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THE INVESTIGATION OF INNATE IMMUNE SYSTEM MEMORY IN *RAG1-/-* MUTANT ZEBRAFISH

By

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The innate immune system in vertebrates is considered to lack specific memory. To investigate innate immune system based immunological protection mediated by cells that are not part of the acquired immune system the Tübingen *recombination activation gene1* (*rag1*)^{t26683} mutant (MT) zebrafish was chosen. Molecular analysis demonstrated MT zebrafish kidney cells expressed Non-specific Cytotoxic cell receptor protein-1 (NCCRP-1) and Natural Killer cell (NK) lysin but lacked T cell receptor (TCR) and immunoglobulin (Ig) VH1, VH2, VH3 and VH4 expression. Differential counts of peripheral blood leukocytes indicated that MT fish had decreased lymphocyte populations (34.7%) compared to *rag1*+/+ wild-type (WT) fish (70.5%), and increased granulocyte populations (34.7%) compared to WT (17.6%). Further, endocytic functions of phagocytes from MT fish were compared to WT fish. No significant differences in the selective and non-selective mechanisms of uptake in phagocytes were observed between MT and WT zebrafish. For the first time it was shown that zebrafish

phagocytes utilize macropinocytosis and Ca^{2+} dependant endocytosis mechanisms for antigen uptake. These characterization studies suggest that MT zebrafish provide a unique model for investigating innate immune responses because fully functional innate defenses are present without the influence of lymphocytes and lymphocyte associated acquired immune responses. To conduct such large scale investigations the first ongoing *rag1*^{t26683} mutant zebrafish breeding colony was established. To meet special husbandry needs of immunodeficient MT zebrafish, standard rearing protocols were advanced and the information was made available to the zebrafish community at: http://www.cvm.msstate.edu/zebrafish/index.html.

Multiple trials were conducted to evaluate the potential for memory of the innate immune system. Significant reduction in mortality was observed in MT vaccinated zebrafish upon secondary exposure to *Edwardsiella ictaluri* when compared to unvaccinated, MT fish. This documents for the first time, that MT zebrafish, lacking an acquired immune system, are able to mount a protective immune response to *Edwardsiella ictaluri* and generate protection upon a repeated encounter to the same pathogen. The observed protection is long lasting and mediated by the innate immune system, but a specific mechanism is not yet defined.

DEDICATION

I would like to dedicate this research to my father, Aloys Hohn and his wife Doris Hohn.

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CHAPTER I

INTRODUCTION

Zebrafish as an immunological model organism

Initially prominent in developmental biology, the zebrafish has been adopted into varied fields of study including immunology [3, 4] and infectious diseases [5].

Advantages and disadvantages. The zebrafish has many advantages when compared to other animal models. Among animals with a fully developed adaptive and innate immune system, the zebrafish is one of the smallest (≤5 cm), allowing large numbers of fish to be housed in a relatively small space. They are prolific, with a given pair able to produce 200–300 new progeny each week. Fertilization of eggs and the subsequent development of embryos occur *ex vivo*, facilitating visual tracking of maturation processes. Furthermore, embryos remain translucent for several days, allowing unprecedented visualization of the development and function of the immune system [6, 7]. Given its small size and rapid organ development, the zebrafish is particularly well suited for chemical screens that interrogate disease mechanisms [8]. These screens can be carried out with relative ease, as chemicals can be simply added to the water [4]. The zebrafish is a genetically tractable model organism. The entire zebrafish genom

has been sequenced and is being assembled (http://www.sanger.ac.uk/Projects/D_ rerio/) [9], and large numbers of genetic markers have been mapped (http://zebrafish.mgh.harvard.edu/). Gene chips containing EST assemblies and unique genes are commercially available for quantitative analysis (Affymetrix, Agilent). Overall zebrafish share many orthologous genes with mouse and man, which gives this species considerable relevance over other traditional developmental models such as *Drosophila melanogaster* or *Caenorhabditis elegans*, which lack genes that are involved in some aspects of neurological processing, adaptive immunity and other functions [3]. Furthermore, recent studies have identified several regions of the zebrafish and human genome that encode the same (or similar) genes [10]. Studies in several species of teleosts have demonstrated presence of all major blood lineages and hematopoiesis is very similar to that in mammals. As teleost do not have bone marrow, blood cells are produced in the kidney [11]. Still, definitive blood cell lineages in zebrafish show a high degree of conservation at the morphological level to their mammalian counterparts [12]. Gene expression studies [13] and functional studies [14, 15] have indicated that the general mechanisms of hematopoietic development and effector cell functions are likewise conserved. Among the disadvantages of the zebrafish model are the lack of antibodies to surface proteins, the difficulty of establishing cell cultures and the lack of conventional knockout technology [4].

TILLING, a reverse genetic mechanism for disease model development. Three main reverse genetics techniques are available to generate zebrafish disease models: targeting induced local lesions in genome (TILLING), gene knockdown with morpholinos, and transgenesis [12]. Conventional knockout technology has not yet been developed in the zebrafish. To circumvent this, TILLING has been adapted to zebrafish biology [1, 16]. Point mutations are induced by N-ethyl-N-nitrosourea or other mutagens in male F0 animals (original generation). Genomic DNA and sperm from F1 males are obtained and organized for high throughput TILLING analysis. Genomic DNA is screened, most often by direct sequencing, to identify missense or nonsense mutations in a gene of interest. Corresponding banked sperm from heterozygous mutant animals is then used to find ‘‘knockout’’ lines (Figure 1.1).

Using target-selected mutagenesis, a series of 15 *rag1* (recombination activation gene) mutations were found in zebrafish. One of the mutations is a single nucleotide polymorphism (SNP) that causes a premature stop codon in the catalytic domain [1]. A functional RAG1 protein is required for V(D)J recombination [17]. Apparently, as in other vertebrates, there is only one functional *rag1* gene; loss of function at this locus results in a complete block of immunoglobulin gene assembly and, presumably, in immunodeficiency [1].

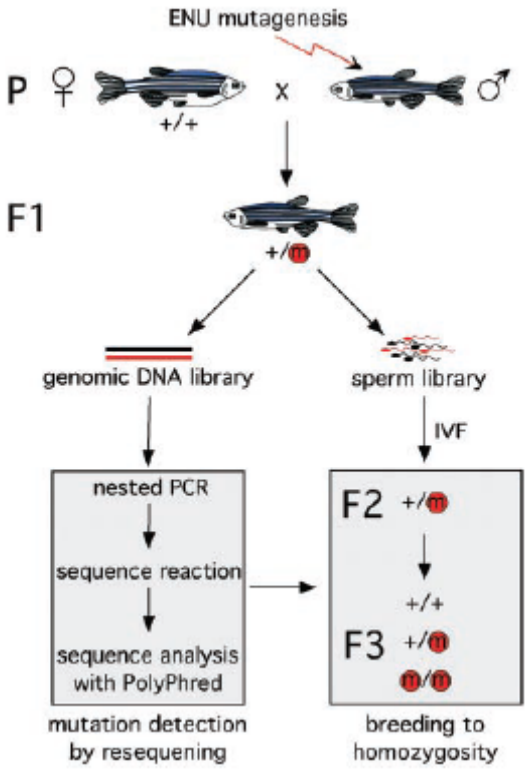

Figure 1.1 Overview of target-selected mutagenesis in zebrafish. Adult male zebrafish are mutagenized with ENU (ethylnitrosourea). Mutagenized males are crossed with wild-type females to produce F1 generation of fish. Sperm is isolated and cryopreserved from fertile F1 males. Genomic DNA is isolated, and screened for mutations by PCR amplification of the target gene and subsequent DNA is sequence analyzed. After a particular mutation is identified, *in vitro* fertilization (IVF) is performed to recover the F2 line carrying the mutation. Finally, mutations can be bred to homozygosity and analyzed for phenotypes (from Wienholds *et al*. [1]).

Overview of cellular immunity in zebrafish

The adaptive and innate branches of the immune system are remarkably conserved across jawed fishes (teleosts) and other vertebrates, including mammals. Studies of hematopoiesis have revealed that most if not all cell types of the human immune system have zebrafish counterparts [18] although sites of maturation differ [4]. In adult fish, the kidney is equivalent to the mammalian bone

marrow as the site of definitive hematopoiesis [19]. Within the kidney marrow, myeloid and erythroid maturation and development occurs with similar intermediate stages as that seen in mammals. Functionally mature myeloid cells retain similar activities and expression profiles as their mammalian counterparts (reviewed in [4]). The development of T cells and the thymus in zebrafish is remarkably similar to that of mammals with the main exception that the zebrafish thymus remains as two discrete bilateral structures [20]. T cell progenitors develop within the kidney marrow and then migrate to the thymus where they undergo differentiation [21]. Zebrafish T cell-specific developmental genes have been cloned and show strong homologies to mammalian counterparts with similar expression and functional profiles. They include *Ikaros*, *lck*, *GATA-3*, *rag-1*, and *rag-2* [12, 22, 23]. The TCRα locus has been cloned and demonstrates mammalian-like variable segments [24], with rag-dependent V(D)J recombination [1]. Upon maturation, T cells exit the thymus and populate various tissues including the kidney, pharynx, intestinal tract, nose, spleen, and skin [7, 25]. B cells are also present in the zebrafish, although there are some notable differences when compared to mammals. Initial production of B cells occurs within the pancreas, and then later shifts to the kidney marrow in the adult [26]. The cells undergo VDJ rearrangements in a rag-dependent fashion but share only IgM and IgD subtypes with mammals [27]. Additionally, a zebrafish-specific heavy chain locus, zeta, is also expressed [28]. Functionally, B cells have an active role in adaptive immunity and zebrafish produce antibodies upon immunization [23]. B cells are found in many organs and tissues with preferential

homing to spleen, kidney, and gut [29]. Bony fish, like mammals, possess cytotoxic cells that include NK cells [30-33]. The molecular mechanisms by which these cells recognize infected or transformed cells remain uncharacterized, and definitive orthologs of the mammalian NK receptor families have not been identified in bony fish [34]. However, teleosts do possess receptors that are structurally similar to mammalian NK receptors [34]. Specifically, fish possess inhibiting and activating novel immune-type receptors which are structurally similar to the Ig-type of NK receptors but cannot be classified as orthologous to any known mammalian gene family [3, 12, 35]. Receptors that appear to possess the intracellular signaling capacity to function as NK receptors are also described [34]. Numerous candidate NK receptors have been identified in bony fish, but this candidacy is based purely on sequence comparisons to mammalian NK receptors [34]. Efforts are currently underway to characterize NK receptors in zebrafish. DAP12, DAP10, CD3ζ and FcRγ adaptor molecules, all expressed by mammalian NK cells, have been identified in zebrafish lymphocytes (purified by forward and side scatter) indicating the presence of NK cells in zebrafish [36].

Like other cyprinid teleosts, adult zebrafish have at least 2 types of granulocytes, a neutrophilic–heterophilic granulocyte and an eosinophilic granulocyte. Zebrafish embryos demonstrate mobilization of peroxidase-expressing cells to a site of acute inflammation within several hours of traumatization, and they confirm the presence of heterophil granulocytes in tissues and circulatory areas proximal to the site of trauma. Hence, it is now accepted that the zebrafish heterophil indeed plays a role analogous to that of

the mammalian neutrophil [15]. The functional role of the zebrafish eosinophil is less certain [15]. In mammals, macrophages, neutrophils, and eosinophils destroy invading bacteria, fungi and virus particles by producing reactive oxygen species (ROS) and it has been shown that phagocytes such as macrophages and neutrophils are the ROS-producing cells in zebrafish [37]. Within seconds following injury, neutrophils and macrophages demonstrate chemotaxis towards the wound site from peri-vascular locations, and macrophages phagocytose debris [38]. Under normal conditions, the leukocyte infiltration resolves by 24 h [39]. Historically, there is good evidence that resolution of inflammation occurs by apoptosis of responding neutrophils [40]. However, real-time imaging in zebrafish indicates that neutrophils and macrophages display directed retrograde chemotaxis back toward the vasculature, indicating a novel aspect of inflammatory response resolution that may complement apoptosis [38, 40].

Rag 1 protein and VDJ recombination

The recombination activating genes encode enzymes that play an important role in the rearrangement and recombination of the genes of immunoglobulin and T cell receptor molecules during the process of VDJ recombination. There are two recombination activating gene proteins known as RAG-1 and RAG-2, whose cellular expression is restricted to lymphocytes during their developmental stages. RAG-1 and RAG-2 are essential to the generation of mature B and T lymphocytes. RAG enzymes work as a multi-subunit complex to induce cleavage of a single double stranded DNA molecule between the antigen

receptor coding segment and a flanking recombination signal sequence (RSS). They do this in two steps. They initially introduce a ‘nick’ in the 5' (upstream) end of the RSS heptamer (a conserved region of 7 nucleotides) that is adjacent to the coding sequence, leaving behind a specific biochemical structure on this region of DNA; a 3'-hydroxyl (OH) group at the coding end and a 5'-phosphate (PO_4) group at the RSS end. The next step couples these chemical groups, binding the OH-group (on the coding end) to the PO_4-group (that is between the RSS and the gene segment on the opposite strand). This produces a 5'-phosphorylated double-stranded break at the RSS and a covalently closed hairpin at the coding end. The RAG proteins remain at these junctions until other enzymes repair the DNA breaks. The RAG proteins initiate V(D)J recombination, which is essential for the maturation of pre-B and pre-T cells [41].

V(D)J recombination is a mechanism of genetic recombination that occurs in vertebrates, which randomly selects and assembles segments of genes encoding specific proteins with important roles in the immune system. This site-specific recombination reaction generates a diverse repertoire of T cell receptor (TCR) and immunoglobulin (Ig) molecules that are necessary for the recognition of diverse antigens from bacterial, viral, and parasitic invaders, and from dysfunctional cells such as tumor cells [41].

In the developing B cell, the first recombination event to occur is between one D and one J gene segment of the heavy chain locus. Any DNA between these two genes is deleted. This D-J recombination is followed by the joining of one V gene, from a region upstream of the newly formed DJ complex, forming a

rearranged VDJ gene. All other genes between V and D segments of the new VDJ gene are now deleted from the cell's genome. Primary transcript (unspliced RNA) is generated containing the VDJ region of the heavy chain and both the constant *mu* and *delta* chains (C_μ and C_δ). (i.e. the primary transcript contains the segments: V-D-J-C_μ-C_δ). The primary RNA is processed to add a polyadenylation (poly-A) tail after the C_μ chain and to remove sequence between the VDJ segment and this constant gene segment. Translation of this mRNA leads to the production of the Ig μ heavy chain protein. The kappa (κ) and lamda (λ) chains of the immunoglobulin light chain loci rearrange in a very similar way, except the light chains lack a D segment. In other words, the first step of recombination for the light chains involves the joining of the V and J chains to give a VJ complex before the addition of the constant chain gene during primary transcription. Translation of the spliced mRNA for either the kappa or lamda chains results in formation of the Ig κ or Ig λ light chain protein. Assembly of the Ig μ heavy chain and one of the light chains results in the formation of the membrane bound form of the immunglobulin IgM that is expressed on the surface of the immature B cell [41].

During T cell development, the T cell receptor (TCR) chains undergo essentially the same sequence of ordered recombination events as that described for immunoglobulins. D-to-J recombination occurs first in the β chain of the TCR. This process can involve either the joining of the $D_\beta1$ gene segment to one of six $J_\beta1$ segments or the joining of the $D_\beta2$ gene segment to one of six $J_\beta2$ segments. DJ recombination is followed (as above) with V_β-to-$D_\beta J_\beta$

rearrangements. All genes between the V_β-D_β-J_β genes in the newly formed complex are deleted and the primary transcript is synthesized that incorporates the constant domain gene (V_β-D_β-J_β-C_β). mRNA transcription splices out any intervening sequence and allows translation of the full length protein for the TCR C_β chain. The rearrangement of the alpha (α) chain of the TCR follows β chain rearrangement, and resembles V-to-J rearrangement described for Ig light chains. The assembly of the β- and α- chains results in formation of the αβ-TCR that is expressed on a majority of T cells [41].

Memory in the innate immune system

Previously, it was observed that larval channel catfish that had not yet developed acquired immunity when first vaccinated demonstrated specific protection upon secondary exposure [42]. This finding suggested that following pathogen exposure in the absence of functional lymphocytes and acquired immune responses, fish innate immunity can provide enhanced defenses against the same pathogen upon re-exposure. This phenomenon is not without precedence. Copepods, fruit flies and bumble bees that do not have lymphocyte-based immune systems [43-45], lampreys that lack lymphoid tissues and *rag 1* and *2* [46] and *rag1* deficient mice [47] demonstrate specific memory in their immune responses.

Significance of research and objectives

Specific memory in innate immunity was observed by others in invertebrates [48-51] and mammals [47]. Motivated by these findings the aim of

this project was to establish a fish model for the investigation of this phenomenon. This model could be used to get new insight in the evolution and function of innate immunity. Since the innate immune system is the first line of defense a better understanding and controlled activation, similar to the effect of vaccination on the acquired immune system, would have many applications. Possible future research could influence veterinary as well as human medicine.

It is hypothesized, that once mutant (*rag1-/-*) zebrafish have been proven to lack functional lymphocytes, they cannot perform lymphocyte driven responses and do not possess acquired immune functions. Therefore this fish line can be used to study innate immunity without the overshadowing effects of acquired immune responses or function.

