-mediated mechanisms that may play a role in bacterial dissemination during infection. One mechanism was the direct transfer of bacteria between two macrophages by pseudopodial membrane tethers. A second mechanism was the phagocytosis of dead infected macrophages by uninfected macrophages, which could lead to dissemination of bacteria within the granuloma.15

Similarly, van der Sar et al.25 used DS-Red labeled S. typhimurium to analyze infection in real time, taking advantage of the transparency of the embryo to visualize infection in the same embryo over time. Injection of a very low dose (approximately 50 cells) of the pathogen into the caudal vein was lethal for the embryos in
30–48 hours postinfection (hpi), while strains with mutations in lipopolysaccharide synthesis genes (LPS) were attenuated for virulence. One important finding from this study was that at later time points of infection, only approximately 20–35% of *Salmonella* were still intracellular with the majority being extracellular in microcolonies, shedding light on a long-standing query in *Salmonella* pathogenesis as to the primary site of bacterial multiplication in vivo. An unusual phenomenon was also observed when the avirulent mutant was injected into the yolk sac, as opposed to the caudal vein. The avirulent mutant was able to proliferate in the yolk sac for 2 days and was then able to disseminate into the embryo. After dissemination of the bacteria, the embryo was killed rapidly. Therefore, if allowed to initially proliferate in the absence of macrophages, the avirulent mutant can kill. The authors suggest that injection into the yolk sac could be used as an in vivo growth control for mutants.25

One of the most important advantages of the zebrafish infectious disease model is the similarity of the zebrafish immune system to that of humans and other vertebrates (see below).33 This is where the zebrafish model surpasses some of the other newly developed models for host–pathogen analysis. While *C. elegans* and *D. melanogaster* do have many aspects of the innate immune system, they lack the components of the adaptive immune system. The zebrafish, on the other hand, has both innate and adaptive immune functions, allowing study of diseases that involve both systems. One infectious disease study capitalized on the delayed development of the adaptive immune system by infecting zebrafish embryos with *M. marinum* at early time points after fertilization. At the time of infection, only the innate immune system had developed, which permitted the researchers to study the contribution of innate immunity alone to granuloma formation.20 The chronically infected embryos were then allowed to develop into adults to study the contribution of the adaptive system at later stages of disease.20

The zebrafish model has also been used to analyze bacterial colonization and disease progression of pathogens that are significant problems in both wild and farmed fish populations. *V. anguillarum* is a gram-negative water-borne pathogen that causes hemorrhagic septicaemia and vibriosis in multiple fish species.34 Using green fluorescent protein (GFP)-labeled *V. anguillarum* and exploiting the transparency of zebrafish larvae, O’Toole et al.23 were able to visualize colonization of the fish in real time. This experiment allowed them to address the question of the mode and site of pathogen entry by observing the fluorescently labeled bacterium in the mouth and gastrointestinal tract of the transparent larvae after 2 hours of exposure and later in the intestine after 6 hours of exposure. Motility was also demonstrated to be a requirement for colonization of the skin of the fish, as a nonmotile mutant successfully colonized the intestine but could not colonize the skin of the zebrafish.23

Another pathogen that causes major problems in aquaculture is *S. iniae*, a gram-positive zoonotic pathogen of both fish and humans. In fish populations, it can cause systemic disease in more than two dozen different species in both fresh and salt water environments, potentially resulting in bacteremia, panophthalmitis and meningitis.35,36 Using adult zebrafish, Neely et al.16 were able to replicate a systemic infection, showing dissemination of bacteria...
from the site of infection to all major organs including the brain, within 22–26 hours. Histologic examination illustrated that infection pathologies closely mimicked that observed in fish infected in aquaculture. Examination of the entire infected fish on a single slide revealed intracellular streptococci in hepatocytes of the liver, a phenomenon involved in the pathogenesis of another Gram-positive systemic pathogen, *Listeria monocytogenes*, but not previously shown for a streptococcal systemic pathogen. Although infection of zebrafish by bacterial pathogens has been successfully demonstrated, can we take what we learn from these analyses and apply it to human infectious diseases? There is great diversity in the way in which pathogens cause disease and, just as many pathogens have developed different ways in which to survive in a host, many hosts respond to pathogens differently. Although mice have been the host model of choice for many years, and have provided us with much valuable information on virulence mechanisms, not all pathogens cause the same type of disease in mice that they do in a human infection. In these situations mice are not providing the analytical tools needed to dissect specific virulence mechanisms. Several studies have demonstrated that not only do the same clinical features of disease occur in experimental infections of mouse and zebrafish, but also in some cases, the zebrafish actually proves to be a better model for mimicking human disease characteristics. One example of such a case is infection of zebrafish by *M. marinum*. *M. marinum* is the closest genetic relative of *M. tuberculosis*, the causative agent of human tuberculosis, and is used as a model to study fundamental *Mycobacterium* pathogenesis. In zebrafish infections, *M. marinum* causes the formation of granulomas much like those found from tuberculous infection in humans caused by *M. tuberculosis* infection. However, the mouse model of tuberculosis (TB) follows a different progression than in humans, causing progressively coalescing, noncaseating lesions illustrating the advantage of using the zebrafish for analyses of *Mycobacterium* granuloma formation.

