Immunity, Vol. 20, 1–20, April, 2004, Copyright ©2004 by Cell Press

The Use of Zebrafish to Understand Immunity

Review

Nikolaus S. Trede,[1,3,*] David M. Langenau,[3] David Traver,[1] A. Thomas Look,[3] and Leonard I. Zon[1,2]
[1]Division of Hematology/Oncology
Department of Medicine
[2]Howard Hughes Medical Institute
Children's Hospital
One Blackfan Circle
Boston, Massachusetts 02115
[3]Division of Pediatric Oncology
Dana Farber Cancer Institute
44 Binney Street
Boston, Massachusetts 02115

For decades immunologists have relied heavily on the mouse model for their experimental designs. With the realization of the important role innate immunity plays in orchestrating immune responses, invertebrates such as worms and flies have been added to the repertoire. Here, we discuss the advent of the zebrafish as a powerful vertebrate model organism that promises to positively impact immunologic research.

Introduction

Ever since Bruton's pioneering description in 1952 of a heritable human immunodeficiency, X-linked agammaglobulinemia (Bruton, 1952), genetics has impacted immunologic research. Cloning of the corresponding gene, Bruton's tyrosine kinase, 41 years later (Tsukada et al., 1993; Vetrie et al., 1993) constituted a first example of forward genetics applied to immunology, where gene discovery is phenotype driven. Reverse genetic approaches in the mouse have provided new models that complement observations made in human patients. However, this approach, where phenotypes result from specific gene inactivation, is biased by its obvious restriction to known genes. Recently, forward genetic screens with an immunologic focus have been carried out in mice (reviewed in Appleby and Ramsdell, 2003). This approach allows an unbiased and genome-wide evaluation of gene function based on detection of phenotypes of interest. However, given the significant space and labor needs required to carry out such screens, they are prohibitive for smaller laboratories. Alternative animal models are therefore needed to complement studies in mice and humans.

The zebrafish offers many advantages over other model systems (Thisse and Zon, 2002), including ease of manual experimentation and drug administration and its prolific fecundity (see Table 1). A great advantage for immunologists is the optical transparency of zebrafish during early development, beyond the onset of T cell ontogeny. Using transgenic zebrafish where T cells are marked with a fluorochrome, early T cell development now becomes accessible to inspection in a living organism. Furthermore, fluorescent T cells can even be visualized in the thymus and gut of adult, live zebrafish (Langenau et al., 2003). This greatly facilitates the readout of the effects of genetic and chemical manipulations, such as in mutagenesis or drug screens for altered T cell function.

In this review, we will introduce some of the many advantages the zebrafish model offers, emphasizing new technologies and approaches to uncover immune genes and their function in health and disease.

*Correspondence: trede@enders.tch.harvard.edu

New Tools for Zebrafish as an Experimental Model System

The zebrafish has exceptional utility as a model system, and forward genetic screens have produced numerous mutants affecting organogenesis and development (Amsterdam et al., 1999; Haffter et al., 1996). Models of human disease have been established in the zebrafish and new genes involved in critical signaling pathways in organogenesis have been identified. Recent progress in several areas has increased the versatility of the zebrafish for immunologic research.

Gene Inactivation

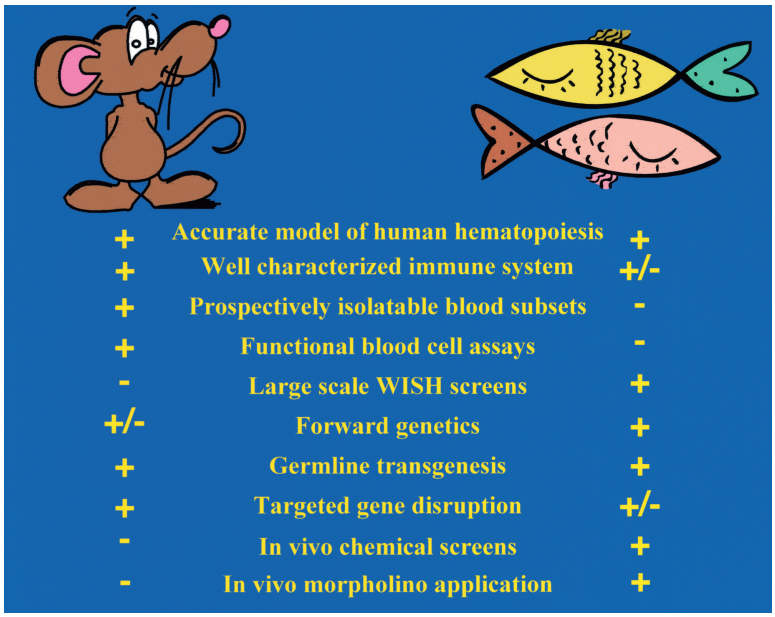
Reverse genetic approaches have been used in zebrafish research. Morpholinos (MOs) are modified antisense DNA oligonucleotides that can be injected into embryos at the one cell stage. By inhibiting either splicing or translation of mRNA, knockdown of gene function can be evaluated for up to 10 days (Nasevicius and Ekker, 2000). Thus, MO effects last long enough to inactivate genes that regulate the immune system. MOs targeting different genes can also be coinjected, thus providing a unique opportunity to test multiple gene functions simultaneously within a living organism. An example of loss of globin production following injection of a GATA-1MO is shown in Figure 1.

In addition to studying transient gene knockdown through use of MO technology, "targeting-induced local lesions in genes" (TILLING) has brought permanent gene inactivation to the zebrafish field. TILLING uses a combination of forward and reverse genetics, allowing for the identification of point mutations that alter function within a gene of interest (Figure 2). In addition to this, attempts are underway to carry out traditional gene targeting approaches in the zebrafish system (Ma et al., 2001). The field awaits the ability to put targeted cells back into the blastula, thereby generating "classic" and conditional knockout animals.

Identification of Blood Cell Lineages through Use of Transgenic Zebrafish

A variety of transgenic zebrafish lines have been generated, which mark specific cell populations through use of a tissue-specific promoter driving expression of fluorochromes. Several of these lines identify hematopoietic populations, e.g., *GATA-1*^{eGFP} (Lagasse and Weissman, 1997) targets green fluorescent protein (GFP) exclusively to erythroid cells, and *rag1*^{eGFP} and *rag2*^{eGFP} are expressed in immature lymphocytes (Jessen et al., 1999,

Table 1. Comparison of Advantages of Mouse and Zebrafish



2001; Langenau et al., 2004). Recently, we have established a transgenic line in which GFP is driven by the zebrafish p56lck promoter (Langenau et al., 2004). This line expresses GFP specifically in T cells (see Figure 3). Use of p56lck^{eGFP} transgenic zebrafish opens the door for in vivo tracking of T cell homing and accumulation in tissues (Langenau et al., 2003, 2004) and permits planning of forward genetic screens for genes causing immunodeficiencies, leukemia, and autoimmune disease (N.S.T., M. Armant, D. Mathis, unpublished data). For example, GFP signals emanating from the pancreas of adult zebrafish are detectable by epifluoprescence microscopy (Traver et al., 2003a). Screening mutagenized fish for accumulation of fluorescent T cells in the pancreas, a hallmark of type I diabetes, could thus lead to the identification of genes involved in the pathogenesis of this autoimmune disease.

