Fishing for novel immune genes

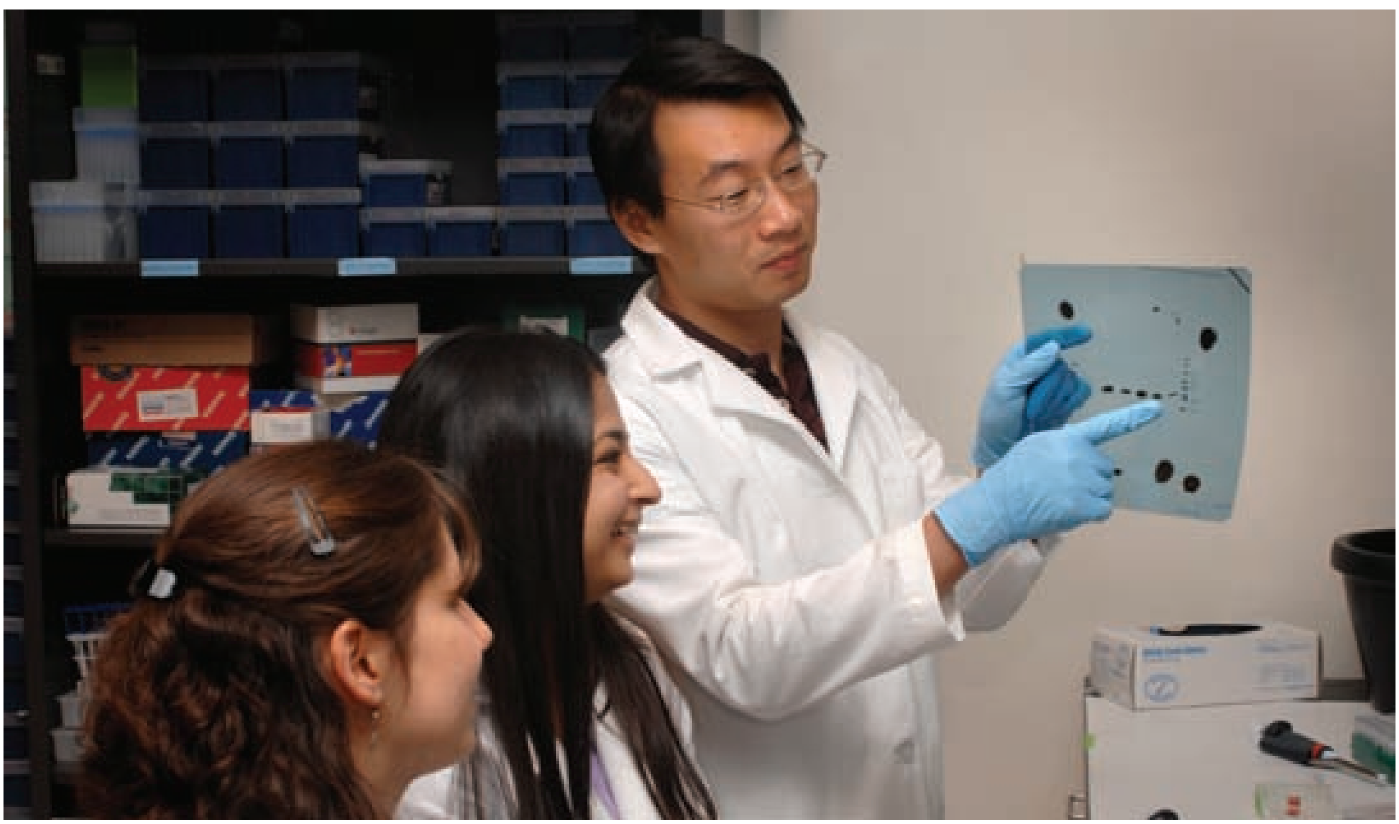
The humble zebrafish embryo may hold clues that can help CVM scientists unlock the mysteries of the immune system

By Jeffrey A. Yoder

The ability to fight off infections falls to the immune system, a complex arrangement of specialized cellular components within the blood that work together to identify, respond to, and help the body recover from diverse pathogens that can cause disease. Scientists in the Center for Comparative Medicine and Translational Research are studying the specific genes that are integral for the efficient functioning of this critical system.