The main objectives of this study are:

1. To establish a breeding colony of mutant (*rag1-/-*) and wild-type (*rag1*+/+) zebrafish.
2. To determine functionality of acquired immunity, in particular absence of functional B and T cells in mutant (*rag1-/-*) zebrafish. Further assess presence and functionality of major innate immune cells, neutrophils, monocytes/macrophages, and natural killer cells.
3. To determine a possible specific memory response of the innate immune system utilizing mutant (*rag1-/-*) zebrafish.

CHAPTER II

BREEDING OF *RAG1-/-* MUTANT ZEBRAFISH

Abstract

Despite its popularity as a model organism, the scientific rigor of zebrafish husbandry techniques is poorly developed. An attempt was made to integrate the available scientific information related to zebrafish husbandry and to advance current protocols to meet special husbandry needs of immunodeficient *rag1-/-* mutant zebrafish. This information was made available to the zebrafish community at: http://www.cvm.msstate.edu/zebrafish/index.html while standardization efforts for rearing and maintenance are currently under way. A flow-through aquatic animal holding system was designed to reduce maintenance stress and the risk of spreading pathogens between tanks.

Using the method described here, the average hatching rate of *rag1+/+* eggs was 69% and 77% for *rag1-/-* eggs. Survival and growth until day 10 post fertilization was similar in both strains with 89% and 6.64 mm in *rag1+/+* and 91% and 6.01 mm in *rag1-/-* fry. At 30 days post fertilization *rag1+/+* fish showed higher survival (82%) and slightly better growth (2.41 cm) when compared to *rag1-/-* zebrafish (73%, 2.13 cm).

Introduction

Given the considerable importance of zebrafish as an experimental model, and the significant economic costs associated with the large-scale use and the establishment and maintenance of culturing facilities, published husbandry standards are wholly inadequate [52]. In a review paper about the husbandry of zebrafish, Lawrence [52] calls for the development of standard protocols to maintain the genetic integrity of lab strains and to improve reproducibility of experiments. The exchange of information about husbandry techniques between the numerous research facilities housing zebrafish has been largely non-existent, and any advances that have been made are often employed in isolation and without the benefits of peer review [52]. While establishing *rag1-/-* mutant (MT) and *rag1+/+* wild-type (WT) fish lines at College of Veterinary Medicine Specific Pathogen Free fish hatchery (CVM SPF fish hatchery), newly developed or optimized rearing protocols were compared to standard published methods [53]. In addition, while CVM SPF fish hatchery houses the first and only breeding colony of *rag1-/-* deficient zebrafish, it was important to compare spawning and rearing success of these MT fish to their WT counterparts. This information was found to be important in perspective to any experiments utilizing these MT zebrafish.

Many companies specialize in offering stand-alone systems to house aquatic animals (Aquaneering Inc. www.aquaneer.com, Aquarien-Bau Schwarz www.aquaschwarz.com, Aquatic Habitat, a division of Aquatic Eco-Systems, Inc. www.aquaticeco.com, Marine Biotech. www.marinebiotech.com). These

commercially available systems work well for larger universities, but for smaller institutes and start-up laboratories these systems may be prohibitively expensive. Therefore a zebrafish rack system was constructed that is useful for small laboratories with easy assembly, low maintenance, and minimal initial cost [2]. Perhaps the most important aim was to construct a system suitable to house immunodeficient fish, focusing on decreased stress and a reduced risk of spreading pathogens.

Materials and Methods

SPF zebrafish facility and holding system. Zebrafish used in this study were propagated and reared in the CVM SPF fish hatchery [54]. Low-cost aquatic animal holding systems were constructed to meet SPF requirements to house immunocompromised zebrafish. Figures 2.1-2.3 show schematics of the rack system, detailed information about rack assembly and lists of parts can be found in Hohn and Petrie-Hanson [2]. Zebrafish were held in 19 L tanks with charcoal filtered de-chlorinated and mechanically filtered (1 μm) municipal water at 27°C. Water was supplied to each tank at a rate of 0.5 L/min in a flow through, single pass system. Air stones were added to each tank to provide aeration. Water quality parameters were 85.5 mg/L calcium carbonate hardness, 15 mg/L chlorides, 85.5 mg/L bicarbonate alkalinity, and pH 7.2.

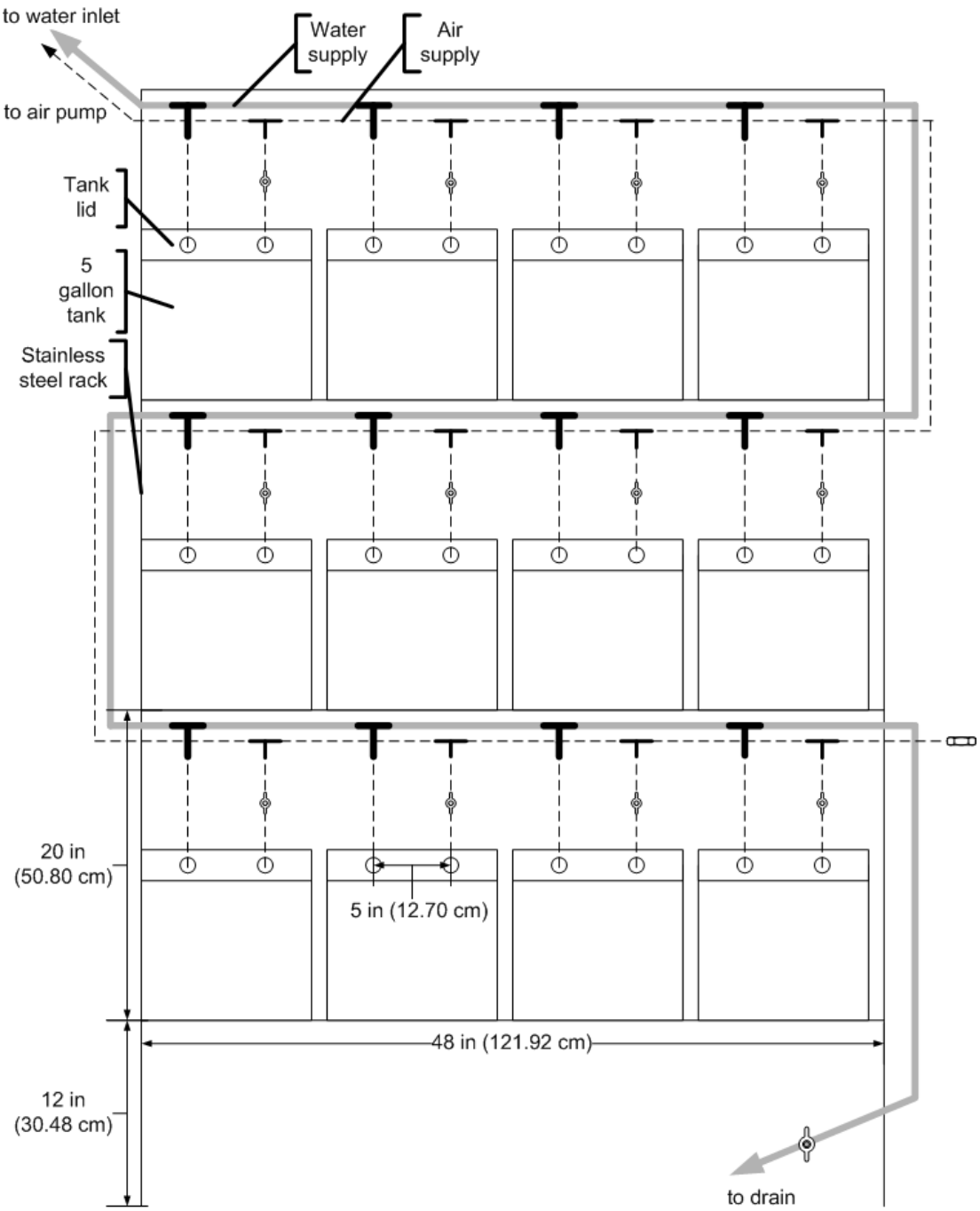

Figure 2.1 Rear view of low-cost aquatic animal holding systems developed at CVM SPF fish hatchery. All tanks are on a single-pass system with individual water inlet and covered by lids to prevent contamination. (Schematics modified from Hohn and Petrie-Hanson 2007 [2]).

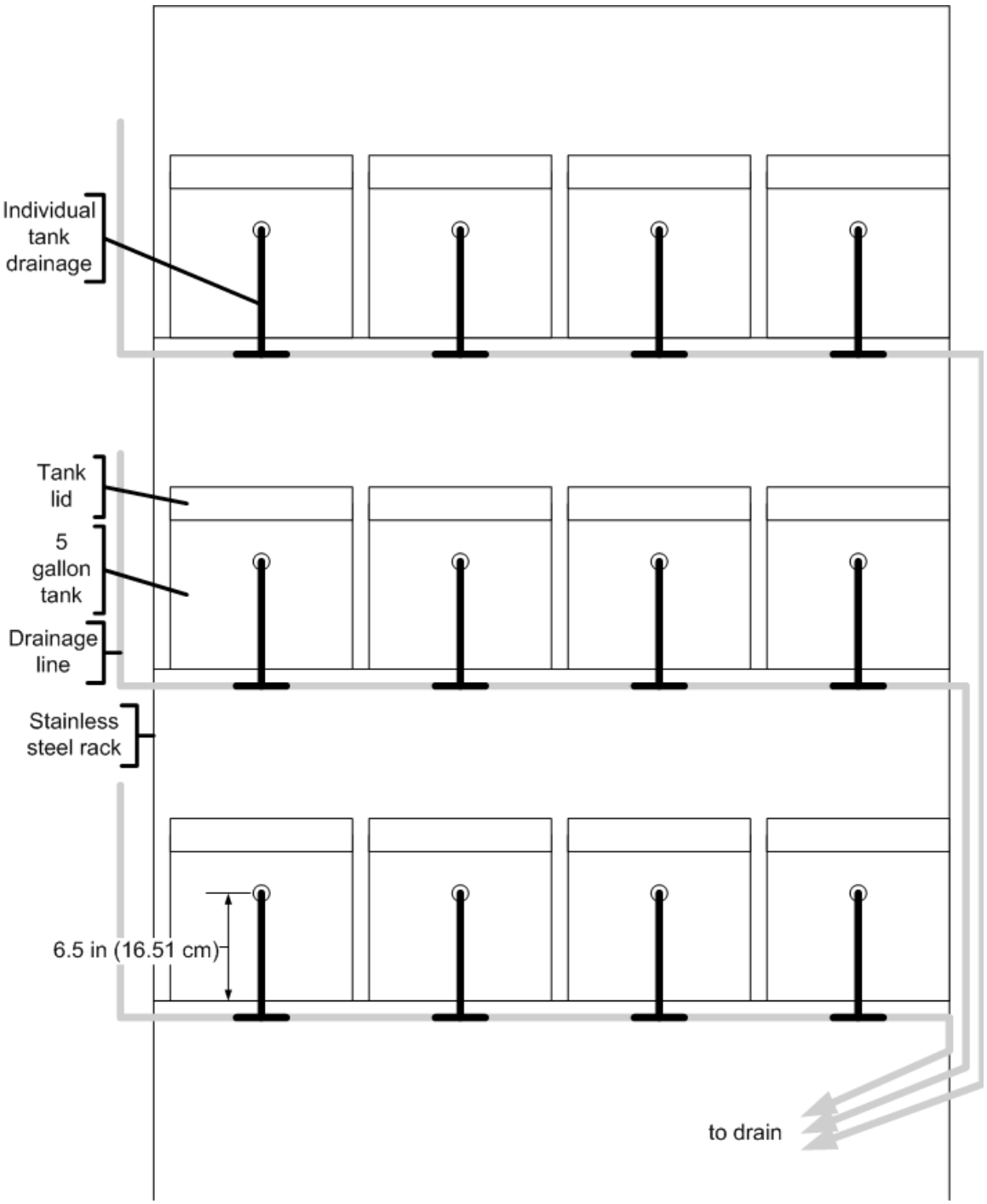

Figure 2.2 Front view of low-cost aquatic animal holding systems developed at CVM SPF fish hatchery. All tanks are on a single-pass system with individual water outlet and covered by lids to prevent contamination. (Schematics modified from Hohn and Petrie-Hanson 2007 [2]).

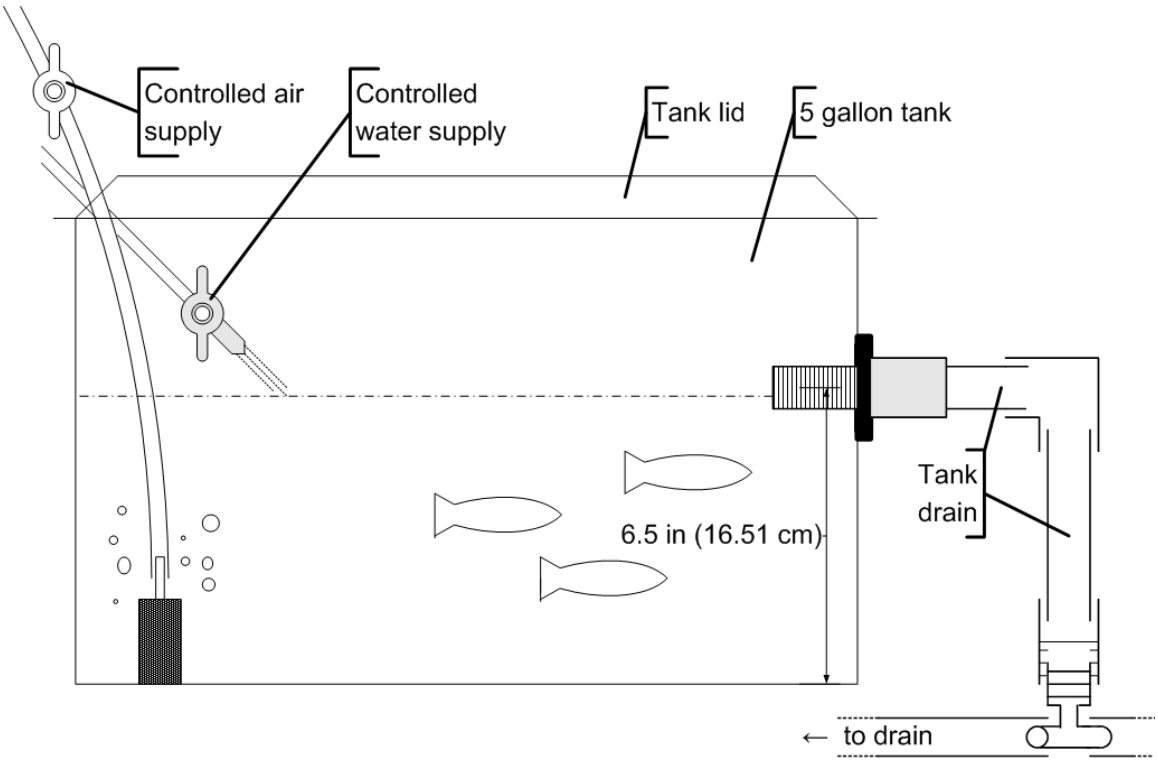

Figure 2.3 Detailed view of fish tank with indicated connection to front and back of the rack system. (Schematics modified from Hohn and Petrie-Hanson 2007 [2]).

Rearing of zebrafish under SPF conditions. General guidelines of the Zebrafish Book [53] for rearing of zebrafish were modified and simplified as mentioned below. A detailed day by day schedule for rearing and maintaining of zebrafish cultures at CVM SPF hatchery can be found at http://www.cvm.msstate.edu/zebrafish/index.html.

Spawning of zebrafish. In preparation for spawning, broodstock was fed twice a day for 5 days with Zeigler® Adult Zebrafish Diet (Aquatic Habitats™, Apopka, FL) and fresh hatched brine shrimp (Artemia International LLC, Fairview, TX). On the afternoon before spawning mating pairs of up to 20 fish

were transferred into a large cage in a clean tank on the top shelf of the flow-through holding system (Figure 2.4). The water temperature was maintained at 28.5˚C, the drain covered with 800 µm mesh, and the air diffuser adjusted to a fine aeration. The following morning the cage containing the fish was removed and the tank disconnected from the water line. Eggs were siphoned into a soft mesh net and rinsed with system water. Spawning success of MT and WT broodfish was monitored over the course of one year. To compare size of hatched fry, 10 MT and 10 WT fry 96 hpf (hours post fertilization), were measured to the nearest 0.01 mm using a dissecting microscope fitted with a calibrated scale in one eyepiece.

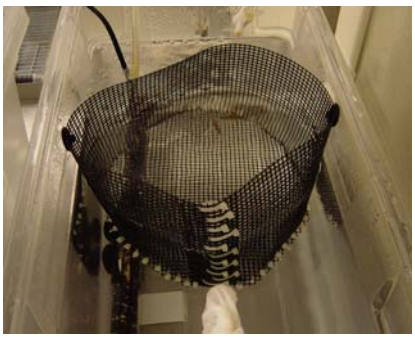

Figure 2.4 Set-up of spawning tank.

Egg treatment trial. The general procedure of treating eggs with sodium hypochlorite was compared to a methylene blue treatment.

Sodium hypochlorite treatment. Eggs were treated as described in the Zebrafish Book [53]. In short, a solution of bleach water was made by placing 0.1 ml of a 5.25% sodium hypochlorite solution and 170 ml of embryo water into a

glass bowl. One hundred eggs were placed into the bleach solution, swirled to allow separation and to allow the entire surface of each chorion to come into contact with the water. After one minute in the solution, eggs were removed and placed into a bowl of embryo water and swirled to allow thorough rinsing. The rinse procedure was repeated and the eggs were placed in 150 ml fresh embryo water, and into an incubator. Three bowls each of MT and WT eggs were prepared and survival and hatching success of embryos was assessed until 4 dpf (day post fertilization).