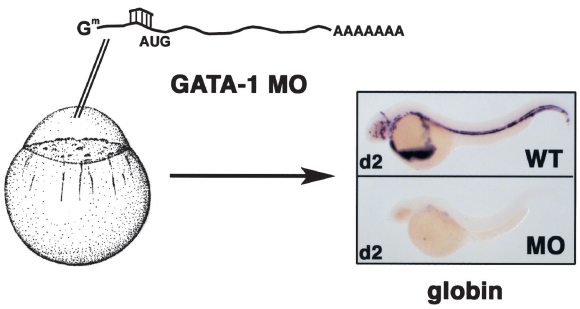


Figure 1. Example of Morpholino Use in Zebrafish

Morpholinos (MO) are modified DNA antisense oligonucleotides that are targeted either to the translational start site or to splice junctions of the gene of interest. After MO injection at the 1 to 8 cell stage, protein is not made correctly for the timespan of MO activity (up to 10 days), and the resulting phenotypes can be observed. For example, a MO targeted to the translational start site of GATA-1 completely suppressed globin production for up to 5 days. Shown here in the picture are day 2 embryos, either wild-type (top) or MO injected (bottom). Note the absence of globin in treated embryo.

FACS-Based Hematopoietic Lineage Identification and Gene Expression Screens

Lack of molecular tools for the analysis of the immune system in zebrafish is a significant stumbling block. Recently, flow cytometric examination of whole kidney marrow (WKM) by light scatter characteristics allowed the separation of the major hematopoietic lineages from total kidney marrow (Langenau et al., 2003) (Figure 4). Combined with T cell-specific transgenic lines expressing GFP, FACS analysis now allows the separation of immature and mature T and B cell populations (Langenau et al., 2004). The ability to obtain sorted lymphoid subpopulations is of great benefit for generating cell type-specific cDNA libraries (see below) and for transplantation (Langenau et al., 2004). We have generated cDNA libraries from highly purified myeloid, lymphoid, and precursor cell populations. We are currently sequencing at least 10,000 clones from each library to identify both novel genes and zebrafish orthologs of known mammalian genes. In an approach pioneered in the zebrafish by the Thisse lab (Donovan et al., 2002), genes of interest will be used to generate in situ probes for high-throughput expression screens during early development. This method will aid in the identification of genes expressed in lymphoid, myeloid, or erythroid cell subsets. Genes with interesting expression patterns can then be tested functionally in gain- or loss-of-function experiments by RNA or MO injections, respectively. The relative ease of testing gene function during early zebrafish development is an important advantage of this model.

Microarray Technology

The zebrafish community has recently created 16,000 gene Affymetrix microarray chips. Microarray technol-

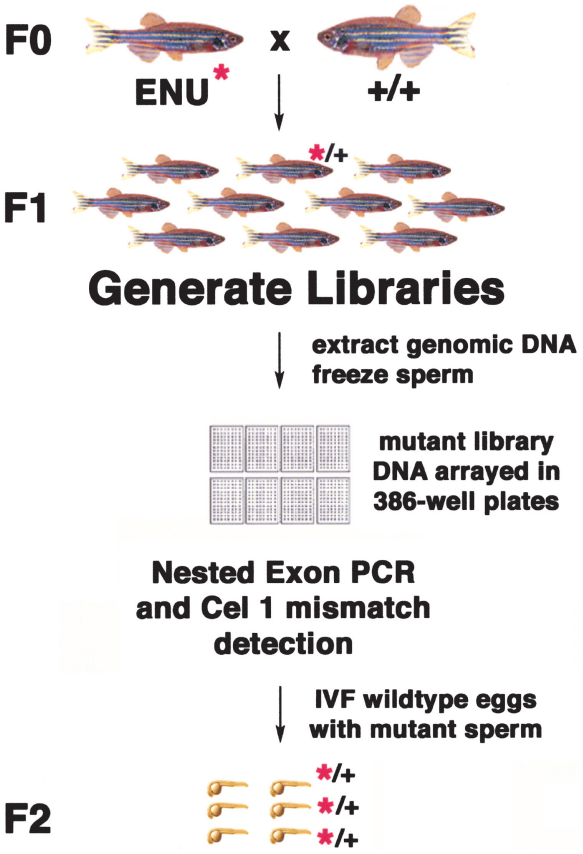


Figure 2. TILLING

Tilling (targeting-induced local lesions in genomes) is a combination of forward and reverse genetic approaches designed to obtain gene mutations. ENU mutagenized males are crossed to wild-type females (F0 generation), and the F1 generation is raised. DNA is prepared from tail clips of F1 males, and DNA and sperm are stored individually. DNA is then pooled in groups of five to ten individuals and arrayed in plates to create a mutant library. Exonic sequence is PCR amplified from pools of DNA that is subjected to a denature-renature step. Mutations (*) are detected through use of the celery mismatch-repair enzyme Cell. Cell is an endonuclease that cleaves DNA at sites of single base pair mismatches, allowing for electrophoresis-based detection of point mutations. Mutations are sequence verified from individual samples in the positive pool. Sperm of the affected F1 male is used to fertilize wild-type eggs in vitro, thus generating the F2 generation. Crossing of individuals in the F2 generation will yield 25% homozygous mutant offspring in the F3 generation.

ogy will be useful for evaluating gene expression in developing zebrafish embryos and adult tissues. These chips are currently being used to identify differences in gene expression between wild-type and mutant embryos, which are deficient in hematopoietic and/or lymphoid development. Gene expression data could also be helpful in the analysis of a mutant with an unknown genetic defect. For example, comparing the gene expression pattern of a novel mutant to the signature expression profile of known mutants may identify common molecular pathways that are disrupted in mutant fish.

In Vivo Chemical Screens

Given their small size, zebrafish embryos can be arrayed in multiwell plates and are therefore ideally suited for in

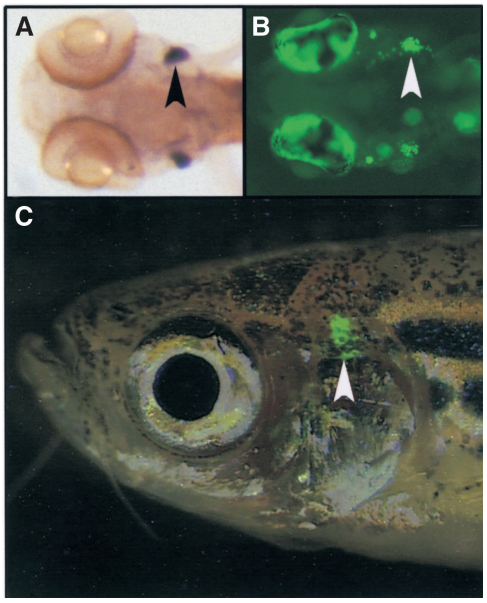


Figure 3. Lck Expression in Zebrafish

$p56^{lck}$ is a tyrosine kinase that is almost exclusively expressed in T cells.

(A) In situ hybridization of a 7-day-old zebrafish larva (dorsal view) using an *lck*-antisense probe. Black arrowhead points to *lck* expression in the thymus, a bilateral organ in the zebrafish.

(B) Thymic expression pattern of GFP driven by the zebrafish *lck* promoter in a 7-day-old larva (dorsal view) is similar to (A). White arrowhead indicates right thymus.

(C) *lck* expression persists into adulthood and is easy to detect using epifluorescence. White arrowhead indicates the left thymus.

vivo small-molecule screens. In a pioneering experiment, embryos were incubated with compounds from a small molecule library during the first 3 days of life (Peterson et al., 2000) and phenotypic effects were visually assessed. Approximately 1% of the compounds caused specific phenotypes, affecting ear, heart, and central nervous system formation, cardiac rhythm, and pigmentation. Using visually based screening methods, up to 400 compounds can be screened each day. However, screening can be made even more efficient by using transgenic fish expressing a reporter gene and robotic plate reading technology. To explore whether this approach could be amenable to probing the immune system, we incubated lck^{eGFP} transgenic larvae at 5 dpf with dexamethasone. All T cells had disappeared within 2 to 3 days (Langenau et al., 2004) in a dose-dependent, mifepristone-inhibitable fashion. This finding illustrates that responses to chemical ablation are evolutionarily highly conserved and lays the ground for in vivo-based drug screens to identify new immunosuppressive molecules in the zebrafish.