The vertebrate immune response is comprised of multiple molecular and cellular components that must interface to provide the host species with an adequate defense against pathogens. Although much information is available on how individual molecules or cells respond to infection, a complete understanding of the whole organism response to pathogen exposure remains unresolved, due to the dynamic complexity of the immune system.

One goal of our laboratory is to gain a better understanding of how vertebrate species respond to and recover from an infection. We are specifically interested in identifying novel genes that are involved in these processes, but have not previously been associated with immunity. For a variety of reasons, we have chosen to use zebrafish larvae as the starting point



Research Associate Poem Turner (left) and immunology graduate students Radhika Shah (center) and Jibing Yang (right) study gene expression data as part of the Center for Comparative Medicine and Translational Research zebrafish investigation.

for these studies with the ultimate goal of our research having an impact on patient health.

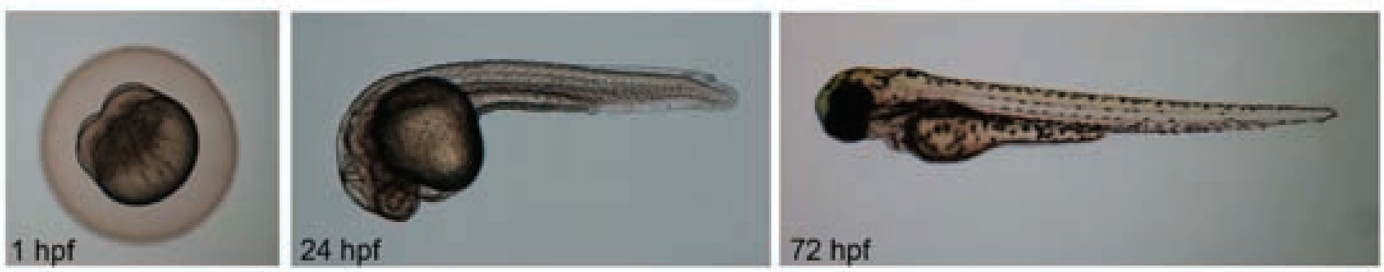
Immunity involves a host (person or an animal) and a pathogen (bacteria or virus) that invades the host and causes disease. The immune system of the host then battles, and hopefully destroys, the invading pathogen. The immune system is comprised of a number of specialized cell types within the blood that work together to fight infections. These cells provide a crucial role in immunity by differentiating between normal cells (self) and bacterial or virally infected cells (non-self) and only targeting the non-self cells for destruction. But how does an immune cell differentiate between self and non-self? The short answer is that immune cells utilize a range of proteins or receptors on their surface that recognize unique structural features of the pathogen. For example, immune cells possess receptors for lipopolysaccharides (LPS), which are a major component of specific types of bacteria and are not present on the host's cells. Once the immune receptor recognizes LPS, it triggers a molecular signaling cascade within the immune cell which leads to the activation of a large number of genes that produce the proteins necessary for the ensuing battle. My laboratory is focused on identifying novel genes that are activated by LPS and other pathogen "mimics."

Why use zebrafish? The zebrafish has become a major vertebrate model organism. One of the main reasons we use zebrafish as a model for human health is that, despite some 400 million years of evolution, zebrafish and mammals share many of the same genes and possess nearly identical immune cells. DNA mutagenesis on zebrafish embryos has been used for large scale screens identifying hundreds of zebrafish mutants with a range of phenotypes that affect development and model mammalian diseases. More recently, chemical genetic screens have identified pharmacological tools that induce or repress abnormal phenotypes in the zebrafish and can serve as lead compounds for drug discovery in mammals. The zebrafish genome is anticipated to be completely sequenced and annotated by the end of the year. The latest assembly of the Zebrafish Genome Project identifies upward of 25,000 genes, a number which is comparable to the latest assemblies of the Mouse and Human Genome Projects that identify more than 24,500 and 23,000 genes, respectively. With this nearly complete genome sequence, advanced molecular analyses of gene activation, such as microarrays, are becoming commonplace for zebrafish research.

Two components of immunity. There are two major components of the vertebrate immune system termed the innate and adaptive immune responses. Adaptive immunity is best known for its production of antibodies by specialized immune cells (B cells) and the hallmark of adaptive immunity is immunological memory. When an organism is infected with a new pathogen, the adaptive immune response can take several days to generate antibodies that are specific for that pathogen. One aspect of immunological memory is that after the first infection of—and recovery from—the pathogen, certain B cells retain the "memory" of how to produce the best antibodies to recognize the pathogen leading to its rapid and efficient destruction during subsequent infections. This is the basis for the well-studied field of vaccine development. My laboratory is interested in how the innate immune system functions in the absence of adaptive immunity and thus in the absence of antibodies.