Methylene blue treatment. This procedure was modified from Goolish *et al.* and McAuley *et al.* [55, 56]. A working solution was prepared by mixing 100 µl of methylene blue (2%) with 3 L of system water. One hundred eggs were placed into 150 ml of methylene blue working solution and placed into an incubator (Figure 2.5). Three bowls each of MT and WT eggs were prepared and survival and hatching success of embryos was assessed until 4 dpf.

Figure 2.5 Incubator for zebrafish rearing. This is an inexpensive alternative assembled from 3 plastic cages used for rat housing and an aquarium heater adjusted to 28.5˚C.

Comparative grow-out trial: General method versus simple method. The general method for raising zebrafish fry as outlined in the Zebrafish Book [53] was compared to a new, simpler method.

General method for raising zebrafish fry (day 2 – day 10). After hatching on 2 dpf, triplicates of 40 MT and WT fish were sorted into glass bowls (470 ml) and placed in incubators. On day 3 pf glass bowls were siphoned and fresh warm system water was added. Starting 4 dpf, fry were fed 300 ml of *Paramecium multimicronucleatum* infusion. Twice a day the water was siphoned from the bowls and replaced with a strong culture of *Paramecium*. Good cultures contained about 30 unicellular protozoan per milliliter. Ten dpf fish length of 10 fish per bowl was recorded as well as survival rate.

Simple method for raising zebrafish fry (day 2 – day 10). After hatching on day 2 pf triplicates of 40 MT and WT fish were transferred to flow-through tanks (Figure 2.3) with a very slow drip (about 1 drop every 3 seconds). Starting on day 3 pf larvae were fed 1 L of *Paramecium* infusion twice a day. The tanks were cleaned by siphoning of debris every other day. Ten dpf total length of 10 fish per tank was measurements to the nearest 0.01 mm using a dissecting microscope fitted with a calibrated scale in one eyepiece.

Comparison in grow-out (day 10 – 30 pf). One flow-through tank each of 100 *rag1-/-* and *rag1+/+* fish were set-up 10 dpf and fish received 50 ml (~1500 nauplii/ml) *Artemia* twice a day in addition to *Paramecium* infusion. Uneaten

Artemia nauplii accumulate on the bottom of the tank, therefore tanks needed to be siphoned once a day. From 10-15 dpf fish were fed solely *Artemia* nauplii twice a day. After day 15 pf, and until 20 dpf, 1/8 teaspoon of shell-free Artemia (Artemia International LLC, Fairview, TX) was added to the diet. At 21 dpf, shell-free Artemia feed was replaced by Golden Pearls Larval Feed 200 – 300 µm (Artemia International LLC, Fairview, TX). At 30 dpf fish were measured with calipers and survival assessed.

Live feed production.

Paramecium culture. Sterile cultures of *Paramecium multimicronucleatum* were obtained from Connecticut Valley Biological Supply Co., Inc. (Southampton, MA). Two lines of *Paramecium* cultures, semi-sterile and non-sterile, were maintained as ongoing cultures. For the semi-sterile culture, system water was autoclaved in 1 L jars and 100 ml of a good *Paramecium* culture was added as well as 3 boiled wheat berries and a 1/4 brewers yeast tablet. Cultures were allowed to grow for 7 days before being used to start a new culture. At least 1 week before a planned spawning a non-sterile culture was started by adding *Paramecium* to 3 L beakers together with at least 20 boiled wheat berries and ½ brewers yeast tablet. When cultures were selected for feeding, they were placed in a warm water bath over-night and filtered through a 150 µm sieve just before the feeding.

Artemia culture. *Artemia franciscana* cultures were maintained as batch cultures. One 2 L culture was harvested each morning and afternoon and fed fresh to fish < 3 month or frozen in ice cube trays as a backup. For the culture, 28˚C aerated municipal water was mixed with 1 tablespoon of Jungle® aquarium salt (Aquatic Eco-Systems, Inc., Apopka, FL) and 2 teaspoons of Great Salt Lake Artemia Cysts 90% (Artemia International LLC, Fairview, TX). After 24 hours of incubation, *Artemia* nauplii were filtered into different size fractions using 300µm, 200 µm, and 100 µm sieves. The smallest fraction was fed to fry younger than 12 days. The other fractions were pooled and suspended in 2 L of system water before being fed.

Statistical analysis. Statistical analysis of data was performed by independent samples T-test with a level of significance at $p \leq 0.05$. Statistical analyses were performed using SPSS for Windows 15.0 (SPSS Inc., Chicago, IL).

Results

Comparison of rearing success of zebrafish under SPF conditions.

Spawning success. It was found that spawning was most reliable when fish where kept under previously described conditions. Conditioning of fish for breeding, as suggested by the Zebrafish book [53], by feeding life feed and additional dry feed resulted in good quality and quantity of eggs. One breeding pair averaged 350 – 500 eggs per spawning using the here described method.

No difference in spawning success was observed between MT and WT broodstock. No significant difference (p= 0.26) in total length of fry (96 hpf) was found. WT fry averaged 3.94 mm (± 0.12) and MT fry averaged 3.88 mm (± 0.11).

Egg treatment. All eggs incubated in methylene blue solution hatched on day 2 pf. Average hatching rate in WT eggs was 69% and 77% in MT eggs (Figure 2.6). WT eggs treated with bleach solution prior to incubation had only a 19% and MT eggs averaged a 42% hatching rate 2 dpf. Hatching rate on 2 dpf in WT fish was significantly higher (p= 0.014) in eggs treated with methylene blue when compared to eggs treated with bleach. Numerical differences between treatments were observed in MT fish. Eggs incubated in bleach solution continued to hatch until 4 dpf. 4 dpf an average of 25% of WT and 61% of MT eggs hatched (Figure 2.6).

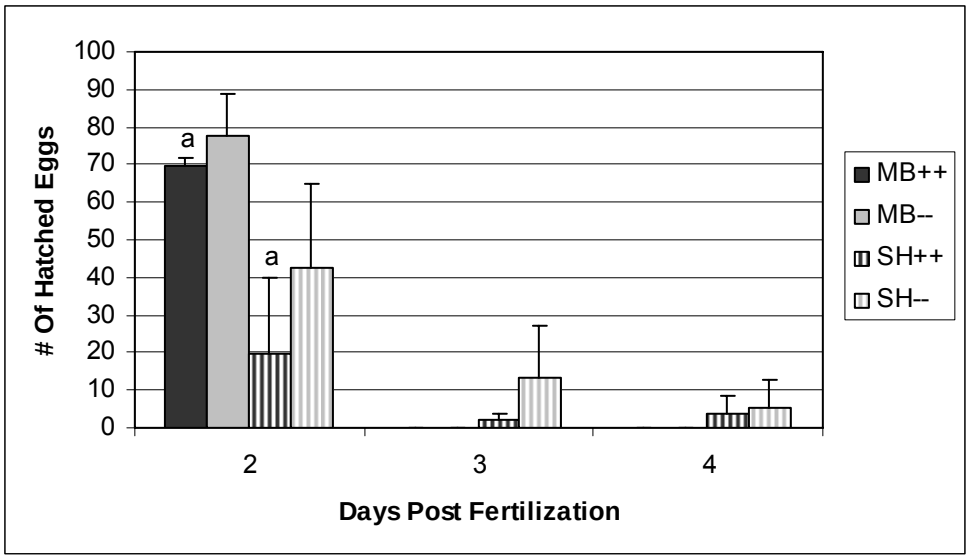

Figure 2.6 Number of eggs hatched (with standard deviation) of WT (++) and MT (--) eggs after methylene blue (MB) incubation or sodium hypochlorite (SH) treatment. Significant difference (p= 0.014) in WT fish between treatments was observed (a).

General method versus simple method in raising fry. The same survival rate of 89% between 2dpf and 10dpf raised by the general method and the simple method was observed in WT fish. MT fish had similar survival rates of 92% when raised by the simple method and 91% raised by the general method. When comparing total growth between the 2 different grow-out methods, WT fry grown in flow-though tanks recorded a significantly higher ($p=0.024$) total length of 6.64 mm compared to fry grown in glass bowls 6.43 mm (Figure 2.7). A significantly lower ($p<0.001$) total growth was observed in MT fry grown under either method when compared to WT fry. MT fry raised by the simple method grew to 6.01 mm and under the general method to 6.12 mm (Figure 2.7).

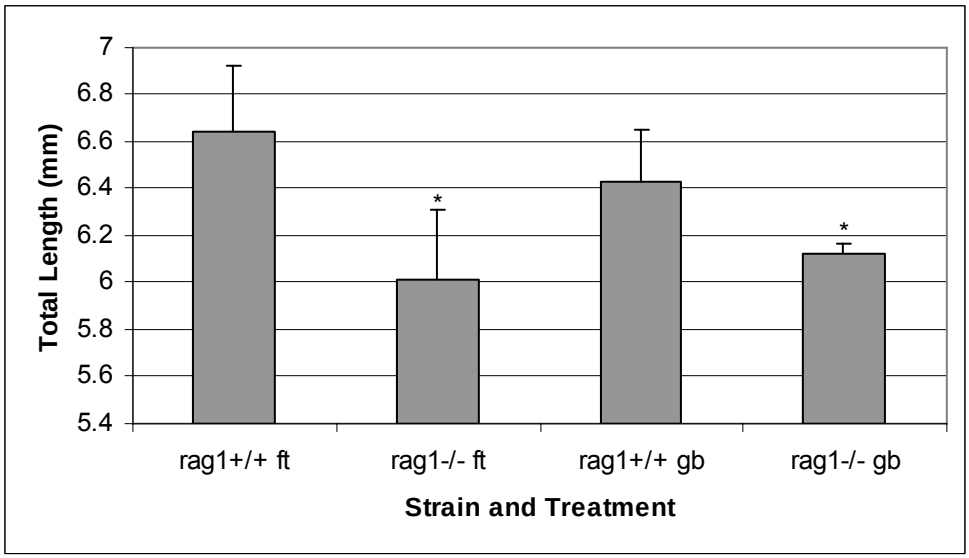

Figure 2.7 Comparison of total length (with standard deviation) of zebrafish fry 10 dpf. WT and MT fry were grown either by the simple method in a flow-through system (*rag1+/+* ft or *rag1-/-* ft) or by the general method in a glass bowl (*rag1+/+* gb or *rag1-/-* gb). MT fish show significant lower growth in the flow-through system and the glass bowl (*) when compared to WT fish.

Grow-out comparison. After 30 dpf, survival rate of WT fish was 82%, and MT fish had a lower survival rate of 73%. Total length was significantly higher ($p=0.006$) in WT fish and averaged 2.41 cm (± 0.25) compared to 2.13 cm (± 0.12) in MT fish.

Discussion

Uncertainty over whether of not *rag1-/-* deficient MT fish can be bred without acquiring diseases at a higher rate [1] and later reports of the unknown cause of death in MT fish that have been tail clipped for DNA analysis [12] initiated the need for improved handling protocols and a SPF zebrafish facility. Commercially available stand-alone zebrafish holding systems were prohibitively

expensive. In addition, these systems are designed to re-circulate the water, an undesired feature when dealing with immunocompromised fish. Therefore, a zebrafish rack system that is affordable for small laboratories, easy to assemble and decreases the risk for spreading diseases by using a flow-through design, was developed [2]. Since the system is designed as single pass, minimal water quality monitoring is required. In addition, the incoming water is filtered (1 µm) to decrease chances of contamination by pathogens and reduce organic pariculates. Covered tank units minimize spread of pathogens between tanks and can be easily removed for cleaning and sanitation. The described system together with IACUC (Institutional Animal Care and Use Committee) approved SPF maintenance Standard Operating Procedures have successfully reduced the spread of pathogens between tanks, minimized loss of zebrafish and enabled successful maintenance and reproduction of WT and MT zebrafish for over 3 years. To date, this is the first recorded *rag1-/-* continuous ongoing zebrafish line.

A single female zebrafish lays between 150 – 500 eggs per spawn [57, 58] the reported 350-500 eggs clutch size is within this range and indicates good husbandry practices. However, zebrafish eggs treated with sodium hypochlorite, the recommended method by the zebrafish book [53] to prevent fungal overgrowth, showed unsatisfactory hatching rates with high variability. It was found that over 50% of the eggs were dead shortly after the treatment and resulting eggs had to be dechorionated due to a tough chorion, a by-product of the bleaching procedure. Several other treatments including washing of eggs in sterile egg water resulted in fungal overgrowth and eggs had to be discarded

(data not shown). Therefore there was a need for a more reliable and time saving technique. Methylene blue treatment of zebrafish eggs was described by only a few investigators [55, 56] and no information was available on hatching rates or hatching time. Our data shows that treating eggs with methylene blue increases hatching rate and decreases hatching time. No tough chorion forms when eggs are treated with methylene blue and hatching is completed 2 dpf. In addition the methylene blue treatment is much less laborious and produced reproducible results. Since system water is used for the methylene blue treatment, no sterile egg water is necessary; this is convenient because most hatcheries, including the SPF hatchery at CVM, do not have sterile water available within the building.

A new grow-out protocol was developed, where 2 dpf fry were transferred into 13 L flow-through tanks. This simple method was compared to a general method in which fry were held in static containers (470 ml). Many protocols are available for raising zebrafish. Traditionally, zebrafish younger than 15 dpf are held under static conditions in containers [53, 59, 60] or in baby tubes, tubes with mesh bottom suspended in larger tanks [61]. Static systems have the advantage that food is not flushed out and is available for continuous feeding. On the downside, overfeeding with *Artemia* may lead to high ammonia and nitrite levels [59]. To decrease the adverse effects of poor water quality on fry growth and survival, frequent water changes are necessary. In addition only a very limited number of fry can be held per static container, usually 100 fry per 1L. This is the main reason why static systems are labor intensive, especially if a high production of zebrafish fry is needed. The new simple method described reduces

maintenance time considerably. The flow-through tanks were set-up on a very slow drip so feed is available until the next feeding time but water is replaced continuously and water parameters remain stable. Feed amount was adjusted for the larger volume and flow-through set-up. To compare the simple method with the general method, the same amount of fry (40/tank) was set-up in static and flow-through tanks. In daily hatchery operations up to 500 fry have been raised in one flow-trough tank following this method, decreasing laborious cleaning and space requirements. The efficacy of a rearing protocol can typically be measured in growth rate and survival of fish [52]. The data presented for both strains shows that survival is equally good in both methods. In general survival of 90% is considered good and inbred strains usually have survival rates of 60-85% (www.neuro.uoregon.edu/k12/FAQs.html#Percent). Published data on fry 10 dpf fed with live feed, gives results well below the growth results presented here. In a study in which different rearing temperatures were compared larvae reached only 3.6 mm when reared at 28˚C [62], in a nicotine study the control group was recorded at a total length of 3.58 mm [63], in another study for gas bladder inflation the control group averaged 5.29 mm [64]. In comparison WT fish grown under the simple method averaged a total length of 6.64 mm and MT fish 6.01 mm at 10 dpf. Zebrafish fry measured just before external feeding at 4 dpf showed no significant difference in length, therefore MT fry have a slightly slower growth rate than WT fry. Reduced growth in mutant lines is observed quite regularly by other investigators (personal communication with David Lains, Zebrafish International Resource Center). Overall, MT fish demonstrate good

survival and growth rates that lie well above published data. The survival and growth data presented supports the successful development of a new simple method for raising zebrafish.

The few published reports of survival rates in the literature are inconsistent. The most detailed information is available form dietary studies. In their evaluation of the performance of larval zebrafish on ten different artificial diets, Goolish *et al.* [55] reported survival rates in the range of 15 – 80 % through 28 dpf, depending on diet and method of food delivery. Mean survival among treatment groups in the study by Önal and Langdon [65] ranged from 40 -98% for a similar time period. Carvalho *et al.* [66] reported mean survival ranging from 55 – 86 % at 27 dpf, again depending upon diet and frequency of application. In this study, survival rates until 30 dpf were in the higher end of reported survival rates by others and therefore are considered good. The total length data of mutant fish after 30 days are significantly lower than the wild-type fish but when compared to the literature these growth data are outstanding. The results of published studies detailing larval growth varied widely. For example in a recent study, Biga and Goetz [67] reported an average total length of 4.7 mm at 28 dpf. These results contrast markedly with those of Carvalho *et al.* [66] who documented average total lengths of up to 14.3 mm for the same time period. In another study by Barrionuevo and Burggren [62] fish grew only 7.2 mm [62]. These differences are remarkable, but are probably indicative of discrepancies in rearing environment, specifically diet. This further highlights the need for establishing standardized, biologically based rearing protocols [52] and diet (D'Abramo, personal

communication). With the data presented here, an attempt to standardize rearing protocols for the CVM SPF fish hatchery was made. To be able to compare data in epidemiological studies or other biomedical and immunological research it is crucial to have standardized protocols to obtain good quality and healthy offspring of laboratory animals on a consistent basis.

In conclusion, rearing protocols have been optimized and standardized to deliver healthy zebrafish, meeting special needs of MT strains, on a consistent basis and to decrease maintenance time. The presented data will aid in the effort to further refine both diet and rearing protocols within the zebrafish community.