Zebrafish Innate Immune System

Teleosts, like mammals, are endowed with primary defense mechanisms against microbial agents. These mechanisms include cytokine and interferon production, complement activation, and stimulation of cellular effectors, such as cells with cytotoxic and macrophage-like activity. The cellular components involved in teleost

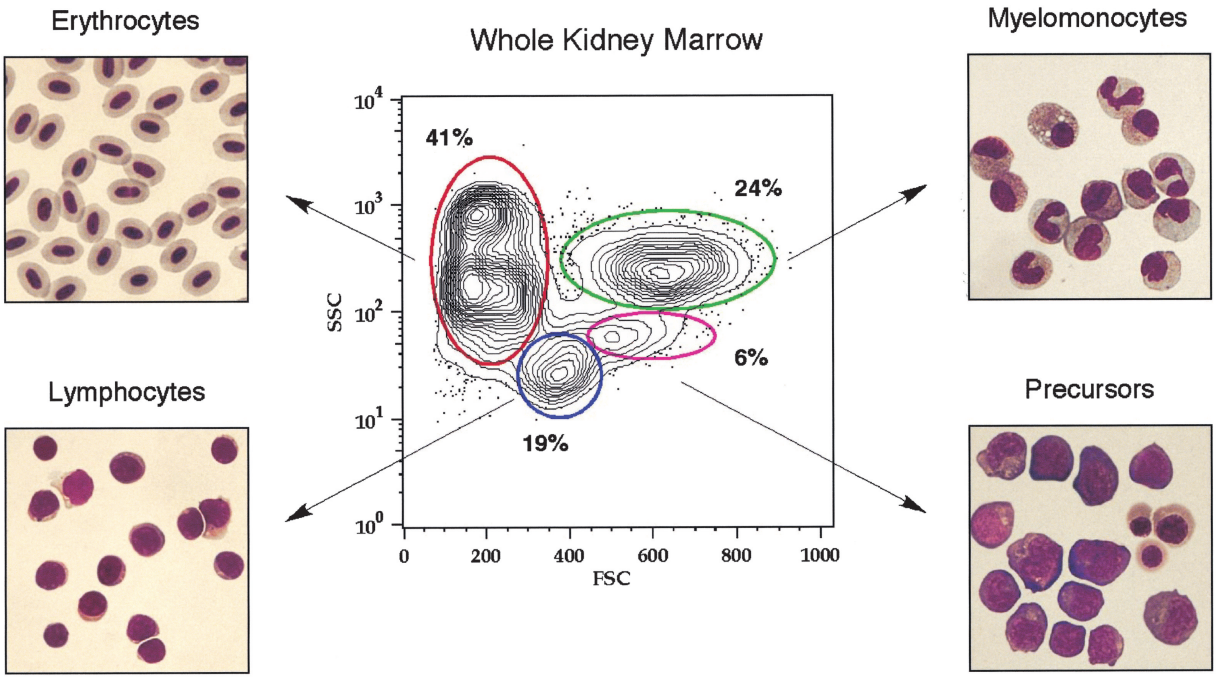


Figure 4. Separation of Definitive Blood Lineages by Flow Cytometry

Suspensions of adult kidney cells form distinct populations when analyzed by size (forward scatter; FSC) and granularity (side scatter; SSC). Sorting of each population reveals that cells within the red gate are comprised of only mature erythrocytes (upper left panel), the blue gate contains only lymphocytes (lower left panel), the purple gate contains immature precursors of all mature blood lineages (lower right panel), and that the green gate contains only myelomonocytic cells (upper right panel). Percentages indicated in each population may vary between different strains of fish and different aquatic habitats. (Taken from Traver et al. [2003b].)

defense against pathogens have recently been extensively reviewed (Traver et al., 2003a) and will not be discussed in this article. Less well studied in zebrafish are two recently discovered types of receptors involved in primary immune response. Toll-like receptors (TLRs) are expressed on the surface of dendritic cells and macrophages and recognize repetitive pathogen-associated molecular patterns (PAMPs) derived from bacteria or viruses. Following PAMP binding, TLRs transduce signals from the cell surface to the nucleus, a process mediated by five adaptor molecules, which share a common Toll/interleukin-1 receptor (TIR) domain with TLRs. This process leads to activation of MAP kinase family members, translocation of NFκB to the nucleus, and secretion of anti-infectious molecules (Akira and Hemmi, 2003). The novel immune-type receptors (NITRs) are characterized by Ig-variable ectodomains and share structural features with mammalian NK receptors. They were recently discovered as a multigene family in several genera of bony fish (Litman et al., 2001; Yoder et al., 2001). Ligands and signal transduction pathways engaged by NITRs are under active investigation.

Toll-like Receptors

TLRs have a long ancestry reaching back to plants. It was therefore expected that primitive vertebrates, such as teleosts, express members of this family. Indeed, in the pufferfish *Fugu rubripes* (Oshiumi et al., 2003) and in the zebrafish an almost complete set of TLRs was recently described (Jault et al., 2004; Meijer et al., 2004). A notable exception is the absence of TLR4 in *Fugu* (Oshiumi et al., 2003), while in zebrafish two copies of TLR4 were discovered. Two fish-specific members (TLR21 and 22) were found in both zebrafish and *Fugu*. In all, 24 putative variants of TLRs have been described in zebrafish (Jault et al., 2004; Meijer et al., 2004), including orthologs of the 10 human TLR families. Some of these represent alternative splice forms of the same gene, as in the example of TLR4.1a and 4.1b (Jault et al., 2004), while others carry the signature of fish-specific gene duplication, as in the case of TLR4.1 and 4.2 (Jault et al., 2004).

In zebrafish, TLRs are zygotically expressed, starting shortly after gastrulation (Jault et al., 2004). To date, five Toll/interleukin-1 receptor (TIR)-containing zebrafish adaptor proteins have been identified (Jault et al., 2004; Meijer et al., 2004). Unlike *Drosophila*, where TLRs and adaptor proteins are maternally expressed and are involved in body patterning, zebrafish TLRs are expressed after body axis determination, and the adaptor proteins, such as MyD88, are expressed even later (Jault et al., 2004). The developmentally earlier expression of the TLRs compared to the adaptor proteins suggests that they may fulfill functions alternative to antimicrobial recognition.

Some TLRs are more tissue restricted; for example, TLR4.2 is expressed in blood, skin, and heart, while TLR3 is expressed ubiquitously.

Meijer et al. have begun to address the functionality of zebrafish TLRs by studying their expression levels following mycobacterial infection. About half of the zebrafish TLRs, including the fish-specific TLR21 and 22 members, were upregulated after intraperitoneal *M.*

marinum inoculation (Meijer et al., 2004). Functionality of the teleost innate immune system can also be inferred from the in vivo observation that *rag-1*-deficient zebrafish that lack adaptive immunity (Wienholds et al., 2002) have a normal life expectancy in fish water, which is notoriously infested with pathogens. In particular, the strikingly high expression of TLRs in the skin of zebrafish (Jault et al., 2004) points to the relevance of innate immune mechanisms in a first layer of defense against microbial pathogens. However, once this first layer of immune protection is compromised, as for example after tail clipping, the *rag-1*$^{-/-}$ fish succumb to infection within 2 weeks (E. Wienholds, personal communication).