Similarly, *S. iniae* causes a systemic infection leading to multiple organ dissemination as well as invasion of the brain and cerebral spinal fluid much like its close genetic relative, the human pathogen, *S. agalactiae*. Animal models for the analysis of meningitis are often very artificial, requiring direct injection into the cerebral spinal fluid or blood stream for access to the brain instead of following a natural route of infection. However, *S. iniae* can be isolated from the zebrafish brain in less than 24 hours from an intramuscular injection into the dorsal muscle or from short-term exposure to the pathogen after dermal abrasion. This model allows the analysis of host–pathogen interactions required for invasion of the brain from a more natural route of infection. One of the advantages of using natural fish pathogens in the zebrafish model, such as *M. marinum* and *S. iniae*, is that over time the host and bacterium have coevolved, with the pathogen adapting to life within their natural hosts, much like human-specific pathogens have adapted to the human host niche. Diseases caused by the fish pathogen often share the same clinical hallmarks of those found in humans from the human-specific pathogen counterpart.

Human-specific pathogens, such as *S. pyogenes*, have also been used in the zebrafish infection model resulting in a fatal infection. While *S. iniae* causes a systemic infection with a large inflammatory response, *S. pyogenes* causes a localized necrotic infection resulting in large areas of necrosis but relatively little to no inflammation (Fig. 2). The surprising absence of inflammatory cells in the midst of large aggregates of bacteria has also been reported for a necrotizing myositis infection caused by *S. pyogenes* in an experimental baboon model. Moreover, a recent report of necrotizing fasciitis in human patients caused by an M14 strain of *S. pyogenes*, the same M type used for the zebrafish infections, found a marked absence of neutrophil infiltration in the necrotic tissue in the presence of large amounts of bacteria. These results demonstrate that the infection of zebrafish by *S. pyogenes* can be used to analyze critical steps in invasive streptococcal infections. Although not all human pathogens will cause a similar disease in the zebrafish, the above examples highlight the
utility of using the zebrafish model to study infection mechanisms relevant to human disease.

Now that many bacterial pathogens have been successfully demonstrated to infect the zebrafish, the truly exciting research will be that which uses the model to ask specific questions about virulence mechanisms. Several studies have used *M. marinum* in the zebrafish model to address specific questions relating to *Mycobacterial* pathogenesis. Two different studies looked at a similar locus, called RD1 for “region of difference 1,” which is approximately 10-kb region conserved in the genome of virulent *M. tuberculosis* but missing from the BCG vaccine strains that are attenuated in the mouse model of TB.\textsuperscript{44,45} Volkman et al.\textsuperscript{20} used zebrafish embryos to look at *M. marinum* mutants that have the entire RD1 locus deleted (ΔRD1). While wild-type formed aggregates within 3–5 days postinfection (dpi), the ΔRD1 mutant bacteria formed only small, transient aggregates and at a much later time point. By injecting fluorescently labeled *Mycobacteria* directly into the hindbrain ventricle, a site devoid of macrophages, they demonstrated that the ΔRD1 mutant recruited macrophages as efficiently as wild type and that subsequent migration of infected macrophages out of the ventricle was also not impaired. The next group of experiments very cleverly exploited the optical

![Histopathologic examination of zebrafish dorsal muscle tissue after injection of streptococci.](image)