CHAPTER III

CHARACTERIZATION OF *RAG1-/-* DEFICIENT MUTANT ZEBRAFISH FOR IMMUNOLOGICAL RESEARCH

Abstract

The establishment of a breeding population of *rag1-/-* deficient mutant zebrafish and an initial evaluation of the leukocyte populations in these fish is reported. Larvae from an F3 spawn of zebrafish heterozygous for a point mutation in the *rag*1 gene were received. These fish were reared for 2 months then DNA from fin clips were evaluated by 2 PCR assays using specific primers: one for the gene point mutation *rag1*^{t26683}, the other for the wild-type allele. Identified *rag1-/-, rag1+/- and rag1+/+* adults were differentially marked by visible implant elastomer (VIE) tagging. These fish served as brooders to establish homozygous *rag1-/-* mutant (MT) and homozygous *rag1+/+* wild-type (WT) populations. Differential counts of peripheral blood leukocytes indicated that MT fish had decreased lymphocyte populations (34.7%) compared to WT (70.5%), and increased granulocyte populations (34.7%) compared to WT (17.6%). Differential leukocyte counts of MT kidney hematopoietic tissue showed 8% lymphocyte, 57% myelomonocyte, and 35% precursor cells, WTs showed 29% lymphocytes, 36% myelomonocytes, 34% precursors and 1% thrombocytes.

Flow cytometric analyses of kidney hematopoietic tissue revealed three leukocyte populations, designated A, B and C. Population A consisted of lymphocytes and comprised 7% of the cells sorted in the MT and 26% in the WT fish. Population B consisted of hematopoietic precursors, and comprised 50% of the gated cells for MT and 53% for WT fish. The C population was monocytes and granulocytes and comprised 34.7% of the gated cells in MT and 17.6% in WT fish. Pooled kidney hematopoietic tissues from 30 fish of each strain were also sorted by a BD FACS Aria high-throughput flow cytometer, and approximately 1.5 million lymphocyte-like cells (Population A) were collected from MT fish.

Analysis of DNA from kidney tissues by RT-PCR demonstrated MT zebrafish expressed Non-specific Cytotoxic cell receptor protein-1 (NCCRP-1) and Natural Killer cell (NK) lysin but lacked T cell receptor (TCR) and immunoglobulin (Ig) VH1, VH2, VH3 and VH4 expression, while WT zebrafish expressed NCCRP-1, NK lysin, TCR and Ig VH1-VH4. Our study demonstrates that a breeding population of *rag1-/-* MT zebrafish can be established and that these fish lack functional B and T lymphocytes yet retain NK cell populations.

Introduction

The zebrafish has many advantages for use in immunological research. Zebrafish can mount innate and adaptive immune responses much like higher vertebrates and they demonstrate a high degree of synteny to the human genome [10, 12]. The zebrafish genome has been sequenced in its entirety, so

zebrafish/pathogen interactions can be readily investigated at the molecular level. Current methods for targeted gene silencing, the presence of many mutant strains and the ease of producing specific mutants make the zebrafish an excellent model for experimental immunology [29]. Zebrafish may prove to be the best model for innate immune responses. The immediate, inducible and nonspecific characteristics make the innate immune response a critical line of defense in all vertebrates. This system is even more important in the protection of ectothermic animals because it is more independent of temperature changes than is the acquired immune response [68].

The development, propagation and characterization of mutant and/or knock out zebrafish, is critical to help define cell and gene functions in zebrafish immunology. Much of the research on inducible components of innate and acquired immunity is based on *in vitro* research utilizing isolated or cultured cells. These types of studies are useful for dissecting specific mechanistic pathways but are artificial, in that unknown cellular interactions that occur in the whole animal are removed from the system. With the production of mutants and specific knock out mice, components of the immune system could be dissected in the whole animal model. The use of SCID and *recombination activation gene 1* (*rag1*) mutant mice has allowed the investigation of the specific contribution of innate defenses in many infectious diseases. Both mutants have normally functioning macrophages, NK cells and neutrophils, but lack T and B lymphocytes [69, 70].

Similar mutants can now be used in fish disease research. Reverse genetics, the ability to inactivate a given gene in an entire animal, is an established technique in zebrafish research [12]. Targeting induced local lesions in genomes (TILLING), an alternative to standard mammalian gene targeting, has proved efficient in yielding mutant zebrafish [1]. TILLING was used to identify a series of 15 *rag1* mutations. One of these is a point mutation that causes a premature stop codon in the catalytic domain of RAG1. A functional RAG1 protein is required for V(D)J recombination when generating functional immunoglobulin and T cell receptor (TCR) genes [17]. As in other vertebrates, there is only one functional *rag1* gene; loss of function at this locus results in a complete block of immunoglobulin gene assembly and, presumably, in immunodeficiency [1]. Further it is stated that these presumably immunodeficient fish survive to adulthood without obvious signs of infectious disease in a nonsterile environment. However, these individuals did not survive fin clipping required for DNA analysis (Wienholds, personal communication).

The aims of this project were to develop non-lethal DNA sampling and tagging identification procedures, confirm the status of the *rag1* point mutation in MT zebrafish, establish a breeding colony, and describe adult MT and WT blood cell populations by DNA, morphological and flow cytometric analyses.

Material and Methods

Mutant and wild type zebrafish production. Zebrafish used in this study were propagated and reared in the College of Veterinary Medicine's Specific

Pathogen Fish Hatchery following our Standard Operating Procedures, 2006, CVM-MSU (http://www.cvm.msstate.edu/zebrafish/index.html), and housed in flow-through zebrafish systems [2]. Briefly, adult zebrafish were held in 19 L tanks with charcoal filtered de-chlorinated municipal water at 25°C supplied to each tank at a rate of 0.5 L/min in a flow through, single pass system. Air stones were added to each tank to provide aeration. Water quality parameters were 85.5 mg/L calcium carbonate hardness, 15 mg/L chlorides, 85.5 mg/L bicarbonate alkalinity, and pH 7.2. Adult zebrafish were fed Zeigler™ Adult Zebrafish Diet (Aquatic Habitats™, Apopka, FL) and Golden Pearls Larval Diet (Artemia International LLC, Fairview, TX) brine shrimp (*Artemia)* nauplii twice daily (Aquatic Eco-Systems, Inc., Apopka, FL).

Non-lethal sampling procedure. The Tübingen 2000 Screen Consortium[1] provided a F3 generation clutch of eggs produced by breeding *rag1* +/- heterozygous zebrafish [1]. This clutch produced embryos with all three expected genotypes: *rag1* +/+ (WT) and *rag1* -/- (MT), and *rag1* +/- (heterozygous).

At 2 months post-hatch (mph) all fish were screened for the point mutation within the *rag1* gene. Approximately ½ of the caudal fin was removed from each

[1] *Max-Planck-Institut fur Entwicklungsbiologie, Tübingen:* Bebber van, F. Busch-Nentwich, E., Dahm, R., Frohnhöfer, H-G., Geiger, H., Gilmour, D., Holley, S., Hooge, J., Jülich, D., Knaut, H., Maderspacher, F., Maischein, H-M., Neumann, C., Nicolson, T., Nüsslein-Volhard, C., Roehl, H., Schönberger, U., Seiler, C., Söllner, C., Sonawane, M., Wehner, A., Weiler, C. *Exelis Deutschland GmbH, Tübingen:* Erker, P., Habeck, H., Hagner, U., Hennen, Kaps, C., Kirchner, A., Koblizek, T., Langheinrich, U., Loeschke, C., Metzger, C., Nordin, R., Odenthal, J., Pezzuti, A., Schlombs, K., deSantana-Stamm, J., Trowe, T., Vacun, G., Walderich, B., Walker, A., Weiler, C.

fish for use in genotyping (Figure 3.1). Each fish was then placed in an individual 1 L container containing recovery water (42 mg/L aquarium salt, and 0.04 g/L Jungle Fungus Eliminator (Aquatic Eco-Systems, Inc., Apopka, FL, active ingredients: sodium chloride, nitrofurazone, furaxolinone, potassium dichromate) in distilled water), and held off-feed for 4 days. Treatment water was replaced daily, and fish were held separately until PCR results were known. Before returning to the general population, fish were implanted with visible implant elastomer (VIE) tags. Different colors were used to identify MT, WT and heterozygous individuals. Tagged zebrafish were transferred to 19 L holding tanks, grouped by *rag1* mutation status, and maintained as breeding populations.

Figure 3.1 At the age of two months post-hatch, half of the zebrafish caudal fin was dissected and processed for DNA analysis. The vertical solid line represents the dissection site.

VIE tag trial. Visible implant elastomer was prepared according to kit instructions (Northwest Marine Technology, Inc., Shaw Island, WA, USA, www.nmt.us). To determine optimal tagging sites, groups of 5 adult zebrafish were implanted with a 5 mm strip of pink elastomer at one of the following sites: abdomen, pectoral fin base, anal fin base, caudal peduncle, dorsal fin base or isthmus (Figure 3.2). Fish groups were held for 20 days, after which tag retention was determined at each site.

To determine optimal tag color, 2 injection sites (dorsal fin base and pectoral fin base) were separately evaluated with 10 fish per site and color group (yellow, red, pink, orange, blue); each fish was implanted with one color at one site. Fish groups were held for 20 days, after which tag color visibility was determined at each site.

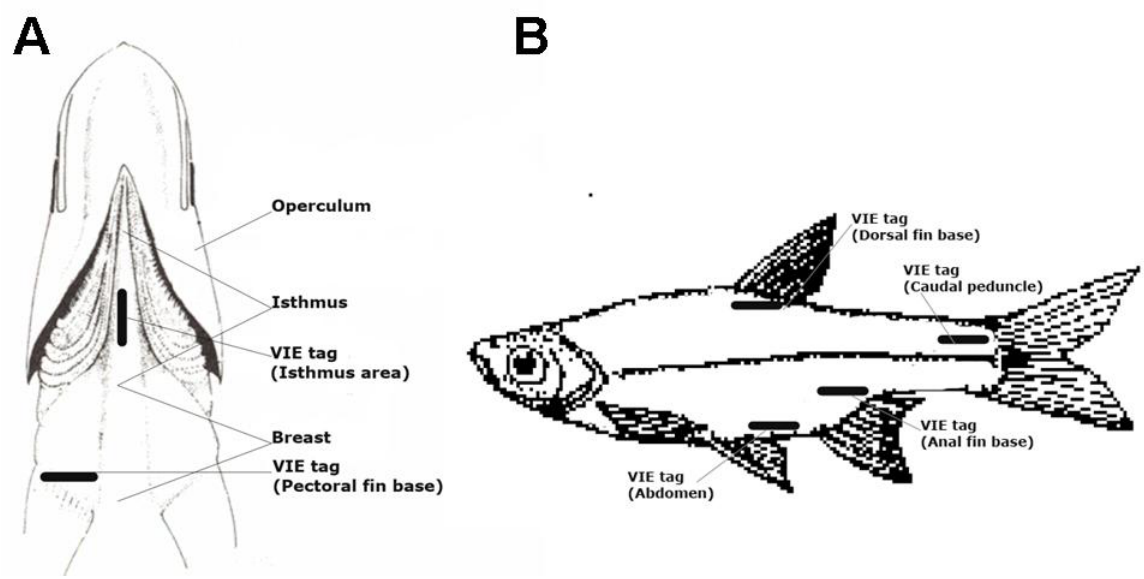

Figure 3.2 Visible elastomer (VIE) tagging sites. A) abdominal view B) side view.

PCR determination of the presence/absence of the *rag1*^{t26683} point mutation. Genomic DNA was isolated from fin clips using a genomic DNA isolation kit (Gentra® Puregene® Tissue Kit, Qiagen Inc., Valencia, CA). The sample was incubated in 600 µl cell lysis solution containing 60 µg proteinase K, and incubated overnight at 50°C. The DNA was then isolated according to the manufacture's suggested procedure and resuspended in 100 µL of hydration

solution. DNA was quantitated using UV spectrophotometry (GeneSpec I, Hitachi Software Engineering Company LTD, Japan).

To identify zebrafish with the *rag1* mutant allele (containing a non-sense point mutation) in the F3 offspring, a PCR based method that was developed to detect single nucleotide polymorphisms by Waterfall & Cobb [71] was modified. Primers were designed using Oligo Primer Analysis Software V6.71 (Molecular Biology Insights Inc., Denver, CO) on Genbank accession NM_131389.1 with manual editing of the C to T for designing the specific primer for the transition mutation (Figure 3.3).

In the first PCR for the semi-nested assay, primers RAG2000U20 and RAG2623L18 were used to generate a 641bp product (Table 3.1 and 3.2, Figure 3.3). This product was then used as template for two PCR assays: the first using primers MRAG2371U19 and RAG2623L18 to generate a 270bp product to evaluate the presence of the mutant allele and the second using primers RAG2000U20 and RAG2389L20 to generate a 400 bp product to evaluate the presence of the WT allele (Table 3.1 and 3.2, Figure 3.3). Takara Hot Start *Taq* polymerase and reagents were used (Fisher Scientific, Pittsburgh, PA) (Table 3.2). The reaction conditions for the first stage were 30 sec at 95˚C, 1 min at 60˚C, 1 min at 72˚C for 32 cycles. For the second stages 4 µL of the product from the first stage was used as template and the appropriate allele specific primer sets were used. The reaction conditions for the second stage were 30 sec at 95˚C, 1 min at 71˚C, 1 min at 72˚C for 36 cycles. Reaction products were evaluated by electrophoresis on 1.5% agarose gels with a 100 bp ladder

(Invitrogen™, Carlsbad, CA) as a size marker. The gels were stained with GelStar® Nucleic Acid Gel Stain (BioWhittaker Molecular Applications, Rockland, ME), UV transilluminated and digitally imaged (ChemiImager 5500, Alpha Innotech Corporation, San Leandro, CA).

Table 3.1 Oligonucleotide pimers used to detect the C to T transition in the *rag1* gene (accession U71093) and product size.

Gene/Fragment	Forward Primer	Reverse Primer	Size (bp)
RAG2000-RAG2623 (first round)	AGCCCAACTCTGAACTCTCC	CATCAGCCGACGAGCATA	641
RAG2000-RAG2389 (second round)	AGCCCAACTCTGAACTCTCC	GCAGAAACACCTTTGACTCG	400
MRAG2371-RAG2623 (second round)	GCAGACGAACTGCGTGACT	CATCAGCCGACGAGCATA	270

Table 3.2 Detailed listing of master mix reagents and volumes used for genotyping.

Reagent/Template	641bp	400bp	270bp
DNA	1 µl (~0.35 µg/µl)	4 µl (641bp template)	4 µl (641bp template)
Water (nuclease free)	18.875 µl	15.875 µl	15.875 µl
10X PCR Buffer (contains 15 mM $MgCl_2$)	2.5 µl	2.5 µl	2.5 µl
dNTP Mixture (2.5 mM each dNTP)	2 µl	2 µl	2 µl
Forward primer (20pmol/µl)	0.25 µl (RAG2000)	0.25 µl (RAG2000)	0.25 µl (RAG2389)
Reverse primer (20pmol/µl)	0.25 µl (RAG2623)	0.25 µl (MRAG2371)	0.25 µl (RAG2623)
Taq HS DNA Polymerase (5 U/µL)	0.125 µl	0.125 µl	0.125 µl
Reaction volume	25 µl	25 µl	25 µl

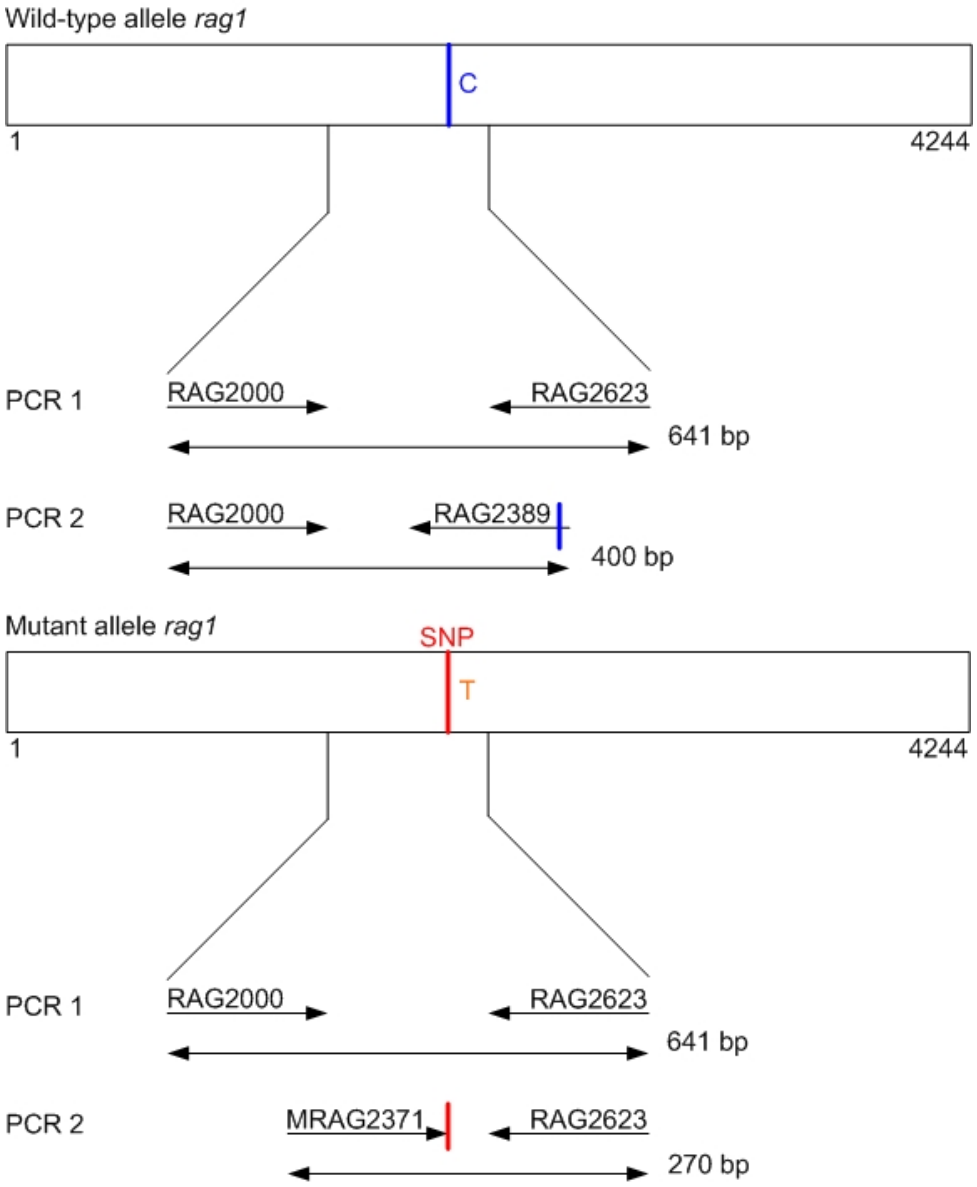

Figure 3.3 Semi-nested PCR to screen for wild-type and mutant allele of sequence U71093 *rag1 Danio rerio*. A 641 bp fragment was amplified from all fish during the first round of PCR. In a second PCR reaction, the wild-type allele produced a 400 bp, while the mutant allele produced a 270 bp product. Indicated are forward and reverse primers for first and second (semi-nested) PCR. Red highlights show site of SNP of interest while blue indicates wild-type nucleotide.