A variety of zebrafish pathogens have been described. Of interest will be the study of pathogen-specific receptor engagement and the ensuing signaling cascade in zebrafish. In particular, it will be appealing to identify unique ligands for the teleost-specific TLR21 and 22, which could provide clues as to evolutionary pressures fish were exposed to in their aquatic environment. Assigning molecular signatures to zebrafish TLRs upon binding of their respective ligands will provide a platform for forward genetic screens designed to identify genes involved in immunity to infection.

Novel Immune-Type Receptors

Zebrafish also have NK cells and NK cell function (reviewed in Traver et al., 2003a). NK receptors effect self/nonself recognition through interaction with major histocompatibility class I molecules. Although there are no true NK receptor orthologs in zebrafish, related structures were recently discovered in bony fish, the NITRs, which comprise 12 distinct families (Litman et al., 2001). These receptors are characterized by an extracellular immunoglobulin-like V domain, a transmembrane region and cytoplasmic tail, containing immunoreceptor tyrosine-based motifs. The precise signals transduced through NITRs are under active investigation (J. Yoder and G.W. Litman, personal communication). Only one NITR, the *nitr5* family, has an activating (ITAM) cytoplasmic motif. All the remaining receptors have inhibitory (ITIM) domains, although the *nitr9* family has been shown to have activating function (J. Yoder and G.W. Litman, personal communication). Similar to TLRs, some NITR family members are ubiquitously expressed, while others are more tissue restricted. However, all are expressed in kidney marrow, pointing to the hematopoietic origin of cells in which they are expressed. Current efforts are underway to determine which lineages of kidney cells express each NITR (J. Yoder, D.T., L.I.Z., and G.W. Litman, unpublished data). Several NITR families are maternally expressed or transcribed early during embryogenesis (J. Yoder and G.W. Litman, personal communication), but they do not appear to play a role in organogenesis. It is therefore tempting to speculate that these receptors play an important role in innate immunity during a phase of development, when adaptive immunity has not yet matured.

Adaptive Immunity in Zebrafish

The origins of *rag*-dependent adaptive immunity reach back 450 million years, coinciding with the emergence of the first jawed vertebrates, the teleost fishes. The thymus, of pivotal importance for adaptive immunity, has its earliest morphological equivalent in pharyngeal lymphoid tissue of the jawless lamprey (Ardavin and Zapata, 1988). Evolution of the structural and functional counterpart of the thymus of higher vertebrates coincides with development of the jaw. The components vital for a fully functional *rag*-dependent adaptive immune system, the thymus, T cells, B cells, highly polymorphic major histocompatibility (MHC) antigens, and enzymes, such as terminal deoxy transferase (TdT), are present in all jawed fishes examined to date, and their functionality has been demonstrated in the teleost species catfish and trout. Thymic organogenesis and lymphoid development are highly conserved from zebrafish to mammals, making the zebrafish an attractive model for screens designed to uncover genes involved in adaptive immunity. *rag*-dependent adaptive immunity in zebrafish has been the subject of several studies (Jessen et al., 1999; Lam et al., 2002, 2004; Langenau et al., 2004; Schorpp et al., 2000; Willett et al., 1997b, 2001) and has been extensively reviewed (Boehm et al., 2003; Schorpp et al., 2000; Traver et al., 2003a; Trede et al., 2001; Trede and Zon, 1998). We will therefore restrict our treatment of this topic to recent developments, highlighting the differences between zebrafish and mammals and emphasizing current controversies and evolutionary implications.

Thymic Development

Overall the processes of thymic development and involution, as well as its compartmentalization into cortex and medulla, are conserved throughout vertebrate evolution (Lam et al., 2002; Manley, 2000). Ectodermal and endodermal germlayers are indispensable for formation of the thymic anlage in teleosts and mammals. For example, zebrafish mutants of endoderm, such as *faust* (Reiter et al., 2001), and hindbrain/neural crest, such as *lazarus* (Popperl et al., 2000), have severe defects in thymic development. Furthermore, the recently cloned *vgo* mutant (Piotrowski et al., 2003), a defect in the *tbx-1* gene, mimics the human DiGeorge syndrome, which is characterized by craniofacial defects, heart defects, and absence of the thymus. Although thymus development is remarkably conserved, there are some noteworthy differences this process in mouse and humans. The most obvious difference is that the thymus in zebrafish remains in continuity with pharyngeal endoderm and therefore persists as a bilateral organ throughout development (Lam et al., 2002; Langenau et al., 2004; Schorpp et al., 2000). Developmentally and functionally the main differences concern timing. Thymic development initiates early in zebrafish ontogeny, at about 48 hr postfertilization (hpf), and the thymic rudiment is formed by 60 hpf (Willett et al., 1999). T cell progenitors start invading the thymic rudiment at 68 hpf. However, no distinction into cortex and medulla, the result of interaction between thymic epithelial cells and T cell precursors, is discernible until 3 weeks of age (Lam et al., 2002). In mouse, evidence for epithelial cells with a medullary phenotype can be detected on E15, and differentiation of thymic stroma into cortex and medulla is completed shortly after birth.

T Cell Development

T cells can first be detected at 72 hr in the thymus by gene expression studies. Intriguingly, *rag-1* and *rag-2* are coexpressed in the olfactory placode of zebrafish

embryos (Jessen et al., 1999, 2001) prior to the onset of T cell invasion of the thymus. Thus the olfactory placode is the first described extralymphoid organ where *rag-1* and *rag-2* are coexpressed. However, the functional significance of this finding is unclear, and histologic analyses (Jessen et al., 2001; Langenau et al., 2004) revealed that cells expressing *rag-1* and *rag-2* in the nose are nonlymphoid in nature.

Population of the thymus with T cell progenitors begins at 3 dpf, around the time of hatching from the protective chorion. However, we were unable to detect T cells outside of the thymus by in situ hybridization or by tracking T cells in transgenic lines (Langenau et al., 2004) for the first 3 weeks of development. Immunocompetence in zebrafish, as measured by humoral response to T-dependent and -independent antigens, is not reached until 4–6 weeks after hatching (Lam et al., 2004). Thus, zebrafish are exposed to environmental pathogens for 4 weeks without a mature adaptive immune system. This contrasts with T cell population of the mouse thymus at midgestation (E10.5), a protective intrauterine environment for the first 3 weeks of life, and circulation of T cells at about day 7 postpartum.

T Cell Repertoire

The gemonic region containing the TCRα locus has recently been identified and sequenced, revealing that the locus contains over 150 nonallelic Vα genes, which can be classified into at least 86 different V families (T. Ota and C.T. Amemiya, personal communication). Genomic organization of the zebrafish locus is very similar to that of the freshwater pufferfish, *Tetraodon nigroviridis* (Fischer, 2002). Tight linkage of TCRδ genes to the TCRα locus in zebrafish is predicted based on findings in the pufferfish. The gene complexity of the zebrafish TCRα locus, however, is considerably greater than that found in either *Tetraodon* or *Fugu* (Fischer et al., 2002). The TCRβ and γ loci have yet to be identified in the zebrafish. Overall, junctional diversity in rearranged TCR genes of jawed fishes appears to be high (Rast et al., 1997).