**FIG. 2.** Histopathologic examination of zebrafish dorsal muscle tissue after injection of streptococci. (A) and (B), 40 hours postintramuscular infection by *Streptococcus iniae* at a dose of $10^3$ cfu. (C) and (D) 40 hours postintramuscular infection by *S. pyogenes* at a dose of $10^5$ cfu. (A) Arrowheads outline a large region of necrosis and square indicates region of magnification in (B) (40× magnification); (B) numerous host inflammatory cells as well as cocci can be visualized in a highly necrotic region (1000× magnification); (C) arrowheads demarcate areas of necrosis and square shows region of magnification in D (40× magnification); (D) Arrow points to a large aggregate of streptococci dissecting along a facial plane (520× magnification).
transparency of the embryos and the ability to fluorescently label the *Mycobacterium* with different fluorophores. Using red-labeled wild-type bacteria and green-labeled ΔRD1 mutant bacteria, they were able to do superinfection experiments to determine the influence of each strain on specific steps of infection. Using this technique they were able to determine that the ΔRD1 infected macrophages actually could be recruited into aggregates if at least a few wild-type infected macrophages were present to initiate a chemotactic gradient to stimulate aggregate formation. Additionally, the RD1 locus was determined to be important for promoting cell death within the aggregates, which in turn leads to intercellular bacterial spread and increased bacterial load. By observing *Mycobacterial* infection of zebrafish embryos in real time the authors were able to discover that granuloma formation is actually a process that is promoted by the pathogen and leads to increased virulence. Also using zebrafish embryos, Gao et al. studied a locus adjacent to RD1, called RD1ext. Their data demonstrated that like the ΔRD1 mutants, when genes in the RD1ext region were disrupted, the resulting mutants were severely attenuated for dissemination and may also be important for growth *in vivo*.

The *M. marinum*–zebrafish model was also used to determine whether the origin of the clinical isolate directly related to its virulence profile. Results from this infection study revealed that the human isolates, from “fish tank granuloma” skin lesions, caused an acute disease in the zebrafish while the isolates from infected fish caused a chronic disease. Using DNA fingerprinting by AFLP, the researchers examined 17 different *M. marinum* strains isolated from various sources. The strains were genetically grouped into two AFLP clusters, with human isolates mainly sorted into one cluster and the animal isolates grouped into a separate cluster. The authors propose that even though humans become infected with *M. marinum* through handling fish, human isolates differ genetically from those that only infect fish. Further studies using the zebrafish model will allow the determination of virulence factors required for survival and persistence in either fish or humans or both.

Given the ease of use and inexpensive cost and maintenance of the zebrafish, another great advantage is using this model for large-scale virulence screens. A recent report documented the use of the zebrafish model in a large-scale screen of streptococcal mutants. Signature-tagged mutagenesis was used to produce a large library of specifically tagged mutants of the virulent *S. iniae* pathogen. In this assay a mixed inoculum of 12 mutants was used to infect individual zebrafish. Screening in this manner greatly increased the number of mutants that could be screened through fewer animals. Isolation and analysis of the hearts and brains of infected adult zebrafish 24 hours after infection identified mutants that could not cause a systemic infection. Further analysis of individual mutants proved the technique to be quite sensitive, as virulence attenuation of the mutants compared to wild type ranged from only 2 fold to over 1000 fold as measured by competitive assay.

**ZEBRAFISH AS A HOST DEFENSE MODEL**

The success of the zebrafish as an effective animal model for the study of vertebrate genomics and development has led to the investigation of the zebrafish immune system. As mentioned earlier, a significant advantage of this model, in terms of relating research to human disease, is that the zebrafish has a fully developed immune system with both innate and adaptive immune responses. This feature of the model provides a means of examining not only the complicated host response but also a pathogen’s ability to counter the innate and adaptive immune defenses. Understanding the zebrafish immune response allows for a full appreciation of the intricate and dynamic interplay between the host and pathogen. The advances that have been made in the study of teleost immunity demonstrate that the zebrafish is an appropriate and highly valuable model for the study of infectious disease. Together immunologic and infectious disease studies provide information that is relevant to the understanding of host–pathogen interactions in the zebrafish that directly relates to human disease.
Innate immunity is present in all multicellular organisms in some form and provides a defense against invasion of a variety of pathogens. The mechanisms of recognition of these pathogens seem to be evolutionarily conserved from invertebrates to humans. Specific structures on pathogens called pathogen-associated molecular patterns (PAMPs) are recognized by receptors on innate immune cells.47–49 These receptors are referred to as pattern recognition molecules.50 The primary function of the innate immune system is to eliminate invading pathogens; however, in vertebrates possessing adaptive immune systems, the innate immune system has an additional purpose of serving as a trigger for the initiation of the adaptive arm of the immune system. The most significant consequences of this initiation are the upregulation of proinflammatory cytokines and modulation of an antigen-specific immune response. Such a response is crucial to combat an invading pathogen unsuccessfully cleared by the early innate defenses.