Differential leukocyte counts. Blood was harvested from adult zebrafish by making a lateral incision just posterior to the dorsal fin in the region of the dorsal aorta, avoiding puncture of the gastrointestinal tract [14]. Blood welling up from this incision was rapidly collected in a heparinized 10 µl micropipette tip and used to prepare blood smears. Smears were fixed and stained using a Hema-3 Stat Pack (Biochemical Sciences, Inc., Swedesbord, NJ) according to the manufacturer's instructions. Blood cells were viewed on an Olympus BH-1 microscope and differential leukocyte counts were performed based on morphological appearance, and were identified based on previous descriptions of zebrafish or comparative teleosts [14, 15, 72, 73]. Differential counts were made on ten MT and ten WT zebrafish. One hundred leukocytes were counted on each slide. Thrombocyte numbers were determined by counting the number of thrombocytes seen while counting 100 leukocytes. Differential leukocyte counts on kidney tissue were done as described above except smears were made from cell suspensions produced from excised kidney tissue as described below.

Flow cytometry. MT and WT kidney interstitial cells were examined by FACS (FACS Calibur, Becton Dickinson) forward scatter (FSC) and side scatter (SSC) analyses. FSC represents cell diameter, and SSC represents cell granularity or complexity. The kidney of each of four MT and four WT adult zebrafish were surgically removed and processed. Each kidney was transferred into a centrifuge tube filled with 200 µl of PBS and 1% FBS. Cells were freed from the tissue by repeated pipetting using a P1000 pipettor. The cell suspension

was strained, using an additional 200 µl PBS and 1% FBS, through a 40 µm Falcon® nylon cell strainer (Becton Dickinson) to eliminate connective tissue. The cell suspension was kept on ice until analyzed by flow cytometry. Mutant and WT kidney cells were also sorted and collected by BD high through-put FACS Aria FSC and SCC analyses, following collecting and pooling of kidney tissue from 30 fish of each strain, as described above.

Identification of mutant zebrafish cells. To determine if the cells that demonstrated lymphocyte morphology were T cells, B cells or NK cells, we performed multiple PCR reactions on adult MT and WT zebrafish. To test for functional B cells, we determined the presence of V(D)J rearrangements of the immunoglobulin (Ig) heavy-chain genes *Igµ* in B cells using rtPCR. Published primers [22, 74] were used in this Ig V(D)J rearrangement study. A PCR product is obtained only if recombination occurs. To test for functional T cells we utilized published primers in rtPCR to screen MT and WT zebrafish RNA for TCR expression [22]. To determine if the MT and WT had NK cells and NCC cells, we designed specific primers for the 3 forms of NK lysin and NCCRP-1.

RNA was isolated from adult zebrafish using TRI REAGENT® (Molecular Research Center, Inc., Cincinnati, Ohio) according to the manufacture's protocol. RNA was quantified using the NanoDrop® ND-1000 Spectrometer (NanoDrop Technologies). Prior to RT-PCR, RNA was DNase treated using the RQ1 RNAse-Free DNase kit (Promega Corporation, Madison, WI). For cDNA synthesis from total RNA, the protocol SuperScript™ III First-Strand Synthesis

System for RT-PCR (Invitrogen Corporation, Carlsbad, Ca) was followed using oligo (dT)20 as primers. After verification of successful synthesis of first-strand cDNA, 5 µl of cDNA was used for PCR amplification of NK lysin form 1 (NK cell lysin 1) (accession number NM_212741), NK cell lysin form 2,3 (NK cell lysin 2,3) (accession number XM690179), NCC receptor protein (NCCRP-1) (accession number NM_130921), and as positive control elongation factor 1-alpha (EF1-α) (accession number NM_131263.1) (Table 2.3). The temperature profile used was 35 cycles of 94° C for 30 sec, 58 °C for 30 sec and 72 C for 1 min.

Table 3.3 Oligonucleotide primers used for RT-PCR.

Gene/Fragment	Forward Primer	Reverse Primer	Size (bp)
NK cell lysin 1	CCAGCTAAAGCAAAACCC	GGAAAGGTGAAACGGAAG	293
NK cell lysin 2,3	TTCATTCATGTTGGCGTGACA	TTGATTTCATCTGGCGTTGAG	450
NCCR	GGAAGCTGGCAGATCACAACA	ACGGTGTCCCAATGCCT	268
EF1-α	ATGGCACGGTGACAACATGCT	CCACATTACCACGACGGATG	392

Results

Non-lethal DNA sampling procedure and confirmation of *rag1* mutation.

Caudal fin sampling of the 2 mph zebrafish with subsequent prophylactic treatment resulted in 100% survival in all groups. Sampling wounds healed after 5 days and after 7 days the fish were tagged and returned to the general MT or WT population. Dissected fins completely re-grew within 1 month. Fin DNA yields ranged from 15-20 µg. Genotyping was performed using this template and identified homozygous WT, homozygous MT and heterozygous fish

(Figure 3.4). The homozygous fish were used as brooders to produce the WT and MT zebrafish used in these studies. When fin-clipped MT fish were not prophylactically treated as described above, or when they were moved from the SPF fish hatchery, they succumbed to secondary bacterial infection.

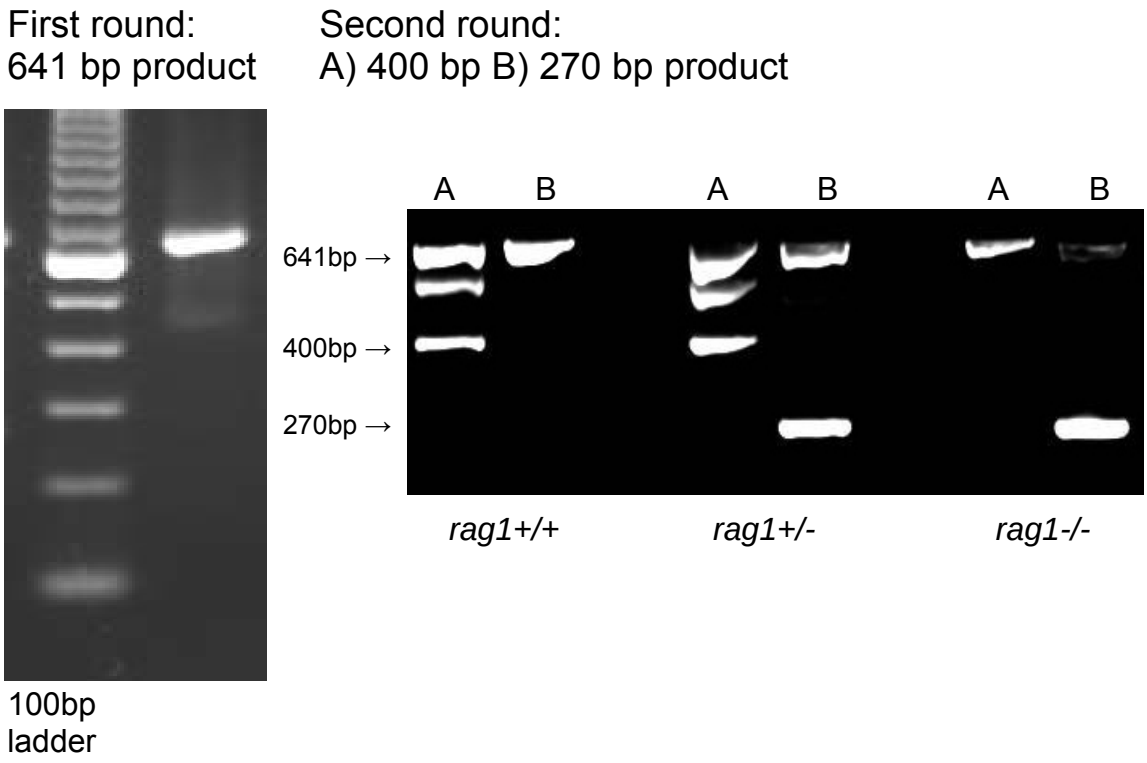

Figure 3.4 Genotyping of broodstock. A 641 bp product is produced first and used as template in a second semi-nested PCR. *Rag+/+* fish produced only a 400 bp product, *rag+/-* fish produced both products, and *rag-/-* fish only produced a 270 bp product.

VIE tag trial. In order to optimize VIE tagging, location (abdomen, pectoral fin base, anal fin base, caudal peduncle, dorsal fin base or isthmus) and color were evaluated in non-screened WT adult zebrafish. In the location study, injection site healing, ease of application, elastomer retention and elastomer

visibility were evaluated. The abdomen was a difficult injection site. One elastomer was lost, 3 were minimally visible, and one was visible. The pectoral fin base was also a difficult injection site. However, all elastomers were retained, all were easily viewed, and the injection site appeared to heal well. The anal fin base injections resulted in 2 lost elastomers, and in 3 fish the injection site remained open and the elastomer was trailing from the site. When the caudal peduncle was injected, all elastomers were lost. Dorsal fin base elastomer injections were all retained and the injection site healed well. However, the tags were very difficult to see. The isthmus was a difficult injection site and only a small amount of elastomer could be injected. All elastomers were retained, but were very difficult to see. Overall, the best injection sites were the dorsal and pectoral fin bases. However, the pectoral fin base was more difficult to inject and if not carefully done can fatally injure the fish. All tags in the dorsal and pectoral fin bases were still visible 6 months after the initial injections.

When different colors were compared (at the dorsal fin bases and pectoral fin bases), all tags were retained and tagging wounds healed. Tags in the dorsal fin base were visible without handling the fish. Yellow and orange as well as pink and red were difficult to distinguish from each other in the dorsal fin base site. Blue was not visible at ambient light but was visible when fluoresced by UV light. The best colors to use in the dorsal fin base were red, pink, orange and yellow.

Peripheral blood differential leukocyte counts. Zebrafish peripheral blood cells included erythrocytes, thrombocytes, monocytes/macrophages,

granulocytes and lymphocytes (Figure 3.5). Erythrocytes were elliptical with the nuclei ranging in shape from round to elliptical, depending on developmental stage. The nuclear chromatin pattern was ribboned and very dense. The cytoplasm was faintly eosinophilic. Thrombocytes had round nuclei and very scant, faintly eosinophilic cytoplasm that narrowed, resulting in a spindle shape. The nuclear chromatin was very dense. Thrombocytes typically rafted in blood and tissue smears. Lymphocytes were round with a high nuclear to cytoplasmic ratio. The cytoplasm stained pale blue and was agranular. Smaller lymphocytes had a very scant cytoplasm. Monocytes were large with a lower nuclear to cytoplasmic ratio. Nuclear shape was round, indented or bilobed, and the cytoplasm was granular, but not frothy. Macrophages were large with a low nuclear to cytoplasmic ratio. The nucleus was irregularly shaped. The cytoplasm was frothy, or vacuolated, and often contained debris, and the margin was often irregular. The granulocyte population was predominately two cell types, a myeloperoxidase (mpo) positive neutrophil and an mpo negative eosinophil. The nuclei of neutrophils were multi-lobed and the cytoplasm stained lilac or pale pink and was not obviously granular by light microscopy. Eosinophils had eccentric nuclei. The cytoplasm stained pale pink and contained larger granules.

Comparisons of PBL differentials from MTs and WTs were 34.7% vs. 70.5% lymphocytes, 12.6% vs. 11.3% monocytes, and 34.7% vs. 17.6% granulocytes (Figure 3.6). Average thrombocyte counts (per 100 leukocytes) were 519 for MTs and 496 for WTs, with standard deviations of 112.545 and 10.583, respectively. Comparisons of kidney hematopoietic tissue leukocyte

differentials from MTs and WTs were 8% vs. 29% lymphocytes, 57% vs. 36% myelomonocytes, 35% vs. 34% precursors and 0% vs. 1% thrombocytes.

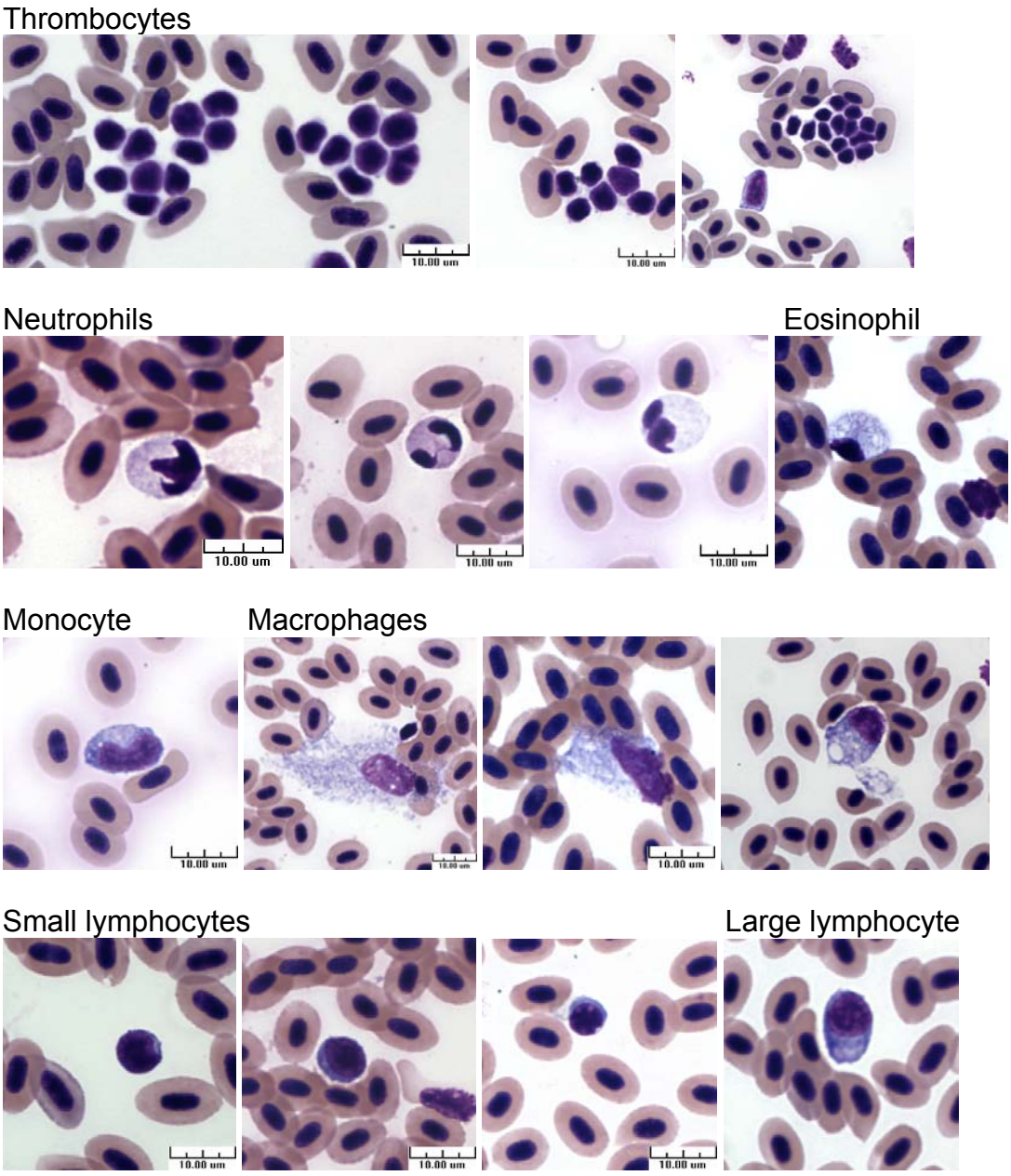

Figure 3.5 Representative zebrafish blood cells stained with Hema-3 Stat Pack. Scale bar= 10 µm