B Cell Development

While T cell development is relatively easy to observe given the superficial location of the bilateral thymi, B cell ontogeny in zebrafish has been more elusive. In mammals, B cells develop in the bone marrow. By analogy, the zebrafish pronephros during larval stages and the kidney marrow in adult fish are the sites of B cell development. We (Langenau et al., 2004) and others (Willett et al., 1997a, 1999) were unable to detect *rag* transcription in tissues other than thymus up to 8 dpf by whole-mount in situ hybridization (WISH). *rag* activity was first detected in the pronephros at 3 weeks postfertilization by in situ hybridization on sections (Willett et al., 1999). More recently, scrutiny of *rag-2*^{GFP} larvae revealed the pronephros as the first extrathymic site of *rag*-expression at 8 dpf (N.S.T., unpublished data). This contrasts with experiments using a highly sensitive WISH technique that detected *rag-1* expression at 4 dpf in the pancreas followed by *Ig*μ at 10 dpf (Danilova and Steiner, 2002). Similarly, *Ig*μ and *rag-1* expression were found in the gut of adult zebrafish (Danilova and Steiner, 2002), while we were unable to detect large numbers of *rag-2*^{eGFP}-positive cells in the gut by anti-GFP staining (Langenau et al., 2004). Several factors may explain the apparent discrepancy between the above observations. The WISH technique employed by Danilova and Steiner may be more sensitive than GFP fluorescence in the pancreas at this time in development. Alternatively, the transit time of B cells through the pancreas may be too short for the GFP molecule to fold properly, thereby precluding its detection by fluorescence. It is also possible that *rag* expression in the pancreas and in the gut is driven by enhancer elements that are not included in the transgenic *rag-2* promoter. Finally, *rag-1* expression, but not *rag-2*, has been detected in the brain (Chun et al., 1991). Thus, it is formally possible that cells in the zebrafish pancreas "misexpress" *rag-1*. In zebrafish, the pancreas develops from two separate buds, which have merged by 52 hpf (Field et al., 2003). It will be interesting to study B cell development in zebrafish mutants, such as *slim jim*, which lack normal pancreatic development (Pack et al., 1996). If the pancreas plays an important role in B cell development, a delay in or absence of B cell ontogeny would be expected.

B Cell Repertoire

Sequencing of Ig VH and Cμ genes from zebrafish revealed similar amino acid sequences to those of other vertebrate species (Danilova et al., 2000). Also, the Ig heavy chain locus of teleosts is organized in a translocon configuration, analogous to mammals (T. Ota and C. Amemiya, personal communication), while the light chain locus is in clustered arrangement, found also in the heavy chain locus of cartilaginous fishes. Diversification of the Ig repertoire is quite inferior in fish species. For example, fish do not use gene conversion (found in birds and rabbits), and somatic hypermutation (found throughout vertebrates) is inefficient. It has been argued that selection of somatic mutations rather than their generation is the reason for the observed poor affinity maturation in lower vertebrates (Wilson et al., 1992). These two processes are mediated, at least in part, by activation-induced cytidine deaminase (AID) and occur in mammalian germinal centers of spleen and lymph nodes. Ectotherms lack germinal centers and the anatomic sites where hypermutation occurs is unknown. Class switching, a process also mediated by AID, further diversifies the immune repertoire in amphibians and mammals. However, this process does not occur in teleost fishes. Fish have only IgM and an IgD isotype equivalents. Given the absence of efficient affinity maturation and class switching, it appeared reasonable to hypothesize that AID appeared in evolution after teleosts branched off from other vertebrates. However, we were able to identify the zebrafish ortholog of AID and identified spliced versions of this gene as early as 2 dpf (N.S.T., unpublished data). This raises the possibility that AID may play a role beyond B cell antigen receptor maturation or that B cells develop earlier in the zebrafish than previously appreciated. It furthermore predicts that AID may also be expressed in more primitive vertebrates, where it could fulfill a role in DNA metabolism. However, despite various approaches, cloning of AID from jawless gnathostomes was unsuccessful (G.W. Litman and N.S.T., unpublished data).

Secondary Lymphoid Tissues

Lymph nodes are absent in fish species and thus appear to have evolved after fishes branched off from other vertebrates. In addition, fishes also do not have organized Peyer's patches but, rather, loose arrays of

lymphoid cells along the wall of the gut (Danilova and Steiner, 2002; Langenau et al., 2004). T and B cells as well as myeloid cells are found in spleen and in kidney, the bone marrow equivalent of adult zebrafish (Danilova and Steiner, 2002; Langenau et al., 2004), suggesting that these organs may serve as major sites for interaction of mature immune cells.

Using *rag-2*^{eGFP} and *lck*^{eGFP} transgenic lines, we evaluated the maturational state of lymphocytes in these organs. In the spleen, 11% of cells fall into the lymphoid gate, and a third of these are *lck*$^{eGFP+}$ lymphocytes. In kidney marrow, approximately 20%–30% of cells are lymphocytes. Of these, 40% are immature B cells, characterized by *rag-2*^{eGFP} positivity and *IgLC3* transcription but lacking *lck* or *TCR*α expression (Langenau et al., 2004). We also detected a *rag-2*-negative population of lymphocytes (45%) that expressed *IgLC3*, thereby qualifying as mature B cells. The remaining 15% of lymphocytes are mature T cells, based on *lck*^{eGFP} positivity, *TCR*α transcription, and lack of *rag-2* expression (Langenau et al., 2004). Peripheral blood contains only a small fraction (0.07%) of *lck*^{eGFP}-positive cells. Taken together, these experiments show that mature lymphocytes preferentially home to spleen, kidney, and gut, and we speculate that they may interact in these organs following an immune stimulus.

Currently, there is no transgenic line where mature myeloid or dendritic cells are marked with fluorescence, and surface markers are lacking as well. However, by morphological criteria, we identified mature myeloid cells in the kidney, spleen, and blood of adult zebrafish (Traver et al., 2003b). Furthermore, kidney marrow cDNA libraries contain transcripts of mature myeloid markers (D.T., N.S.T., and L.I.Z., unpublished data). In conclusion, all three cell types participating in immune reactions in secondary lymphoid organs in mammals are found in kidney, spleen, and gut of zebrafish, making these the major secondary lymphoid organs.

The Balance of Innate and Adaptive Immunity—An Evolutionary Perspective

Vertebrates adapt to their respective microbial environments by adjusting the balance between innate and adaptive immunity. As discussed above, lower vertebrates rely heavily on innate immunity, while their adaptive immune responses mature later (Lam et al., 2004), and humoral responses lack class switching and efficient affinity maturation. Additionally, T cell responses are particularly affected in fishes by their lower body temperature (Q10 effect). While terrestrial mammals are protected during early development by maternal immunity, the highly vascularized gills of fishes are constantly exposed to millions of different microbes (Bergh et al., 1989; Breitbart et al., 2002) starting immediately after hatching from their protective chorions. The most efficient way to deal with these circumstances is to rapidly distinguish self from nonself by recognizing pathogen-associated molecular patterns (PAMPs). Thus, while poikilothermic fishes have a well-developed adaptive immune system, innate immunity may be the most rapid and efficient response to microbial exposure. The increased number of TLR receptors coupled with highly polymorphic NITRs, NK cells (Shen et al., 2004; Shen et al., 2002), and complement-dependent phagocytosis (Secombes and Fletcher, 1992) support this notion. Given the very potent innate immunity in fishes, selective pressure to fine tune adaptive immunity may not have been very high. Thus, there was no "need" for lymph nodes, germinal centers, or implementation of an efficient machinery for antibody diversification. When vertebrates became terrestrial, infection-induced selective pressures changed. Furthermore, endothermic metabolism accelerated T cell responses, leading to the development of a more elaborate adaptive immune system, including optimizing somatic mechanisms to improve the immune response.