While the adaptive immune response is a vital part of the host immune system, the early events in the innate immune response are equally significant and deserve attention. The antigen presenting cells and effector cells of the innate immune system are the first factors to come into contact with and recognize an invading pathogen. Although professional antigen presenting cells, or dendritic cells, have not yet been identified in zebrafish, dendritic cells have been suggested to be present in rainbow trout.51 Zebrafish and other teleosts do have antigen-presenting cells, including monocytes and tissue macrophages, which are very similar to mammalian monocytes and macrophages in both appearance and function. The major histocompatibility complex (MHC) molecules are responsible for antigen processing and presentation to lymphocytes. The MHC class I, II, and III gene loci have been identified and characterized in zebrafish.52–54 In histologic sections of adult zebrafish kidneys and spleens macrophages are easily identified.55,56 Distinguishing characteristics of these cells include their large size and their vacuolated, agranular cytoplasm.56,57 While the bone marrow is the source of blood cells in mammals, myelopoiesis occurs in the kidney of the adult zebrafish, the primary adult hematopoietic organ in zebrafish.58,59

Zebrafish macrophages are capable of engulfing foreign material, cellular debris and invading microorganisms (Fig. 3). A number of studies have shown engulfed remnants of apoptotic erythrocytes in phagosomes in the process of being degraded.26,60 Macrophages can be observed in embryos as early as 15 hours postfertilization (hpf)56 and Lieschke et al.56 also established that embryonic macrophages were capable of removing carbon particles from the circulation by phagocytosis within 1 hour after microinjection of embryos at 2 dpf. After phagocytosis, even early embryonic macrophages are capable of killing pathogens, where large phagosomes containing degraded material can be observed.26 Adult and embryonic zebrafish macrophages and granulocytes have been shown to be reactive oxygen species-producing cells, which contribute to their ability to kill phagocytosed pathogens.61

Herbomel et al.26 examined macrophage activity of 30 hpf embryos challenged with either E. coli or B. subtilis using videomicroscopy. E. coli was easily cleared from circulation completely within 3 hours, with the degradation of the bacteria observed over the next few hours. Low doses of B. subtilis were eliminated in a short amount of time as seen with E. coli infections. However, when embryos were challenged with higher doses of B. subtilis, at a concentration equal to the E. coli experiments, the macrophages became highly vacuolated and activated, but were not able to clear the bacteria. Individual phagosomes rapidly fused, forming single large phagosomes that contained intact bacteria. The bacteria remained intact for a long period of time suggesting the macrophages were having difficulty eliminating the gram-positive bacteria. By 5 hours postinjection, only two of seven embryos had cleared the circulation of bacteria. These two embryos were eventually able to kill and degrade the bacteria and survive, while the other five embryos died as a consequence of being overrun with B. subtilis. In an additional experiment, Herbomel et al.26 illustrated that macrophages are not only able to clear bacteria from the circulation, but these cells will also migrate to a site of infection in an area not nor-
mally occupied by embryonic macrophages. Similar to mammalian macrophages, the entire population of zebrafish macrophages exhibited an activated state during an infection despite the fact that only a portion of the macrophage population migrated to the site of infection. The interaction between embryonic macrophages and the bacterium *M. marinum* has also been examined. *M. marinum* causes a chronic infection of macrophages resulting in granuloma formation, mimicking some aspects of the pathology seen in mammalian tuberculosis. Davis et al. demonstrated that embryonic zebrafish macrophages could be used to study *M. marinum* and granuloma formation, clarifying a problem observed in the mouse model of tuberculosis, in which aggregates of macrophages are contaminated with CD4-positive T cells at early stages of granuloma formation. The T-cell contamination of the aggregates makes it difficult to differentiate the roles of individual host factors and cells that mediate granuloma formation. Because mature lymphocytes are not present until 4 weeks post-fertilization in zebrafish embryos, the researchers were able to determine how early innate responses contribute to the infection and the initiation of granuloma formation.

Granulocytes are another cell type that an invading pathogen will encounter early in the infectious process. The zebrafish granulocytes reside in the kidney and circulation. Two separate granulocyte lineages have been identified in zebrafish to date, heterophils (or neutrophils), which appear to be functionally orthologous with mammalian neutrophils, and a unique granulocyte. This unique granulocyte seems to exhibit both basophil and eosinophil characteristics, and is differentiated by a granular cytoplasm with a small non-segmented, peripherally located nucleus (Fig. 3). Basophils have been identified in other teleost species, but in zebrafish a distinct basophil lineage has not yet been identified. Zebrafish eosinophils/basophil granulocytes are not well characterized, and because there are significant morphologic differences between this zebrafish granulocyte and mammalian granulocytes, further analysis examining gene expression patterns and functional studies are necessary to determine the physiologic role this cell plays in innate immunity.