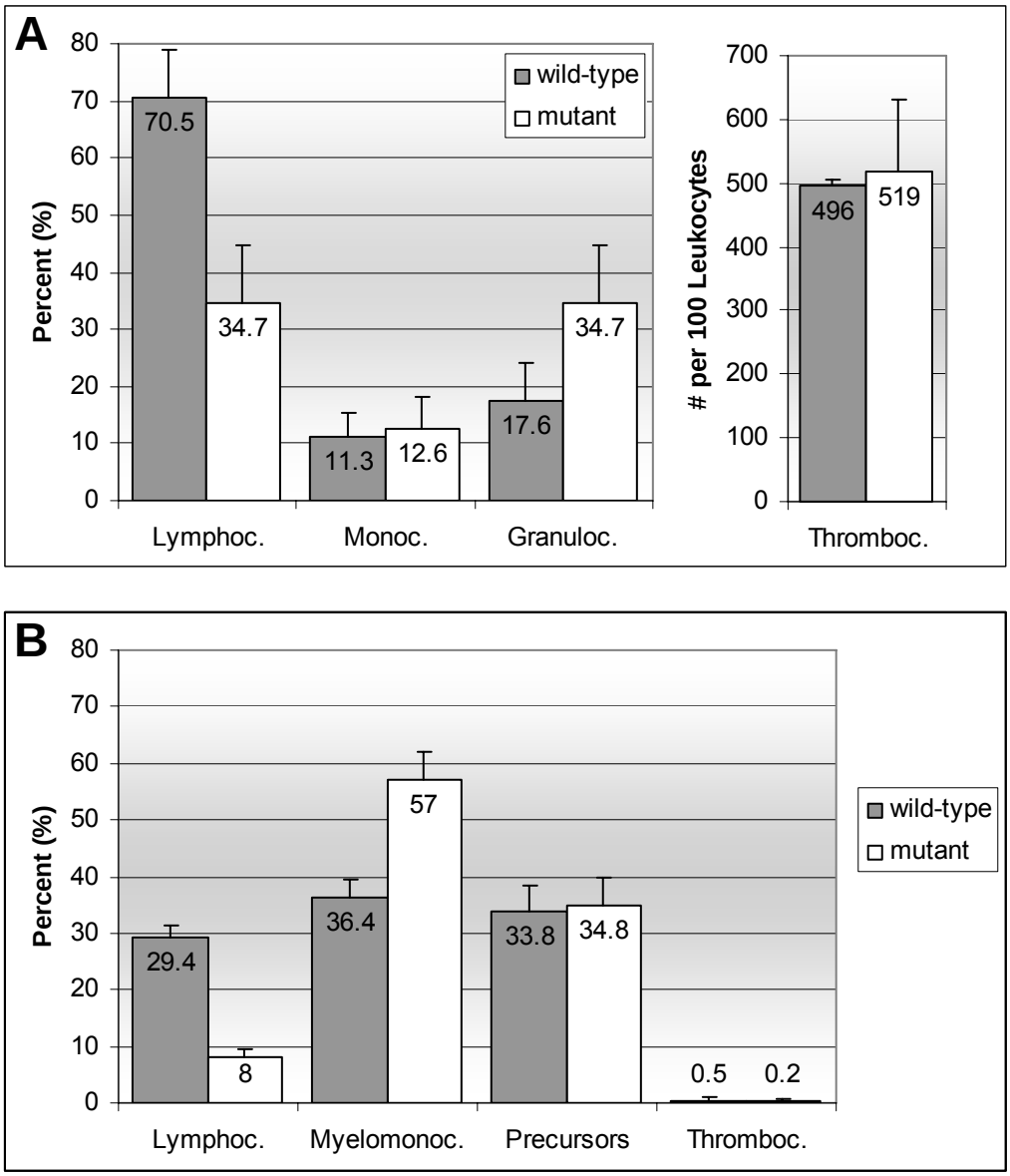

Figure 3.6 Leukocyte differential counts from (A) whole blood smears and (B) whole kidney smears of wild-type (*rag1*+/+) (n=10) and mutant (*rag1*-/-) (n=10) zebrafish.

Flow cytometry. Examination of WT and MT kidney interstitial cells by FACS analysis on the Calibur and the high through-put Aria demonstrated three main cell populations (Figure 3.7). On the basis of forward scatter (FSC) and side scatter (SSC) properties and location in a FSC vs. SSC plot, these populations

were identified as lymphocytes and designated C, hematopoietic precursors designated B, and monocytes/granulocytes/myelomonocyte precursors, designated A. Thresholds were set to exclude erythrocytes. Interestingly, differential counts of kidney hematopoietic tissue indicated thrombocytes were not present, or present at less than 1% (Figure 3.6 B). On the Calibur, the number of cells with the characteristics of small lymphocytes was significantly reduced in MT tissue: 7% of gated cells comprise the lymphocyte population in MTs whereas WTs have a 26% lymphocyte population. We also observed an increased population of myelomonocytes in MTs (43%) over WTs (21%). Similar percentages were collected on the Aria. Approximately 1.5 million lymphocyte-like cells were obtained from 30 pooled MT kidneys.

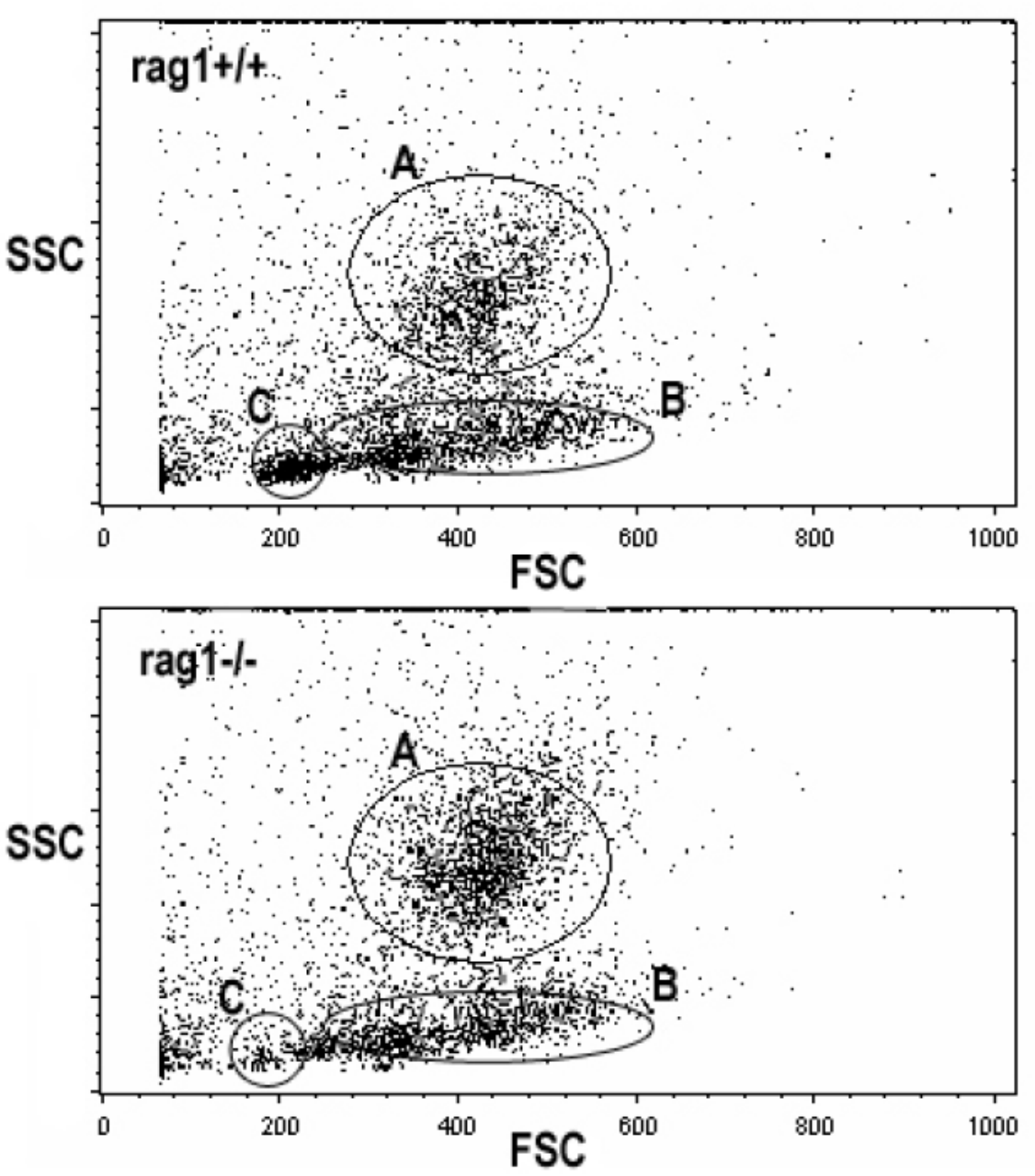

Figure 3.7 Forward scatter (FSC) and side scatter (SSC) of whole kidney cell lysate differentiates 3 distinct cell populations in wild-type (*rag1*+/+) and mutant (*rag1*-/-) zebrafish: A: macrophage/monocytes and granulocytes B: precursors C: lymphocytes. Note the reduction in $SSC^{low}FSC^{int}$ cells (gate C), characteristic of small lymphocytes, in mutant (*rag1*-/-) fish.

PCR identification of MT and WT zebrafish cell populations. To determine if the cells that demonstrated lymphocyte morphology were functional B cells, MT fish were evaluated for V(D)J rearrangements of the immunoglobulin (Ig) heavy-chain genes *Igμ* in B cells. Published primers [22, 74] were used in this Ig V(D)J rearrangement study. A PCR product is obtained only if recombination occurs. No recombination occurred in MT fish (Figure 3.8). Therefore immunoglobulin (VH_1) cannot be expressed and the MTs lack functional B cells. Additional PCR determined that MT fish lacked VH_1, VH_2, VH_3 and VH_4. *Tcr* genes in zebrafish also undergo V(D)J recombination [24]. To test for functional T cells, published primers were utilized in RT-PCR to screen mutant and wild-type zebrafish RNA for TCR expression [22] and found only expression in WT fish (Figure 3.9). These data indicate that MT fish lack functional T and B cells.

To determine if NK and NCC cells were present, specific primers were designed for NCCRP-1 and 3 forms of NK lysin. All RT-PCR assays demonstrated similar amounts of product for mRNAs encoding all NK and NCC specific proteins in both WT and MT zebrafish (Figure 3.10).

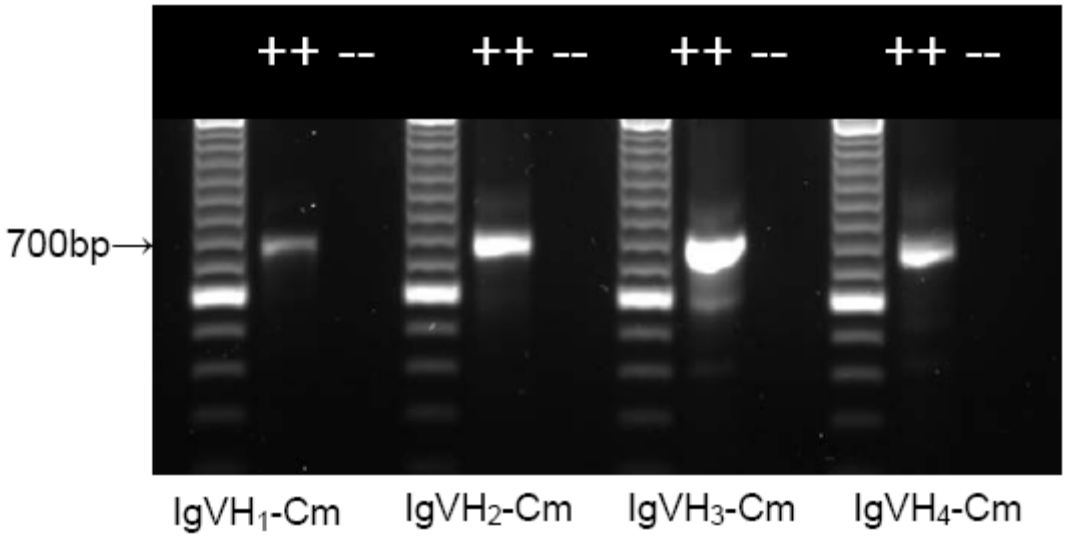

Figure 3.8 VDJ-Cm recombination in B cells occurs in wild-type fish (++) and leads to PCR amplification (Ig VH_1-VH_4). In mutant fish (--) the priming sites are too distant to yield a product and demonstrates lack of functional B cells.

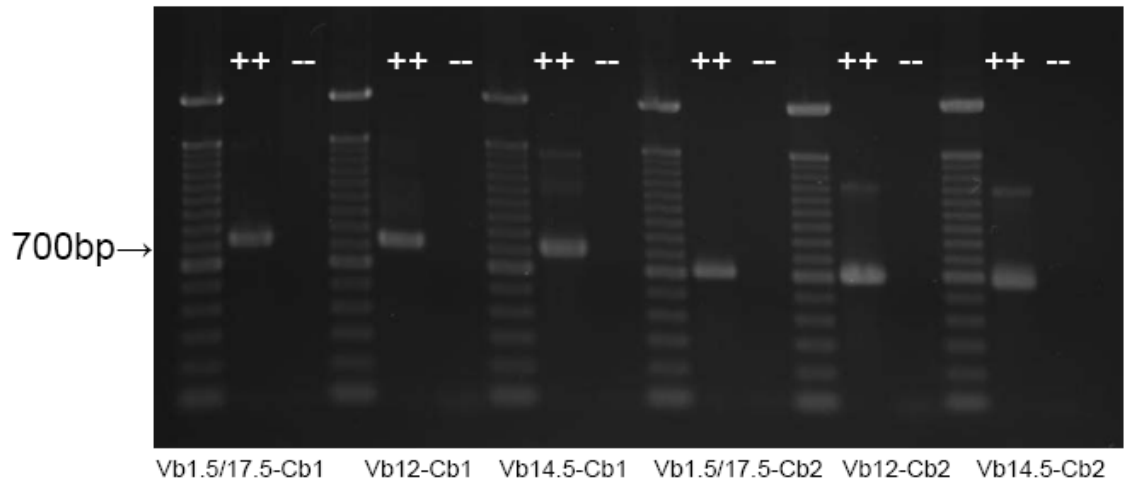

Figure 3.9 TCR V(D)JC recombination in T cells occurs in wild-type fish (++) and leads to PCR amplification. In mutant fish (--) the priming sites are too distant to yield a product and demonstrates lack of functional T cells.

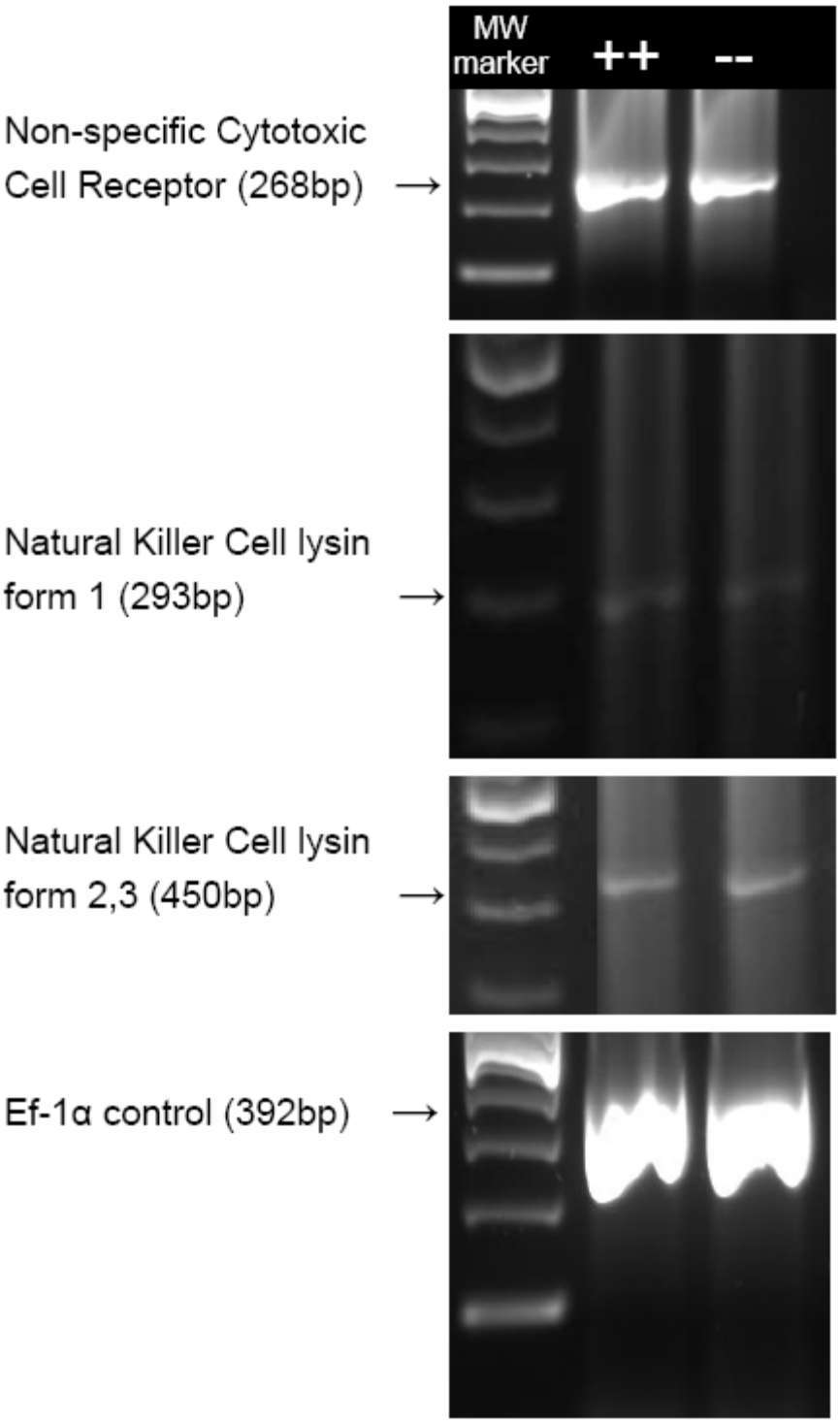

Figure 3.10 RT-PCR was used to analyze mRNA expression for NCC cells (NCCRP-1) and NK cells (NK lysin). Data demonstrates that wild-type (++) and mutant (--) fish have functional NCC and NK cells.