We speculate that teleosts and terrestrial mammals have been exposed to different selective pressures that were exerted by differences in the types of microbes and their abundance in their respective habitats. The dissimilar environments during early development (extrauterine development versus protected intrauterine milieu) may have accentuated the selective pressures and led to the refinement of mammalian adaptive immunity. In addition, compared to fishes, higher vertebrates in general have fewer offspring and require a longer time to reach reproductive age. This exerts additional pressure for the development of the very efficient, individualized adaptive immune response found in mammals. Nevertheless, as shown above, the teleost adaptive immune system is ontogenetically highly conserved, and zebrafish therefore present an excellent opportunity to further our understanding of the immune system.

Transplantation Immunology in Zebrafish

To enable prospective isolation strategies, test autonomy of mutant gene function, and analyze immune cell function, zebrafish hematopoietic cell transplantation (HCT) has been recently developed (Langenau et al., 2004; Traver et al., 2003b). Efficient HCT in mammals requires ablation of the host hematopoietic system to create niche space for the engraftment of donor cells and to prevent their rejection by host immune cells. Transplantation protocols in both mouse and man have taken advantage of the fact that the minimum lethal dose of γ-irradiation specifically ablates the blood-forming system and that the ensuing hematopoietic failure can be rescued by a bone marrow transplant. Recent studies have shown that despite 450 million years of evolutionary divergence between mammals and teleosts, the minimum lethal dose of γ-irradiation leads to death and hematopoietic ablation with similar kinetics. Transplantation of 1×10^6 WKM cells (approximately one kidney equivalent) was sufficient to rescue over 75% of irradiated animals over the 30 day window defined as radioprotection in the mouse (D. Traver et al., submitted). Similarly, long-term ($>$6 months posttransplant) hematopoietic cell engraftment into adult recipients has been successfully accomplished using sublethal doses of irradiation (Langenau et al., 2004). In contrast to transplant studies in inbred mouse strains, transplant engraftment requires ablation of the immune system by γ-irradiation in adult zebrafish, where purebred lines are not available.

Analysis of transplantation kinetics was aided by the use of lck^{eGFP} and erythroid-specific *GATA-1*^{eGFP} donor

cells. Continued production of lck^{eGFP+} or GATA-1^{eGFP+} cells from transplanted kidney marrow serves as a surrogate marker of donor-derived hematopoietic stem and progenitor cell activity. Long-term repopulation of transplant recipients by either GATA-1^{eGFP} or lck^{eGFP} transgenic donor cells have shown that HSCs are contained within the adult kidney (Langenau et al., 2004; Traver et al., 2003b). By contrast, transplantation of GFP-positive thymocytes from lck^{eGFP} transgenic fish into irradiated recipient fish resulted in only short-term repopulation of the thymus. Taken together, these results suggest that thymic progenitor cells arise in the zebrafish kidney and are likely derived from HSCs. These thymic progenitor cells migrate from the marrow and seed the thymus throughout the life of the animal but do not have the capacity for self renewal. These new transplantation technologies now permit the testing of cell-autonomous defects in existing erythroid and lymphoid mutants and allow prospective isolation strategies for HSCs or specific immune cell subsets to be tested.

We hypothesized that transplantation in 2-day-old embryos, before the onset of adaptive immunity, may lead to neonatal tolerance induction similar to that shown in mice over 40 years ago (Medawar and Woodruff, 1958). At this stage embryos are also transparent, allowing easy visualization of transplant success by identification of circulating GFP^{+} cells. Transplantation of WKM cells from *GATA-1*^{eGFP} adults rescued multilineage hematopoiesis for at least 6 months in *gata-1*$^{-/-}$ mutant embryos, which normally die by 2 weeks of age of erythropoietic failure (Traver et al., 2003b). Transplant experiments in embryos also allow for the rapid assessment of homing properties of FACs sorted cell populations. For example, we have shown that GFP-positive thymocytes home to the thymus by 24 hr posttransplant (Langenau et al., 2004).

Disease Models in Zebrafish

The Use of Zebrafish as a Model for Immunity to Infections

One of the major challenges facing modern immunologic research is the identification and unraveling of molecules and genetic factors influencing the immune response to infections and the differential susceptibility to microbial challenges. The discovery of a CCR5 allele conferring relative resistance to HIV infection (Liu et al., 1996; Samson et al., 1996), and the description of the first patients with a defect in IRAK-4, rendering them susceptible to pyogenic infections (Picard et al., 2003), are prime examples for mendelian genetic contributions to susceptibility to infection. Despite these advances, a more systematic approach to this problem is needed. Several animal models have been established, such as *C. elegans* (Aballay et al., 2000) and *Drosophila* (Pukatzki et al., 2002). Despite evolutionary conservation of the major innate immunity pathways, the extrapolation of results from these invertebrates to mammals is not always straightforward. For example, activation of *Drosophila* Toll is different from that of mammalian TLRs, as interaction occurs with a cleaved cytokine in the former and directly with microbial inducers in the latter (reviewed in Hoffmann, 2003). On the other hand, vertebrate models, such as goldfish (Talaat et al., 1998) and frogs (Ramakrishnan et al., 1997), have the disadvantage of genetic intractability. As a vertebrate genetic model, the zebrafish presents itself as an attractive alternative for the study of genetic modifiers of the immune response to infections.

To date, no systematic studies of experimental infections have been carried out in fish. However, an eclectic array of reports suggests that similarities in the immune response to infections exist between zebrafish and mammals. Among the effectors of primary defense are macrophage-like cells, first seen at 25 hpf (Herbomel et al., 1999) and granulocytes at 48 hpf (Lieschke et al., 2001). Both cell types are endowed with chemotactic homing abilities to sites of infection. In addition, cytokines have been identified in fish species. For example, the pufferfish class II helical cytokines were identified (Lutfalla et al., 2003) and are likely involved in protection against infection. Similarly, interferon-stimulated pufferfish kidney cells induced the antiviral MX gene. An interferon gene (Altmann et al., 2003) and an interferon-inducible MX gene (Altmann et al., 2004) were also discovered in zebrafish. Zebrafish have functional IFN proteins, as demonstrated by their protective effect on zebrafish cell lines infected with a fish rhabdovirus (Altmann et al., 2003). At the subcellular level, zebrafish and trout cells respond to various infectious stimuli similarly to mammals, suggesting that the interferon-eIF2α kinase-eIF2α axis is conserved from teleosts to mammals (Garner et al., 2003).

Zebrafish and mammals can be infected by similar pathogens. For example, zebrafish are susceptible to bacteria pathogenic in mammals, such as *L. monocytogenes* (Menudier et al., 1996) and *S. pyogenes* (Neely et al., 2002). Fish can also be infected by that are normally nonpathogenic in humans. Two recent in vivo studies examined the response of embryos (Davis et al., 2002) or adult zebrafish (Prouty et al., 2003) to *M. marinum* infection. Labeled mycobacteria were injected into embryos at 32 hpf, which permitted the real-time observation of the immune response in a living vertebrate (Davis et al., 2002). Formation of macrophage aggregates with pathological hallmarks of granulomas were observed and were accompanied by upregulation of inducible granuloma-specific mycobacterium genes. Additionally, *E. coli* are rapidly cleared from circulation after injection into the axial vein of 28 hpf zebrafish (van der Sar et al., 2003). These data demonstrate that in zebrafish innate immune mechanisms are at work well before maturation of *rag*-dependent adaptive immunity.