In mammals, granulocytes have a significant role in acute inflammation. Lieschke et al. demonstrated that zebrafish heterophil/neutrophil granulocytes also play a role in acute inflammation. Using 2 and 6 dpf embryos, myeloperoxidase activity was assayed before and after the tip of an embryo’s tail was sectioned. Myeloperoxidase is a lysosomal enzyme present in the mammalian neutrophil primary granules and eosinophils. This enzyme is part of the primary defense system of the cell. Myeloperoxidase activity was not evident until 8 hours after trauma. Heterophil/neutrophil granulocytes were visible by electron microscopy in close proximity to the trauma site, and immature heterophils were found within vessels between muscle fibers. Under normal circumstances, these granulocytes are not found in the muscle tissue, which suggests that the cells sensed trauma and migrated through tissues and toward the site of inflammation. Taken together, the morphologic and functional similarity of zebrafish heterophil/neutrophil granulocytes and mammalian neutrophils, emphasizes the usefulness of the zebrafish as a model for the study of human disease.

Cytotoxic cells are a third effector cell type of the innate immune system that plays an important role in the clearance of invading microorganisms. Research suggests that zebrafish and other bony fish possess two cytotoxic cell lineages that are involved in innate immunity, which include natural killer (NK) cells and nonspecific cytotoxic cells (NCC). Both cell types appear to be NK-like and have cytotoxic function. In bony fish, NCCs have been referred to as the bony fish equivalent of NK cells. However, there is evidence suggesting that NCC do not represent the NK lineage. Shen et al. has developed multiple cytotoxic catfish cell lines that are similar to human NK cells and have been shown to be distinct from NCC. NCCs are able to recognize protozoan parasites and are morphologically small and agranular; more closely resembling monocytes rather than NK cells, which are large and granular. A great deal of functional analysis of
these cell lineages is still necessary to fully understand the function of both cell types.

Although, there are no true NK receptor orthologs in zebrafish, there are related structures recently discovered in bony fish, the novel-immune-type receptors (NITRs). The function of NITRs has not been definitively shown in fish, but it has been shown that NITRs can stimulate the same signaling pathways as NK receptors when transfected into human NK cells. In addition, NITR genes are expressed in many of the catfish NK-like cell lines. Taken together, these studies suggest that NITRs may serve as functional orthologs to mammalian NK receptors.

In addition to cellular components of innate immunity, bony fish also possess effector molecules that are highly important to the function of the innate immune system. Teleost species have a conserved, functional complement system that is similar to higher vertebrates. As with mammals, fish complement can be activated by three pathways, the classical pathway, the lectin pathway and the alternative pathway. Complement has a critical role in host defense against invading pathogens, participating in producing opsonizing molecules, inducing inflammatory responses and forming the membrane attack complex. A number of the complement proteins have been identified in various bony fish species, and many of these factors have homologies to their equivalent mammalian proteins (reviewed in Holland and Lambris). Specifically in zebrafish, complement proteins such as factor B, C2, C3, and C4 have been identified. Although C5 has not yet been examined in zebrafish, this factor has been identified in both rainbow trout and carp. C5a is a small peptide that is a very important mediator of inflammation and phagocyte recruitment. Boshra et al. has demonstrated that C5a in rainbow trout is capable of inducing leukocyte migration and contributes to inflammation. This study and others suggest that C5a has a conserved function in both humans and teleost species. There is additional evidence that zebrafish C5a is very similar to human C5a. In an adult zebrafish model for necrotizing fasciitis, a C5a peptidase insertional mutant of Streptococcus pyogenes, a
strict human pathogen, was found to be completely attenuated (M.N. Neely and D.R. Runft, unpublished data). The data suggest that because *S. pyogenes* C5a peptidase is necessary for survival in the zebrafish that the zebrafish C5a complement protein can be cleaved by the peptidase, as it is in humans. However, further analysis is necessary to determine whether *S. pyogenes* C5a peptidase is acting directly on the zebrafish C5a complement factor.

Cytokines and chemokines are important signaling molecules that link the immune system. These molecules act in a paracrine manner to affect the behavior of cells from both arms of the immune system. Altmann et al. ⁸² was the first to clone and characterize a type I interferon (IFN) from zebrafish. Interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α), have also been identified and examined in the zebrafish. ²² These cytokines have important roles in fighting against invading pathogens. IFN has specific antiviral activity, while TNF-α and IL-1β contribute to protection against a broader range of pathogens that include viruses, bacteria, and parasitic infections in mammals.