Discussion

The Tübingen *rag1* mutant zebrafish survived and were fertile in standard zebrafish housing [1] but did not survive the DNA sampling procedure (E. Wienholds, personal communication). Therefore, sampling procedure were optimized to use minimum amount of sample so the fish suffered minimal physical damage, and individual treatment and recovery chambers were used to optimize survival. When clipped fish recovered within these chambers, sampling wounds healed well, and the genotyped MT brooders were typically prolific.

The PCR based method to detect the presence of the single base pair mutation in the *rag1* gene was efficient and easy to use. The assay utilized a region specific PCR to generate sufficient template from the small amount of genomic DNA provided from the small fin clips. Then two specific PCRs identified the presence of the mutant and wild-type alleles. The PCR specific for the wild-type allele had a general *rag1* gene upstream (upper) primer and the 3' end of the lower primer at the site of the point mutation with G, the complement of the wild-type nucleotide. The PCR specific for the mutant allele used an upper primer with the 3' nucleotide being the T, representing the mutant sequence and a general *rag*1 lower primer. By having primer sets for both forms we could easily distinguish all three genotypes (homozygous +/+, homozygous -/-, and heterozygous +/-).

Zebrafish tagging was necessary to ensure that separate MT and WT breeding populations were maintained. The challenging characteristic of zebrafish for tagging purposes is their small size. Tagging methods previously

used with other fish species include anchor tags that are punctured into the epidermis of the body or a fin (percutaneous), visible implant (VI) alpha numeric tags that are implanted under the skin (subcutaneous) and passive inducer transmitters (PIT) tags that are invasively placed under the skin or within the abdominal cavity (internal). Anchor and pit tags were too large for use in zebrafish. The injection force of skin tattooing, a less invasive marking method, killed channel catfish fry the size of zebrafish (unpublished data). VI tags have very small numbers that are difficult to discern by the unaided eye. Visible Implant Elastomers (VIE) were the only logical choice for use in zebrafish. This procedure utilizes an inert, non-immunogenic polymer that is injected under the skin. Multiple colors are available and are easily discerned by the unaided eye. Different colors or color combinations and different anatomical locations can identify treatment groups. Successful tagging regimes are species variable.

The VIE method was successfully used with mummichogs [75], rainbow trout *Oncorhynchus mykiss* [76], small perch (*Perca fluviatilis*) and common bully (*Gobiomorphus cotidianus)* [77], and Colorado squawfish [78]. Sites that were more easily injected in larger fish were difficult to inject in zebrafish. However, following injection, zebrafish demonstrated excellent survival and good tag retention times. Interestingly, the tag visibility in zebrafish was very good, but zebrafish retention times were not as long as those reported in larger fish species cited above. Another study also found that small tropical fish were more difficult to inject with the elastomer, yet retention and visibility were good [79]. Survival and growth were not affected by the tagging method. Elastomer tag detection

rates in fingerling rainbow trout were low (29 to 33%) approximately 2 years after the tagging [80]. An explanation for these findings may be that operator fear of injuring smaller fish during elastomer injection results in a small amount of elastomer being delivered.

Differentiations of color visibility and tag retention rates by anatomical location on fish were specifically addressed in several studies. In Silver perch, (*Bidyanus bidyanus*) and Australian bass (*Macquaria novemaculeata*) tag visibility was good in most locations. The front dorsal tagging showed the least visibility with less than 90% after 8 months in both species [81]. The best tag locations for both species were behind the dorsal fin and adjacent to the anal fin. Combinations of four colors and three locations were used with perch and common bully [77]. In perch, tag retention was 100% throughout the study, but in bully, originally high tag retention decreased to 72% after 125 days. In barbel (*Barbus barbus*), VIE tags in the postorbital adipose tissue were retained at a lower rate (48%) than in the scalp and caudal and anal fin bases (82%) [82]. In bluegill however, none of the scalp tags were retained [83].

In barbel, the caudal site had the best retention, but was the most difficult to inject so the authors recommended a more practical tag site [82]. In the current zebrafish study, tag locations with the best retention were more difficult to inject or difficult to easily view on live fish. The dorsal and pectoral fin bases offered a good compromise between ease of injection, good visibility and good tag retention. Multiple studies determined the best colors to use depended on the natural pigmentation of the fish being tagged [76, 80]. Red was the best color to

use in seabream [84], and red and orange were the best in seahorses *Hippocampus guttulatus* [85]. Red and pink were found to be the most visible in zebrafish, and orange and yellow also worked well. However, in specific tagging sites of zebrafish, yellow and orange were difficult to distinguish from each other, as were pink and red. Curtis [85] observed confusion between orange and red, orange and pink, and green and yellow in seahorses.

Blood smears of MT zebrafish revealed significantly reduced lymphocyte populations and significantly increased granulocyte populations. Hematological findings of the WT fish are comparable with published values for zebrafish [86]. Published peripheral blood differential results from zebrafish [12] reported 81% lymphocytes, 8% monocytes and 11% granulocytes, and these were comparable to WT fish in this study, with 71% lymphocytes, 11% monocytes and 18% granulocytes. Zebrafish granulopoiesis and myelopoiesis have been well described [87]. The morphologic and functional characterization of macrophages and neutrophils (heterophils) have been further described [15]. Although leukocyte cell counts were significantly different between MT and WT, cell morphologies and staining characteristics were comparable with each other, and other published data. The thrombocyte populations in MT and WT peripheral blood were statistically the same.

Kidney hematopoietic tissue was used for flow cytometry, so there was a greater hematopoietic precursor cell population than would be found in peripheral blood. Gate values and cell populations agree with published data [12, 22, 88]. Flow cytometry findings corroborate the leukocyte differential counts that

demonstrated a significantly reduced lymphocyte population in the MTs. Flow cytometric quantifications of brown trout and rainbow trout peripheral blood identified three cell populations that consisted of erythrocytes, lymphocytes plus thrombocytes and neutrophils [89]. Thrombocyte percentages from zebrafish kidneys in this study were <1%.

Primitive hematopoiesis occurs in zebrafish and includes myelopoiesis that forms the myelomonocyte precursor that differentiates into two myeloid lineages, granulocytes and monocytes [90]. The granulocytes further differentiate into heterophils and eosinophils [91]. The cell populations we identified appeared to directly correlate with four main cell populations identified by Traver [92]: erythrocytes, lymphocytes, precursors, and myelomonocytes. In flow cytometric analyses of fish peripheral blood, thrombocytes are included in the small lymphocyte fraction. However, kidney tissue was utilized for flow cytometry to omit this problem. Further, the erythrocyte population was gated out of the flow cytometry results of kidney tissue, and the A population was NK cells and NCCs in MT fish, and NK, NCCs and lymphocytes in WT fish.

The presence of V(D)J recombination in WT fish and the lack of identifiable V(D)J recombination demonstrated functional *rag* expression in WT fish and a lack of a functional *rag*1 gene in the MTs. These findings confirm the original results of Wienholds et al [1]. The RT-PCR findings further demonstrated that NK and NCC cells were present in MT fish. These would account for the lymphocyte-like cell population observed in the flow cytometry findings. Fish possess both NK-like cells, and NCCs [35]. MT zebrafish lacking *rag1* expression

demonstrated a marked reduction or absence of lymphoblasts in the thymus [29]. *Rag1* deficient mice also lack functional T and B cells [70], but have functional NK cells [47]. Mammalian NK cells are large, granular (atypical) lymphocytes that have unique surface markers (CD3-, CD56+ and CD16+) and do not have recombined *TCR* or *Ig* genes [35]. The cytoplasmic granules present in NK cells categorize them as a cytotoxic cell, and these granules are used to kill other cells. These killing mechanisms are similar to cytotoxic T cells, but the mechanism of target or antigen identification is different. A channel catfish NK cell line has been developed [32]. Further studies strongly support a distinct NK lineage in fish [93, 94]. Cell surface markers or receptors for fish NK cells have not yet been determined. However, in a different study, catfish granzymes, NK-lysin and perforin, and zebrafish NK-lysin have been identified [35].

Rag1^{t26683} mutant zebrafish provide a unique model for investigating innate immune responses because fully functional innate defenses are present without the influence of lymphocytes and lymphocyte associated acquired immune responses. The *rag1* MT zebrafish provide the model to elucidate mechanisms of the innate immune system and developmental immunology in fish. Future research will provide new tools to direct the level and type of fish immune defenses against pathogens. More specifically, *rag1* MT zebrafish provide the platform to investigate the roles of NK cells, NCCs, macrophages, neutrophils and eosinophils in immune responses.

CHAPTER IV

MEMORY IN THE INNATE IMMUNE SYSTEM PROVIDES PROTECTION IN *RAG1-/-* DEFICIENT MUTANT ZEBRAFISH

Abstract

This is a report of a novel observation of memory in the innate immune system in a vertebrate. *Rag1* (*recombination activation gene 1*) deficient mutant zebrafish lacking functional B and T lymphocytes demonstrated protection upon secondary bacterial exposure. *Rag1-/-* mutant (MT) and *rag1+/+* wild-type (WT) zebrafish were intra-muscularly vaccinated with 10^2 colony forming units (CFU) *Edwardsiella ictaluri* (*E. ictaluri*)/fish and reinfected with 10^4 CFU *E. ictaluri*/fish either 30 or 60 days later. Naïve groups of MT and WT fish were also injected with 10^4 CFU *E. ictaluri*/fish and mortality rates compared to vaccinated fish. Following the secondary or protection exposure 30 days after vaccination, a significant reduction in mortality of 42% was observed in MT, vaccinated zebrafish when compared to unvaccinated, MT fish. Mortality in WT fish was reduced by 51% in the vaccinated group. When 60 days were in between vaccination and protection exposure a significant 35% mortality reduction in MT, vaccinated zebrafish was observed. WT fish in the same trial

showed a significant reduction in mortality from 41% in naïve fish to 26% in vaccinated fish. When antibiotics were orally administered during the 30 or 60 days between vaccination and protection exposure, decreases in mortalities in MT, vaccinated fish were 17% and 6% respectively and decreased mortalities in WT, vaccinated fish were 14% and 12% respectively. Sham-injected and non-injected controls did not suffer mortality.

This documents for the first time, that MT zebrafish, lacking an acquired immune system, were able to mount a protective immune response to the catfish specific pathogen *E. ictaluri* and generate protection similar to WT zebrafish upon repeated encounter to the same pathogen. The observed protection is long lasting and mediated by the innate immune system, but a specific mechanism is not yet defined.

Introduction

The zebrafish, one of the favorite animals of developmental biologists, is being increasingly used as an infectious disease model [95]. Several advantages have been noted for the zebrafish model that are specifically favorable in epidemiological studies. The small size makes maintenance and pathogen challenges possible in limited space and stand alone systems are readily available or can be easily built [2]. A relatively rapid life cycle and ease of breeding guarantees availability of different age classes throughout the year. Other features such as ex-utero development makes genetic manipulations easy, and optical clarity of early embryos gives the possibility to analyze infections by

using fluorescent microorganisms, are complemented by ever-expanding genetic and genomic resources [3, 29, 96]. Numerous regions of synteny conserved among the genomes of zebrafish and human have been identified [97] making it more likely to translate new immunological findings in zebrafish to higher vertebrates and mammals. Because the zebrafish model bridges the gap between important lower animal models, like *Drosophila*, and well studied mammalian models, it is important for infectious diseases and immunological research [95]. So far, zebrafish have proven a useful model to study infectious diseases caused by human-specific pathogens like *Salmonella typhimurium* [98], *Edwardsiella tarda* [99], *Streptococcus pyogenes* [100] and fish-specific pathogens like *Mycobacterium marinum* [101], *Streptococcus iniae* [100] and for vaccination study against viral haemorrhagic septicemia virus (VHSV) [102].

Through target-induced local lesions in genomes (TILLING), a powerful methodology for creating gene knock-out models in zebrafish, *rag1-/-* mutant (MT) zebrafish have been successfully created by a reverse genetic approach [1, 16, 103, 104]. These MT zebrafish lack T and B cell lymphocytes, and they cannot perform lymphocyte driven responses, leading to a loss of acquired immune functions [105]. Therefore, this fish line can be used to study innate immunity without the overshadowing effects of acquired immune responses.

Current immunological dogma considers the vertebrate innate immune system to lack specific memory largely because there is no evidence of mechanisms that could provide such memory. Previous research has demonstrated that larval channel catfish (*Ictalurus punctatus*) that had not yet

developed acquired immunity when first exposed to *Edwardsiella ictaluri* (*E. ictaluri*) demonstrated protection upon secondary exposure to the same pathogen (Dr. Petrie, personal communication) [42]. This indicates a possible adaptable innate immune system that may be an important component of vertebrate immunity. *Edwardsiella ictaluri,* a gram negative intracellular bacterium, causes enteric septicemia of catfish (ESC), one of the most important diseases of farm-raised channel catfish. In 2003 52% percentage of reported losses in channel catfish fry were due to ESC [106].

E. ictaluri, was chosen for this study because of the previous findings of protection in catfish larvae. It is also an extensively studied pathogen of channel catfish (*Ictalurus punctatus*) [107-111] and grows at a temperature suitable for use in zebrafish (22-28˚C). *E. ictaluri* infection induces a comparable acute inflammatory response and chronic manifestation in zebrafish, expressing some of the same pathological behavior and signs as channel catfish when infected [112]. Typical clinical signs demonstrated by zebrafish include tail chasing behavior due to the presence of the *E. ictaluri* in the brain, and hanging in the water column with the head up and tail down. Epidermal petechiation was concentrated in the ventral or belly region, abdominal ascites, and exophthalmia (pop-eye) were also present. The liver demonstrated characteristic pale areas of tissue destruction (necrosis) or a general mottled red and white appearance. Petechial hemorrhages were found in the muscles, intestine and fat of the fish, and the intestine was often filled with a bloody fluid.

In the current study, *rag1-/-* MT zebrafish were utilized to investigate specific memory in the innate immune system in the absence of lymphocyte-driven acquired immunity. *E. ictaluri* was used for vaccination and protection challenges. Time intervals of 4 and 8 weeks between vaccination and protection exposures were used.

Materials and Methods

Zebrafish care. Zebrafish were housed in the CVM-MSU specific pathogen free (SPF) fish hatchery. Maintenance and propagation of fish were performed according to modified standard protocols [53] and are posted at : http://www.cvm.msstate.edu/zebrafish/index.html.
All experimental animal protocols were approved by the Institutional Animal Care and Use Committee at Mississippi State University.

Bacterial strain and media. *Edwardsiella ictaluri* strain 93-146 was originally isolated from an ESC outbreak in a commercial channel catfish pond in Louisiana and biochemically confirmed. Bacteria were re-isolated from a single symptomatic fish, inoculated into brain heart infusion (BHI) medium and incubated in a shaker incubator at 30˚C overnight. Logarithmic phase cultures were obtained by dilution of the overnight culture 1:10 and grown for another 3 to 5 hours. Bacterial concentrations were determined by optical density (OD) at 540nm where 5.9 x 10^8 colony forming units (CFU) per ml read at 0.4 OD. Purity of *E. ictaluri* culture was assessed by plating serial dilutions on tryptic soy agar

(TSA) + 5% sheep blood plates with incubation for 2 hours at 37˚C and overnight at 30˚C.

Antibiotic feed. To make oxolinic acid medicated feed, 0.7 g oxolinic acid (Sigma-Aldrich Inc., St. Louis, MO), was mixed with 15 ml distilled water and evenly sprayed on Zeigler™ Adult Zebrafish Diet pellets (Aquatic Habitats™, Apopka, FL) (recommended by Dr. Gary Aukes, www.fishyfarmacy.com/articles.html). Pellets were air-dried at room temperature for 2 days and were then kept at -20˚C until use.