Although these studies yield important insights into the first steps following (myco)bacterial infection, injection of the microbes into embryos is artificial and cumbersome. This type of strategy is therefore not amenable for genetic screens designed to uncover genetic response modifiers. In order to make the zebrafish a fully viable screening tool for genetic modifiers, suitable infectious agents for natural immersion infection have to be identified. Recently, immersion infection of zebrafish embryos was reproducibly achieved with both viral (*spring viremia* of carp virus) and bacterial (*Edwardsiella tarda*) strains (C. Kim, personal communication). Decreased or increased susceptibility to infection can now be read out either by direct observation of survival of mutagenized embryos or by assessing clearance of fluo-

rescently labeled microbes. Cloning of the affected genes could yield new clues into molecular pathways involved in immunity to infection.

Leukemia Models in the Zebrafish

Mouse models of T and B cell malignancies have provided unique insights in the collaborating events that lead to cancer formation. For example, viral insertion screens and mating strategies have defined numerous genes involved in enhancing and suppressing the onset of disease (Alt et al., 2003; Blyth et al., 2001; Fanidi et al., 1992; Haupt et al., 1991; Scheijen et al., 1997; Shinto et al., 1995; Strasser et al., 1990). However, a limitation of these studies is that viral insertion is not random and largely identifies activating mutations, while mating strategies require transgenic and knockout mice that have disruption of known gene products. Forward genetic screens in zebrafish prone to cancer may help to uncover new genetic lesions that alter cancer-associated phenotypes.

Using transgenic strategies, we have developed a model of T cell acute lymphoblastic leukemia (T-ALL) in which leukemia onset and infiltration can be monitored in real time (Langenau et al., 2003). An EGFP-mouse cMyc fusion transgene (Yin et al., 2001) was targeted to the developing lymphocytes through use of the zebrafish *rag2* promoter (Jessen et al., 2001). Stable transgenic zebrafish lines were established that develop clonal T cell leukemia with a mean latency of 22 dpf and a mean survival of 82 dpf. Leukemias develop in the thymus, spread locally into surrounding tissues, and eventually invade skeletal musculature, visceral organs, and regions adjacent the eye. Leukemias can be transplanted into irradiated recipients, have increased DNA content, and are oligoclonal as determined by *TCR*-α gene rearrangements. Based on these data, leukemic cells exhibit characteristics of T cell malignant transformation.

Similar to a subtype of human T-ALL (Ferrando et al., 2002), both *scl* and *lmo2* are coexpressed in Myc-induced T-ALL in the zebrafish. In human patients, this subgroup represents the most common and most treatment-resistant form of this disease. Use of our zebrafish T-ALL model in genetic screens may lead to new understanding of the molecular basis for this disease in humans.

Additional models of leukemia have been described using the zebrafish (Kalev-Zylinska et al., 2002). For example, transient expression of the human RUNX1-CBF2T1 transgene resulted in defective hematopoiesis, reduced circulation, and internal hemorrhages in the central nervous system and pericardium. Transiently injected embryos also had dysplastic erythroid cells and accumulation of immature hematopoietic precursors in blood-forming organs. Developing stable transgenic zebrafish lines which express this RUNX1-CBF2T1 fusion will be useful for dissecting the molecular pathways that lead to leukemogenesis in this subtype of AML. Taken together, these results highlight the similarities between mouse and zebrafish models of this disease.

Several challenges face the development of the zebrafish as a bona fide leukemia and lymphoma model. First, conditional transgenic strategies need to be developed to facilitate the propagation of aggressive cancer-prone lines. New technologies using estrogen-responsive oncogenes (for example, MYC-ER^{TM}), tetracycline-activated transgenes, or CRE or FLIP recombinase strategies will need to be developed in the fish. Second, antibodies to blood cell markers are lacking, making phenotypic characterization of blood cell tumors incomplete. Finally, it is important to show that the mechanisms underlying tumor formation are similar to those found in humans and mice. Because of this, new zebrafish models must be created that mimic both the human disease and known mouse models of cancer.

Although the zebrafish cancer model faces many challenges, the power of this model lies in its ability to perform forward genetic and chemical modifier screens and to visualize the onset of tumor formation through use of GFP-transgenic technology. ENU-induced mutational screens will likely lead to a new understanding of inactivating mutations that suppress or enhance oncogenic transformation, identifying novel drug targets for the treatment of cancer. Similarly, transgenic zebrafish predisposed to developing leukemia or lymphoma may also be a useful model system for use in chemical suppressor screens (Figure 5).

Zebrafish Screens Probing Immune Function

Forward Genetic Screens for Lymphoid Mutants

Zebrafish lymphopoiesis occurs relatively late compared to other developmental processes, including hematopoiesis. A screen for immunodeficiencies should be carried out at 5 dpf, when T cell progenitors have robustly colonized the thymic anlage. Screens for defects in immune function or autoimmunity have to await functional and morphologic maturity of the zebrafish immune system, which occurs at 4–6 weeks pf (Lam et al., 2004). In this setting, two types of screens are feasible, "classic" F2 screens or early pressure (EP) screens. Space requirements of F2 screens, where 5000 F2 families have to be raised to approach saturation, appear prohibitive when screening for a restricted number of genes that affect only lymphopoiesis and should be done in conjunction with other screens (Habeck et al., 2002). EP screens exploit the opportunity the zebrafish offers to create gynogenetic diploid embryos, where both alleles of a gene are derived from either the wild-type or the mutated, meiotically duplicated maternal chromosomes. Resulting embryos are viable and fertile and may show the gene defect in its homozygous manifestation (Beattie et al., 1999; Patton and Zon, 2001; Traver et al., 2003a). EP screens for lymphoid mutants have been successfully completed in Boston and Freiburg, Germany. These screens have yielded interesting mutants affecting pathways in thymic and T cell development (Schorpp et al., 2000; Trede et al., 2001; Trede and Zon, 1998). To date, the readout was restricted to WISH, using for the most part the robust *rag-1* probe. This approach is time consuming and requires fixing of the embryos, thus precluding observation during later parts of development. The advent of transgenic lines expressing fluorochromes under the control of a T cell-specific promoter (Langenau et al., 2004) promises to streamline the screening process, as mutants can be read out by simple inspection of living larvae. Furthermore, mutants can be followed throughout development and allowed to screen adults for physiologic (Traver et

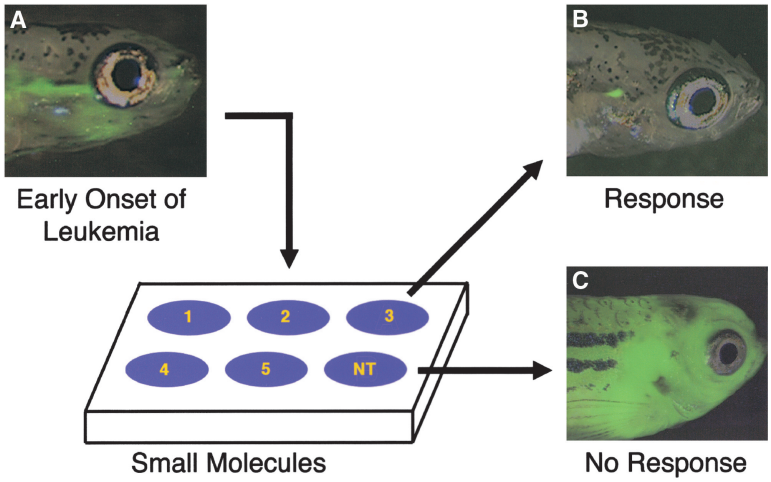


Figure 5. Small Molecule Treatment

Three to four leukemia-prone fish that are transgenic for a lymphoid promoter driving GFP are grouped together in 6 well plates at the beginning of disease manifestation ([A]; increased signal in thymus and surrounding areas). Compounds from a small molecule library are added to the wells, and changes in fluorescent signal intensity is observed and compared to untreated fish (NT) or wells containing inactive compounds (C). A compound that is active against the process of leukemogenesis (well 3) can be read out as a decrease in signal intensity (B).

al., 2003a) or abnormal accumulation of T cells, as for example in leukemia (Langenau et al., 2003) or in autoimmunity.