IFN synthesis can be stimulated by either viral infection or a synthetic double-stranded (ds) RNA molecule. Upon binding to the appropriate receptor, IFN induces a number of antiviral proteins that are responsible for the degradation or prevention of synthesis of viral RNA. ³³ These proteins include 2′-5′-oligoadenylate synthetase, protein kinase p68, and Mx. Altmann et al. ⁸² examined IFN activity by using a construct of the Mx promoter linked to a reporter gene. Expression of zebrafish IFN could be stimulated by synthetic dsRNA, as demonstrated by the upregulation of the IFN-inducible Mx promoter. IFN-like activity had previously been studied by looking at the induction of the Mx protein by IFN in salmonid cells. ³⁴ These functional investigations serve to illustrate that type I IFN function is conserved between mammals and fish. In an additional experiment conducted by Altmann et al. ⁸² zebrafish hepatocytes were transfected with zebrafish IFN, and then challenged with snakehead rhabdovirus (SHRV). IFN transfected cells showed a reduction in plaque formation as compared to control cells. This study was further supported by an experiment conducted by Phelan et al. ²⁹ in which reverse transcription-polymerase chain reaction (RT-PCR) analysis of adult and embryo zebrafish infected with SHRV showed upregulation of both IFN and Mx mRNA. The antiviral activity of zebrafish IFN again demonstrates that the cytokine serves a similar function in fish and mammals.

TNF-α has an important role in initiating an inflammatory response once a pathogen has penetrated the host. IL-1β also participates in the initiation of the nonspecific inflammatory response. Together these proteins work to start a cytokine cascade that ultimately results in inflammatory cell recruitment to the site of infection, activation of these cells, and finally the response prompts activation of the adaptive immune response. Using quantitative real-time PCR analysis, IL-1β and TNF-α mRNA expression levels were found to be upregulated in embryos and adult zebrafish infected with *E. tarda*. ²² In a recent publication, Yazawa et al. ⁸⁵ used a construct of the Japanese flounder TNF promoter linked with GFP to examine TNF induction. The construct was introduced into zebrafish embryos by means of microinjection. When these embryos were exposed to lipopolysaccharide (LPS) GFP expression was observed, demonstrating the functionality of the zebrafish regulation of the TNF promoter in response to LPS. These experiments suggest that zebrafish IL-1β and TNF-α function to stimulate the immune response as it does in mammals.

The toll-like family of receptors (TLRs) is an ancient pattern recognition family, responsible for the detection of conserved markers on the surface of pathogens. These conserved structures distinguish foreign markers from the host, and as mentioned above, are called PAMPs. ⁴⁷–⁴⁹ Upon TLR binding of PAMPs, adapter molecules are recruited to the TLR complex. These adapter proteins share a common Toll/interleukin-1 receptor (TIR) domain with TLRs. Signaling through adapter proteins and subsequently through downstream signaling molecules leads to the activation of mitogen-activated protein (MAP) kinase family members and translocation of NFκB to the nucleus, and ultimately results in the transcription of proinflammatory cytokines. ⁸⁶–⁸⁸
In total, 24 putative variants of TLRs have been described in zebrafish.\textsuperscript{89,90} The zebrafish carries one or more copies of orthologs of at least 10 of the human TLR genes. Only one copy of each of four TIR-containing adapter proteins has been identified in zebrafish,\textsuperscript{89,90} including MyD88, MAL (TIRAP), TRIF (TICAM), and SARM. There is one additional adapter protein gene in humans that has not yet been described in zebrafish, TRAM (TICAM-2). Using RT-PCR analysis Meijer et al.\textsuperscript{90} confirmed expression of all the predicted TLR genes in adult zebrafish, except for the genes TLR19 and TLR20b; both genes appear to be fish-specific TLR genes and may be expressed only in a developmental stage. This investigation also confirmed the expression of IL-1 and IL-18 receptors and the four predicted TIR domain adaptor genes. To further examine TLR expression in adult zebrafish, the response to \textit{M. marinum} infection was analyzed. RT-PCR analysis demonstrated that the homologues of human TLR1 (TLR1 and TLR18 in the zebrafish), TLR2, TLR5, and TLR9 genes were expressed at higher levels in the infected fish compared with the controls. While the increased expression of TLR1 and TLR2 are part of a typical host response to \textit{Mycobacterium} infection, the expression of TLR5 and TLR9 are less easily explained. TLR9 is induced by unmethylated CpG dinucleotide motifs that are commonly found in viral and bacterial genomes. Zebrafish TLR9 may have been stimulated by detection of CpG motifs of \textit{M. marinum}. In mammals, TLR5 responds to bacterial flagellin, but \textit{M. marinum} does not produce flagella. The zebrafish TLR5 may have ligand specificities that differ from mammals, but additional function analysis is necessary to determine the role of TLR9 and TLR5 in pathogen recognition in zebrafish. The zebrafish TLR5 may have ligand specificities that differ from mammals, but additional function analysis is necessary to determine the role of TLR9 and TLR5 in pathogen recognition in zebrafish. The zebrafish TLR20 and TLR22 also showed increased expression, perhaps providing a fish-specific response to bacterial infection. Expression levels of the IL-1 and IL-18 receptor genes were unaffected by the infection. The zebrafish homologue of MAL showed increased expression in the infected fish, while the expression levels of the other putative TIR domain adapter genes were not responsive to \textit{Mycobacterium} infection. Despite some differences, it appears that the function of TLRs of mammals and teleosts are likely to be highly conserved.