Innate immunity in the absence of adaptive immunity. During vertebrate embryogenesis there is a period of time before B cell maturation when the immune response relies solely on the innate immune system. This timeframe provides a unique opportunity to assess the innate immune response in the absence of a functional adaptive immune response. This stage of development can also provide a simplified model for examining the vertebrate immune response from a whole organism perspective. This developmental stage in mammals occurs in utero making infection studies challenging. In zebrafish embryogenesis occurs ex utero so the zebrafish is an ideal vertebrate model for deciphering the innate immune response in the absence of adaptive immunity.

In vivo studies versus cell-based studies. Another advantage to using zebrafish larvae for our research is the ability to monitor the whole organism's response to an infection rather than just a cellular response. The majority of previous studies evaluating how infection influences



At one hour post fertilization (1 hpf), the embryo is at the 4-cell stage. At this stage the embryo is inside a protective membrane. At 24 hpf, the embryonic head and tail are visible. At 72 hpf, the zebrafish begins swimming in a start and stop fashion.

gene expression have been conducted using purified blood cells in Petri dishes. These studies have provided an amazing amount of information about how isolated individual blood cell types respond to infection. The studies fail to address, however, how these blood cells respond to an infection in the context of a whole organism. We have chosen to use the three-day-old zebrafish larvae for gene expression, or microarray, studies to identify novel innate immune response genes. Our approach is to expose three-day-old larvae with compounds that mimic an infection (LPS) and then evaluate which genes are turned on and off at multiple time points after exposure. We are using microarray technologies which allow us to evaluate the levels of expression for some 43,000 zebrafish gene sequences simultaneously.

Our initial studies with exposing zebrafish larvae to LPS indicate that more than 800 genes are influenced by exposure to LPS during five different time points. We also observed that in addition to immune response genes, LPS induces the expression of metabolism genes—a likely result of the digestion of LPS into sugars and lipids. In order to focus on only the immune response genes, we are repeating these experiments with other pathogen mimics that are structurally unrelated to LPS and should not activate these other metabolism genes. Once these experiments are complete, we can then select only those genes that are activated or turned on by multiple pathogen mimics. By identifying the genes which are turned on by pathogen

Graduate student Amy Heffelfinger microscopically assesses the quality of three zebrafish embryos (on computer screen).

A model species

Briton Francis Hamilton first described zebrafish (Danio rerio) in his 1822 government-commissioned report, "An Account of the Fishes Found in the River Ganges and its Branches," as a species with no economic worth.

Indigenous to India and surrounding regions, zebrafish can be found living in streams and flooded regions including rice paddies. They prosper in a range of water conditions, which may be why they have become a popular fish for freshwater aquariums.

The fish is named for its five uniform, pigmented, horizontal blue stripes on the side of the body. Males are torpedo shaped and have gold stripes between the blue stripes; females have a larger, whitish belly and have silver stripes instead of gold. The zebrafish grows to 2.5 inches, lives for around five years, and produces 300 to 500 eggs per spawning.

More than 20 years ago, George Streisinger, a developmental biologist at the University of Oregon, looked to develop a new model for vertebrate embryology using a species that developed ex-utero, or outside of the mother. His interest in tropic fish led him to select the zebrafish as a model for a variety of reasons, including their small size, ease for mating in the laboratory, the large number of embryos generated by a single mating pair, and the optical clarity of the early zebrafish embryo, which provides a "window" into the processes of organ formation within a vertebrate species.

Within 24 hours post fertilization or hpf, the zebrafish embryo possesses a beating heart, circulation, neural networks, eyes, and much more. At 28 hpf, the embryo utilizes early macrophage cells to recognize and kill bacterial infections. Although by 72 hpf the zebrafish embryo is predicted to have a completely functional innate immune system, the adaptive immune system is not completely functional until a few weeks after fertilization.

Zebrafish have the ability to regenerate fins, skin, the heart, and the brain (in larval stages) and have also been found to regenerate photoreceptors and retinal neurons following injury. The mechanisms of this regeneration are being researched and may lead to a better understanding of healing and repair mechanisms in vertebrates.