Screens for Mutants in Innate Immunity

Given the optical transparency of the zebrafish during early development, defects in innate immunity could be addressed in forward genetic screens. This could be accomplished by visualizing altered immune responses to infection using labeled pathogens (Figure 6A), as elegantly demonstrated for *M. marinum* infections (Davis et al., 2002). In this case, loss-of-function mutations would lead to increased proliferation of microbes, while a gain-of-function mutation may decrease the bacterial load. Alternatively, pathogen-responsive promoters could be identified and used to create stable transgenic zebrafish lines. In this case, embryos would be infected with unlabeled microbes and response to infection could be read out through a fluorescent reporter (Figure 6B). Here, loss-of-function mutations may decrease fluorescence emission, while a gain-of-function mutation is expected to increase the number and/or size of the fluorescent signal. Similar screening approaches have been successful in *C. elegans* (Kim et al., 2002) and in the fruitfly (Braun et al., 1997; Elrod-Erickson et al., 2000). Identification of mutants and cloning of the corresponding genes has the potential of uncovering novel participants in pathways involved in vertebrate innate immunity.

Remaining Challenges

The zebrafish is a relative latecomer to the field of immunology. Consequently, many of the tools accessible to researchers using more established animal models are often not available to zebrafish researchers. While anti-

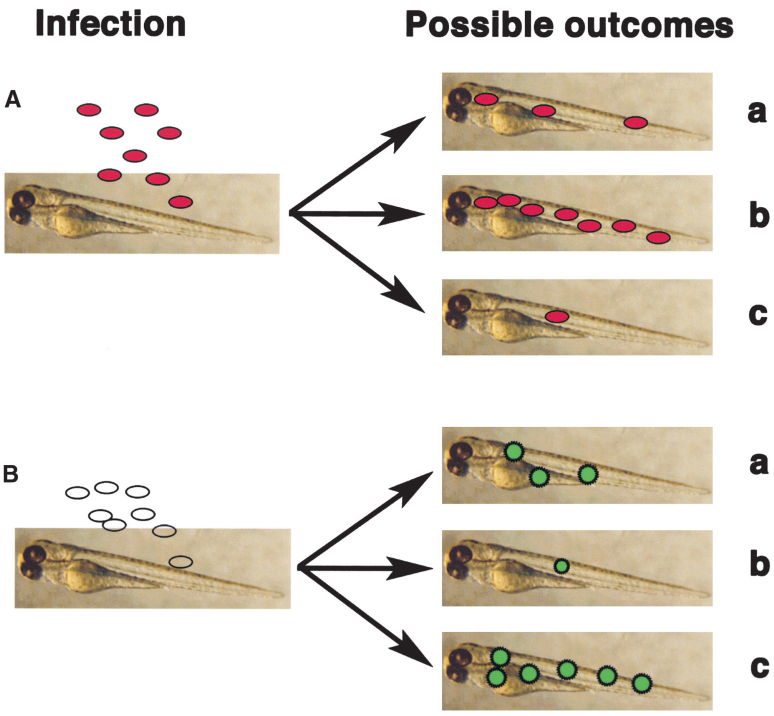


Figure 6. Schematic Representation of Screens for Alterations in Innate Immunity

The optical transparency of zebrafish allows visualization of infectious outcomes using either fluorescent microbes (red ovals in [A]) or transgenic fluorescent reporters driven by promoters of genes that are actively transcribed during infection (green circles in [B]). a, wild-type; b, loss-of-function mutant; c, gain-of-function mutant. LOF mutants in (A) are permissive for unchecked microbial expansion, while GOF mutants could potentially reduce the number of microbes more efficiently than wt. LOF mutants in (B) have reduced numbers of cells activated by microbial infection (Bb). GOF mutants (Bc) here would have increased numbers and/or signal intensities following microbial challenge.

bodies directed to human proteins that crossreact with zebrafish proteins have been described (Guyon et al., 2003), anti-human antibodies generally fail to crossreact with zebrafish cytokines or cell surface proteins on immune cells. There is even little crossreactivity between related teleosts; for example, an anti-catfish-Ig antibody failed to bind to zebrafish B cells (D.T., A. Winzeler, L.W. Clem, and N.W. Miller, unpublished data). Additionally, raising antibodies to specific immune receptors has largely been unsuccessful. After immunizing mice with zebrafish kidney cell or membrane preparations, antibodies were readily obtained. However, Endo-H treatment of cells prior to incubation abolished any binding of the antibody to zebrafish cells. This result implies that surface receptors of zebrafish blood cells are glycosylated in a manner sufficiently different from mice to elicit a potent antisugar immune response. Other strategies are currently underway to circumvent this problem. Since completion of the zebrafish genome sequencing project by the Sanger Center, in silico cloning of molecules of interest has become a successful strategy. However, due to a high degree of divergence, homology cloning of cytokines and their receptors is notoriously difficult in lower vertebrates, and the zebrafish is no exception. Current attempts rely on cloning these molecules from tissue-specific libraries either by recognizing signature sequence motifs or by expression pattern. Identification of these molecules will allow the establishment of lymphoid cell lines and carrying out of in vitro differentiation and functional studies.

Finally, successive inbreeding leads to infertility in lower vertebrates, making it difficult to create truly inbred zebrafish lines. Intrastrain genetic heterogeneity poses potential problems for positional cloning, and hematopoietic cell transplantation can be hampered by these differences. For example, transplantation of T cells from AB-strain fish into irradiated AB-strain recipients can cause a lethal GvHD-like syndrome (D.L., N.S.T., and D.T., unpublished data).

Conclusion

Historically, the major strength of the zebrafish has been the opportunity it offered to carry out forward genetic screens in a vertebrate organism in a relatively restricted space. The mutants resulting from these screens have revealed a number of genes, some novel or with novel functions affecting virtually all biological processes. Despite some interesting differences, the innate and adaptive immune systems of the zebrafish are highly conserved when compared to mammals. Thus, screens in zebrafish promise to uncover genes involved in T cell development, thymic organogenesis, and in immunity to infections. Coupled with novel approaches probing autoimmunity and leukemogenesis, zebrafish will be instrumental in elucidating physiology and pathology of the immune system. Rapid progress in zebrafish genomics and availability of functional genomic instruments, such as lineage-specific cDNA libraries and microarray chips, are among the latest additions which speed up cloning of mutant genes and probing gene function. Genes identified in these screens will then be available for more in-depth studies in mouse, where the arsenal of molecular tools available to the immunologist is still leagues ahead of the zebrafish. Recent technical advances, such as hematopoietic lineage labeling, morpholino gene knockdown, and gene-inactivation through TILLING, will be conducive to tailoring the zebrafish into a versatile tool for immunologic research. Establishing the zebrafish as a model system provides an alternative and complementary tool to the use of mice and will further our understanding of the immune system.

Acknowledgments

We would like to thank Louis DuPasquier, Gary Litman, Bob Finberg, Georg Holländer, Carol Kim, and Hans-Reimer Rodewald for invaluable and stimulating discussions; Matt Keefe for expert technical assistance; Jenna Galloway, Dave Ransom, and Nelson Hsia for help with graphic design. N.S.T. and L.I.Z. are supported by a grant from NIDDK R21DK63660, N.S.T. by NIH K08HL04233, A.T.L. by grant CA-68484, D.M.L. is a NSF predoctoral fellow, and D.T. is a fellow at the Irvington Institute for Immunologic Research.

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