In a recently published study, Meijer et al.\textsuperscript{91} went on to examine the response of adult zebrafish to \textit{M. marinum} infection by microarray analysis, with a particular interest in looking at the host immune response. Infected fish were sacrificed after 8 weeks postinfection, at which time the infected zebrafish were exhibiting signs of fish tuberculosis. Overall, the data suggested that both an innate and an adaptive immune response had been initiated, with increased expression of a number of complement components, immunoglobulin heavy and light chains and T-cell–specific genes. A number of the upregulated genes included factors involved in the inflammatory response, many of which have been shown to contribute to the response to mycobacterial infections in other mammalian systems. Conversely, there were also several immune related genes that did not show a change in expression. The expression profile may indicate that there is a specific immune response induced by \textit{M. marinum}. Some of the genes not induced include important signaling molecules of the TLR cell-signaling pathway, MyD88, ECSIT, TRAF6, and TAB1. Interestingly, the signaling molecule IRAK-4 was induced in this study. IRAK-4 associates with TRAF6, leading to activation of NF\textsuperscript{κ}B, in response to bacterial pathogens (see below). Additional analysis is necessary to determine if IRAK-4 is associating with a different signaling molecule other than TRAF6 in response to mycobacterial infection. Furthermore, a number of significant immune-related genes were not present on any of the three microarray genechips employed for this investigation. Some of the missing genes included the adapter proteins MAL, TRIF, SARM, and all of the zebrafish TLRs except TLR21. Taken together, this study adds a great deal to the understanding of host–pathogen interactions between \textit{M. marinum} and the zebrafish, but due to the lack of representation of some immunity-associated genes on the microarray chips, as well as some inherent problems with microarrays in general, there are some important questions yet to be addressed.

In a second study that supports the idea that zebrafish TLRs function and signal in a con-
served manner as compared to mammalian TLRs, an orthologue of mammalian TLR3 and two important TLR signaling molecules IRAK-4 and TRAF6 were identified and characterized. In humans, the adapter proteins contain a death domain that can associate with the death domain of IL-1R–associated kinase (IRAK). Subsequent autophosphorylation of IRAK enables its interaction with tumor necrosis factor-associated factor-6 (TRAF-6), ultimately leading to the activation of nuclear factor κB (NFκB) and MAP kinase. In the interest of examining the mechanisms of the zebrafish TLR signaling pathways, adult zebrafish and embryos were infected with either SHRV or E. tarda. TLR3 is induced during antiviral responses in mammals, resulting in proinflammatory cytokine production by signaling through TRAF6. In fact, TRAF6 is involved in all TLR signaling pathways. However, IRAK-4 has a more specific role in the signaling pathway of TLR4 in response to bacterial pathogens. By looking at TLR3, IRAK-4, and TRAF6, the researchers were able to investigate separate pathways involved in recognizing bacterial and viral pathogens. An NFκB-luciferase reporter plasmid was constructed to measure the activation of NFκB in zebrafish liver cells. Overexpression of zebrafish IRAK-4, TRAF6, or TLR3 was able to stimulate NFκB activation in zebrafish liver cells, confirming the involvement of these factors in the activation of the transcription factor NFκB, ultimately resulting in transcription of immunomodulatory genes. In addition, messenger RNA expression profiles of each gene in zebrafish embryos and adults were examined by quantitative real-time PCR after infection with SHRV or E. tarda. As anticipated, subsequent exposure to SHRV resulted in upregulation of only TLR3 and TRAF6 mRNA transcripts. However, infection with E. tarda resulted in a surprising increase in mRNA expression of TLR3, as well as the predicted upregulation of IRAK-4 and TRAF6 mRNA transcripts. The TLR3 results with E. tarda may again indicate an additional recognition role of zebrafish TLR3 not found in mammals.