Following Dr. Streisinger's death in 1984, his pioneering use of the zebrafish in research has spread to an estimated 300 developmental and genetics labs in more than 30 countries. Many of the mutant strains produced in the Streisinger lab are still alive and well in research labs throughout the world and are being used towards providing answers to human and animal health issues.

The extensive research involving zebrafish—one of the few fish species to have been flown into space—has led to the creation of the Zebrafish Information Network (ZFIN), an online database of zebrafish genetic, genomic, and developmental information.

Candidate biomarkers for infection

As an offshoot from the zebrafish research project described here, Dr. Yoder's laboratory has identified a number of genes that are turned "on" after immune stimuli. He and Dr. Adam Birkenheuer in the CVM Department of Clinical Sciences are collaborating on an investigation funded by the Center for Comparative Medicine and Translational Research (CCMTR) which is designed to determine if any of these genes might be useful biomarkers for canine patients presenting with infection.

Drs. Yoder and Birkenheuer are applying multiple experimental strategies to validate the immunological relevance of these genes in zebrafish and mice prior to studies in canine patients. Since they want to develop an easy assay for analyzing the expression of genes from patients' blood samples, they sort through the hundreds of genes that are increased in expression after LPS exposure in the zebrafish larvae and, for this study, only focus on genes known to be expressed in the blood. They then establish that each gene is expressed at higher levels after LPS exposure in mice and after bacterial and/or viral infection in adult zebrafish. Only those genes which meet the preceding criteria are investigated as possible biomarkers for infection in patients.

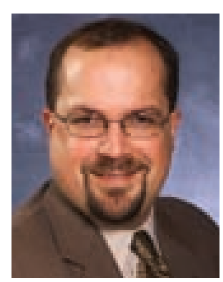
This is one of the first studies to utilize the new CCMTR Clinical Studies Core for identifying appropriate canine patients, obtaining owner consent, and collecting blood samples. The laboratories of Drs. Yoder and Birkenheuer are currently using these blood samples to evaluate the expression levels of multiple genes in healthy dogs, dogs with infection, and dogs with cancer. The hope is to identify a gene that has increased expression—that is turned "on"—in only the dogs with infections. The results of these experiments may yield a rapid, new methodology for screening patients suspected of harboring an infection.

One-cell zebrafish embryos being aligned for micro-injection with anti-sense agents to disrupt the function of candidate genes.

mimics, we can then focus on genes that are well conserved between zebrafish and mammals and are novel or functionally uncharacterized. Finally, we can validate that these genes do respond to infection by determining if they are also turned on after different types of infection in adult zebrafish and mice. Our goal is generate a short list of approximately 20 genes that are 1) novel or uncharacterized, 2) well conserved between zebrafish, mice, and humans, and 3) respond to infection stimuli in both zebrafish and mice.

Function in fish. Although the genes in our short list are activated by an infection, does that mean the gene is actually functionally relevant to immunity? In order to address this question, our laboratory is combining a genetic strategy that disrupts gene function within the zebrafish larvae with bacterial infections. The overall approach is to disrupt each gene from our short list in the zebrafish larvae and then assess the ability of these animals to recover from a bacterial infection. We expect that the disruption of at least a few of the genes from our short list will result in the larvae's inability to recover from infection, indicating that those genes are necessary for recovery. Once we identify one to three of these helpful genes, we will then be challenged with determining their specific function. This broad-based strategy is relatively unbiased in that we may end up identifying genes that encode cell surface proteins, or encode proteins that are secreted by the cell, or encode proteins that regulate the expression of other genes—all of which are associated with an immune response.

What lies down the river? Once we have identified a few genes from our short list that are required for recovery from infection, we will begin the process of employing molecular biology, cell biology, biochemistry, and genetics to define the role or roles these genes play in immune response and recovery. The outcome of these future experiments may determine that novel proteins play important roles in known immunological pathways. Alternatively, these studies may uncover novel immunological pathways. No matter the outcome, these studies will provide a better understanding of how a whole organism responds to and recovers from infection and may lead to new targets for drug strategies to expedite the recovery time after a patient is infected.



Dr. Jeffrey A. Yoder is an assistant professor of innate immunology in the Department of Molecular Biomedical Sciences and a member of the Center for Comparative Medicine and Translational Research. In December of 2007 he was profiled by Genome Technology Magazine *as one of "Tomorrow's PIs." He was recently awarded a grant from the National Institute of Health to pursue the studies noted in this article.*