A number of studies have provided evidence that TLRs serve as an important link between innate immunity and adaptive immunity in mammals. The TLRs recognize a specific invading pathogen, and are capable of inducing the correct cell signaling that results in upregulation of costimulatory molecules and inflammatory cytokines to drive an appropriate adaptive immune response. Functional studies to examine adaptive immune responses in zebrafish, specifically looking at activation through TLR signaling, are feasible because teleosts have a developed adaptive immune system, which includes features such as immunoglobulin (Ig) genes expressed by B cells and T-cell receptor (TCR) genes expressed by T cells. For both mammals and zebrafish it has been shown that Ig and TCR genes are encoded by multiple gene segments distributed in arrays along the chromosome. As B and T lymphocytes develop, an intricate system of gene segments encoding multiple variable (V), diversity (D), and joining (J) regions of Ig or TCR are joined together by somatic rearrangement, also referred to as V(D)J recombination, to form a continuous functional gene encoding an antigen receptor. Both B immunoglobulin and TCR genes in the zebrafish go through V(D)J recombination. The recombination activating genes, rag1 and rag2, catalyze the somatic rearrangement of B immunoglobulin and TCR genes, and both genes have been identified in zebrafish. These genes have served as markers to follow development of organs containing lymphocytes. Wienholds et al. was able to generate an inactivated rag1 mutant in the zebrafish. This investigation showed that a homozygous rag1 mutant demonstrated loss of function at this locus that resulted in a complete block of V(D)J joining. Despite this severe immunodeficiency syndrome, these mutants were able to survive into adulthood and were fertile. The significance of this study is not only in the functional analysis of the zebrafish rag1 gene, but it also demonstrates a technique to generate loss of function mutations. Such mutations in immune genes are highly useful for understanding the function of the gene and how a particular immune factor impacts the progression of infectious disease.

Overall, T cell distribution and function seem to be conserved in comparison with mammals. T cells are found in the kidney, phar-
ynx, intestinal tract, nose, and spleen and under the skin. Functional and morphologic maturity of the zebrafish immune system occurs at 4–6 weeks postfertilization. Teleost show graft rejection and produce antibodies after immunization. The first immunoglobulins can be detected at approximately 4 weeks postfertilization. Similar to mammals, the major lymphoid organs in the zebrafish include the thymus and gut-associated lymphoid tissues, but the zebrafish anatomy differs in that the fish do not have lymph nodes or bone marrow.

Although the adaptive immune systems of zebrafish and humans are very similar there are some significant differences worth noting. For research examining a pathogen at a stage in which adaptive immune defenses are engaged, it is important to be aware of these differences. While teleost have been shown to have an antibody response to immunization, illustrating a functional humoral response, the immunoglobulin heavy chain classes encoded on the zebrafish genome differ somewhat from both humans and mice. Previously it was thought that the zebrafish immunoglobulin heavy chain locus only encoded two heavy chain classes, immunoglobulin μ and δ. However, recently Danilova et al. examined the complete immunoglobulin heavy chain locus and identified a third heavy chain class, immunoglobulin ζ. Furthermore, unlike mammals, class switching does not occur in zebrafish B-lymphocytes. An important difference between teleost and mammals is that while both possess activation-induced cytidine deaminase (AID) homologues, which contribute to receptor diversity caused by somatic hypermutation, class-switch recombination does not occur in zebrafish. The possibility exists that mammalian AID serves a dual function that does not exist in teleost species.

CONCLUSION

Even though the use of zebrafish as an infectious disease model is still in the early stages, great strides have already been made. A number of pathogens have been shown to successfully infect and cause disease in the zebrafish host. However, we have only scratched the surface given the vast array of pathogens that could potentially be studied in the zebrafish. The type of questions that can be addressed regarding infectious disease is also just beginning to be explored. For instance, not every pathogen must cause a fatal infection to provide important information on host-pathogen interactions. The zebrafish would be an ideal host in which to study commensal relationships. Such an investigation has recently been shown with the development of the gnotobiotic zebrafish model to determine the influence of indigenous microflora on development.

A great number of advances have been made in the study of the immune system of zebrafish as well. Future studies will continue to provide important information on the specific immune defenses stimulated during infectious disease. In turn, infectious disease investigations will be able to examine the specific mechanisms employed by a pathogen to counter those host defenses. Given the availability of two stages of development, the transparent embryo with only innate immunity that develops into the adult with both adaptive and innate immune functions, the types of questions that can be investigated are limitless. However, because the zebrafish model is relatively new to the laboratory, there is still a need for reagents and tools to conduct these studies, as well as the continued discovery of important immunity factors. For instance, the identification of additional cell surface markers, like CD3, CD4, and CD8, would aid in the distinction between lymphocyte cell types in both immunological and infectious disease studies using zebrafish. Cytokines play a main role in stimulating and linking host immune responses. The presence of key cytokines like IFN-γ, IL-10, and IL-12 in other bony fish suggests that these molecules may be present in zebrafish as well. Defining whether these cytokines and others are expressed in zebrafish and if these molecules serve the same important functions in zebrafish as seen in mammals will further our knowledge about conserved vertebrate immunity. The identification of dendritic cells in the zebrafish, as has been done in the rainbow trout, with specific cell markers will also aid
in determining early innate immune functions. The considerable knowledge already available from the zebrafish development community as well as the number of tools recently developed gives this new model a generous head start. As the model develops, new reagents and cell-specific markers will greatly aid in elucidating specific host–pathogen interactions.

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