Exposure by injection. Adult zebrafish (6-9 month old) were anesthetized in MS-222 [53], a stock solution of anesthetic was prepared by combining 400 mg of Finquel® (Argent, Redmond, WA), 97.9 ml double distilled water and 2.1 ml TRIS base (1M). The pH of the stock solution was adjusted to ~7. Just before use 4.2 ml of the stock solution was mixed with 100 ml system water. Zebrafish were IM injected using an insulin syringe (Figure 4.1). Depending on the treatment, fish where injected with *E. ictaluri* at concentrations of 5.9 x 10^4, 5.9 x 10^3, 5.9 x 10^2 CFU *E. ictaluri*/fish (from here on referred to as 10^4, 10^3, or 10^2 CFU *E. ictaluri*) delivered in a total volume of 10µl. Sham injected fish were received 10 µl sterile physiological saline (0.85%). After recovery from anesthesia, each group of fish was moved to tanks in the flow–through system and maintained at 26˚C±1. All fish, except trial 2, were held under the same conditions during the duration of the individual experiments. For trial 2, naïve fish were kept under SPF conditions until injection with 10^4 CFU *E. ictaluri*. Fish were

observed 2x a day for clinical signs of disease, moribund fish were euthanatized with MS-222 (300 mg/l), and bacteria isolated as described later.

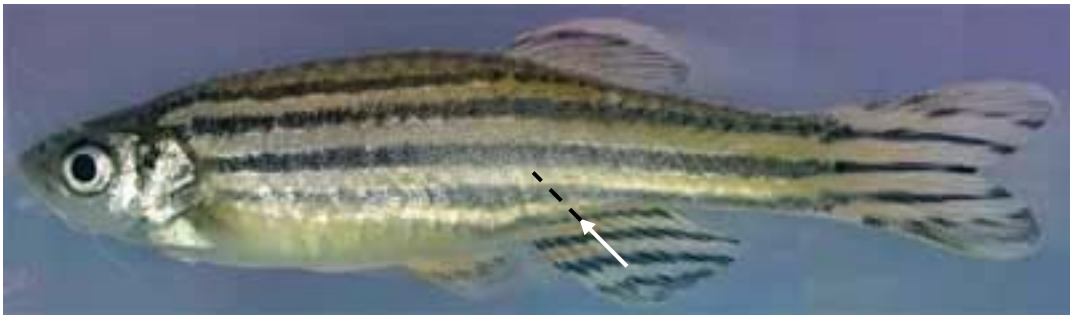

Figure 4.1 IM injection of adult zebrafish. The white arrow indicates injection site. Dashed line indicates approximate depth of injection.

Experimental design of individual trials.

Trial 1: LD determination. Fish where injected with 10^4, 10^3, or 10^2 CFU *E. ictaluri* to determine $LD_{<25}$ as a vaccination dosage in the primary exposure, referred to as vaccination exposure, and $LD_{>80}$ as the protection dosage for the secondary exposure, referred to as protection exposure. Injections of MT and WT fish with *E. ictaluri* were performed in triplicate with 15 fish per replicate and in addition 15 control fish per strain were sham injected. Mortalities were recorded for 10 days post injection (dpi).

Trial 2: 1 month. For the vaccination exposure, 140 MT and 120 WT fish were injected with 10^2 CFU *E. ictaluri*. 1 month post vaccination exposure, the previously vaccinated fish were injected with 10^4 CFU *E. ictaluri* in triplicate with 24 fish per replicate. In addition naïve MT and WT fish were injected with 10^4 CFU *E. ictaluri* in triplicate with 24 fish per replicate. Three controls per strain

were set-up: 24 fish were sham injected at the vaccination and protection exposure, 24 fish received 10^2 CFU *E. ictaluri* at the vaccination exposure and were sham injected at the protection exposure and 24 fish were not injected at all.

Trial 3: 1 month with antibiotic feed. Set-up like trial 2 except that eight replicates with 10 fish per replicate were used per group. Oxolinic acid feed was administered for 10 days during 10 dpi-20 dpi.

Trial 4: 2 month. Set-up like trial 3, but no oxolinic acid feed was administered.

Trial 5: 2 month with antibiotic feed. Set-up like trial 3.

Reisolation of bacteria. Mortalities after vaccination and protection exposure were recorded and individual fish brains plated on TSA + 5% sheep blood plates. After 48h at 30˚C bacterial colonies were tested with oxidase reagent (Becton Dickinson, Sparks, MD). *Edwardsiella ictaluri* identification was confirmed by biochemical analysis using the bioMerieux api20E strip (BioMerieux, 69280 Marcy l'Etoile, France), code 4004000.

Statistical analysis. Data were expressed as daily specific mortality (# of dead fish per day), mean specific mortality (average # of dead fish) per tank with standard deviation and overall specific mortality (total # of dead fish) with mortality rate (%). Mortality data of treatments between groups was analyzed by

one-way analysis of variance (ANOVA) with post hoc LSD correction (least statistical significance) for multiple comparisons with a level of significance at $p \leq 0.05$. Statistical analyses were performed using SPSS for Windows 15.0 (SPSS Inc., Chicago, IL).

Results

Most fish that died between the time of injection and up to 2 dpi showed no presence of *E. ictaluri* in their brain tissue. These fish were not included as mortalities. Starting at 2 dpi mortalities were caused by the injected pathogen, as *E. ictaluri* was reisolated from the brain tissue of moribund and dead fish. Dying fish manifested behavior and external signs associated with ESC infection including hanging in the water column with head up and tail down and erratic swimming or swirling. *E. ictaluri* bacteria were successfully isolated from treatment fish. No control fish died due to injection stress or manifested symptoms of ESC and randomly selected control fish were negative for *E. ictaluri*.

Trial 1: LD determination. MT and WT adult zebrafish were injected with 10^4, 10^3, 10^2 CFU *E. ictaluri* to determine $LD_{<25}$ as a vaccination dosage in primary exposure and $LD_{>80}$ as a highly lethal dosage in secondary exposure. Over the course of 10 days MT and WT fish, injected with 10^2 CFU, had a 9% and 13% mortality rate respectively. Mortality rates increased to 33% for the MT and 38% for the WT fish when injected with 10^3 CFU. After injection with the highest dosage of 10^4 CFU *E. ictaluri*, 62% of the MT and 87% of the WT fish

died (Figure 4.2). No significant difference in specific mortality per tank was found between MT and WT fish (Figure 4.3). Nevertheless, numerical differences show that MT zebrafish have a decreased mortality rate compared to WT fish during the first 10 dpi with *E. ictaluri* (Figure 4.2). Specific mortality followed similar patterns between the two groups of fish, highly depending on the injection dosage (Figure 4.3). Highest mortalities were recorded on days 3 and 4 post injection with 10^4 CFU *E. ictaluri* (Figure 4.3c). It should be noted that in WT fish, after a phase of die-offs during days 1-10 pi, no more mortalities were observed until 18 dpi (Figure 4.5). Low grade mortalities in MT fish due to *E. ictaluri* infection were recorded until the last day of observation. Based on these findings, the following *E. ictaluri* exposure challenges were carried out using 10^2 CFU *E. ictaluri*/fish for the vaccination exposure followed by 10^4 CFU in the protection exposure.

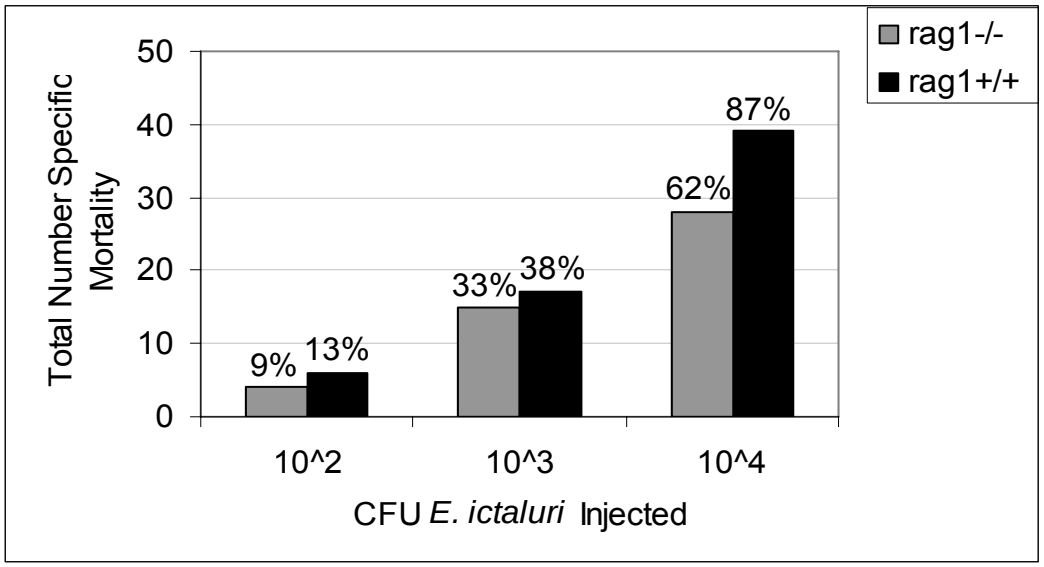

Figure 4.2 Specific mortality and corresponding mortality rate for *rag1-/-* MT and *rag1+/+* WT zebrafish 10 dpi with 10^2 CFU, 10^3 CFU, and 10^4 CFU *E. ictaluri*/fish.

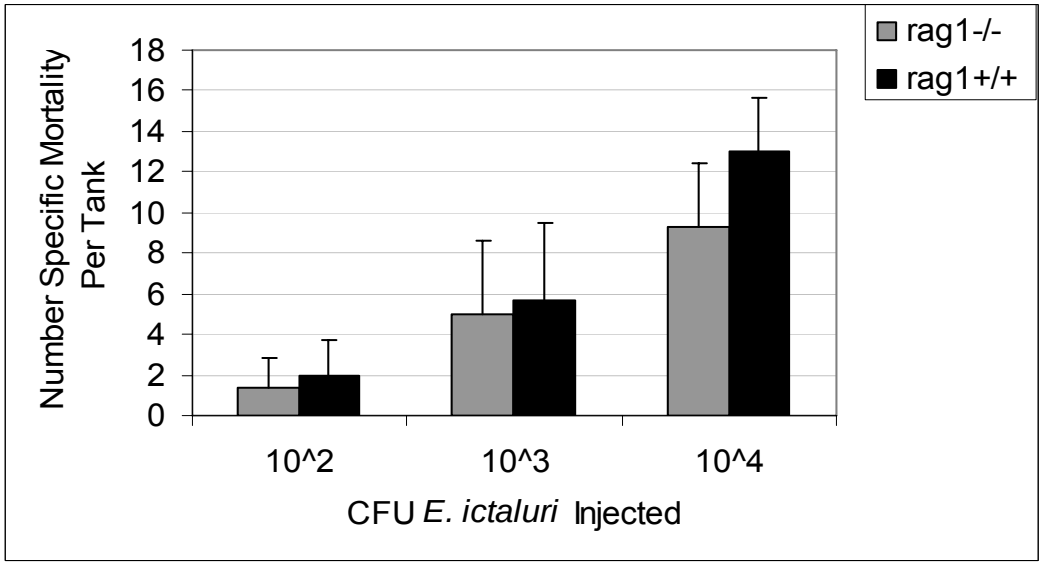

Figure 4.3 Specific mortality per tank for *rag1-/-* MT and *rag1+/+* WT zebrafish 10 dpi with 10^2 CFU, 10^3 CFU, and 10^4 CFU *E. ictaluri*/fish. Error bars indicate standard deviation between tanks (n=3).

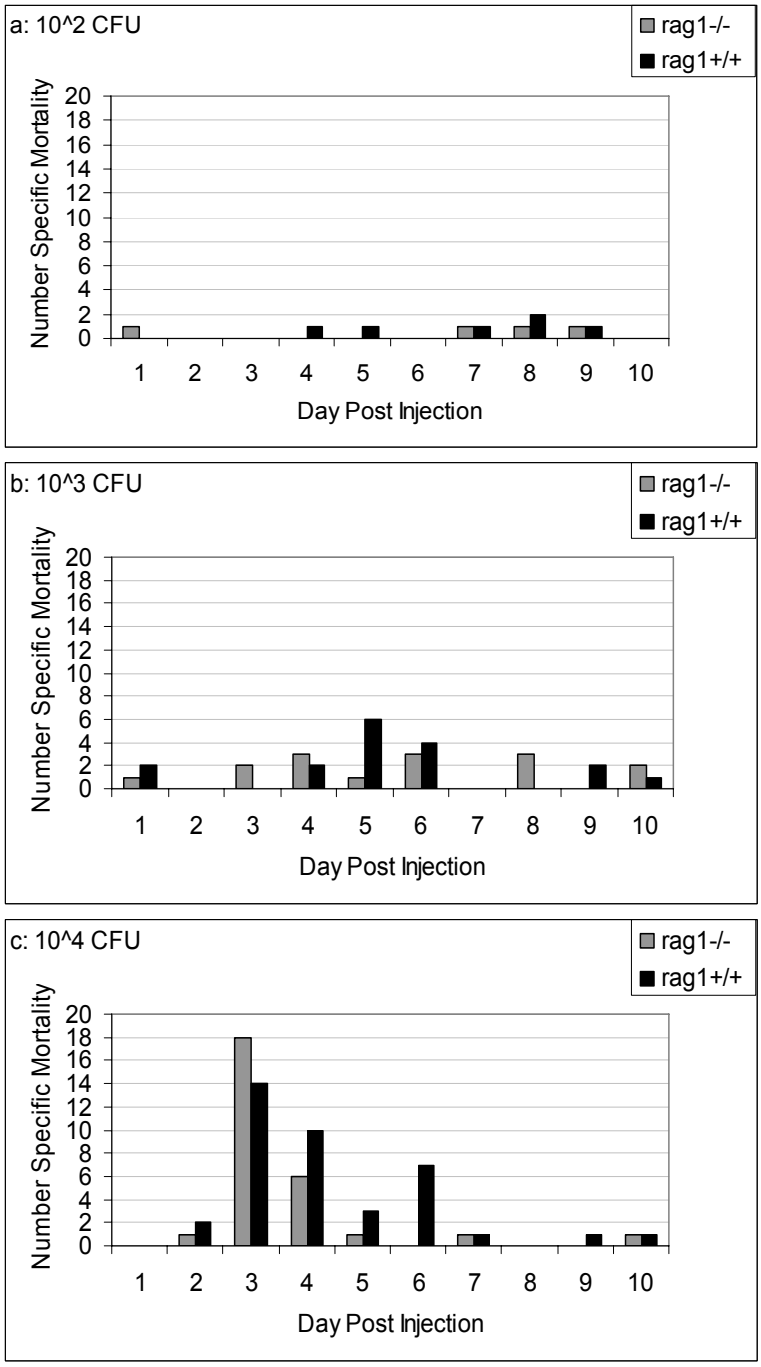

Figure 4.4 Specific mortality for *rag1-/-* MT and *rag1+/+* WT zebrafish over the course of 10 dpi. Three different concentrations of *E. ictaluri* were compared: a) 10^2 CFU *E. ictaluri*/fish, b) 10^3 CFU *E. ictaluri*/fish, and c) 10^4 CFU *E. ictaluri*/fish.

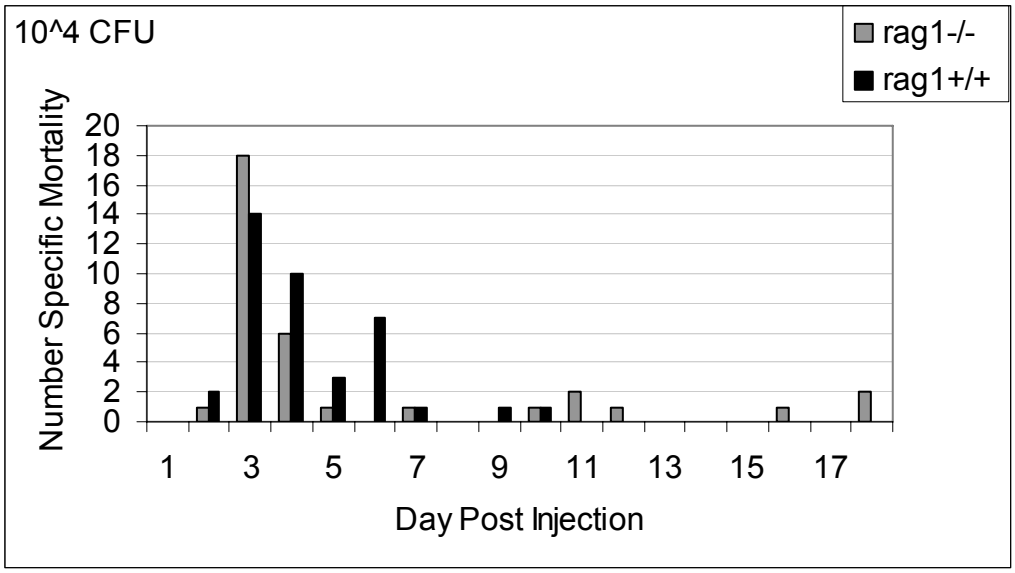

Figure 4.5 Specific mortality for *rag1-/-* MT and *rag1+/+* WT zebrafish over the course of 18 dpi. All fish were injected with 10^4 CFU *E. ictaluri*.

Trial 2: 1 month. Upon exposure to 10^4 CFU *E. ictaluri* naïve MT and WT fish followed same mortality patterns as observed in trial 1, where most fish died at 3-4 dpi followed by a steady decrease in mortalities until 10 dpi (Figure 4.6). Cumulative mortality in these naïve MT and WT fish was 67% and 70% respectively (Figure 4.7). As expected, mortality rates of vaccination exposed WT fish decreased to 19% after protection exposure. Interestingly, vaccination exposed MT zebrafish also showed a decrease from 67% to 25% in cumulative mortalities upon protection exposure (Figure 4.7). The decrease in mortality rate between naïve and vaccination exposed fish is significant for MT ($p < 0.001$) and WT fish ($p < 0.001$) (Figure 4.8).

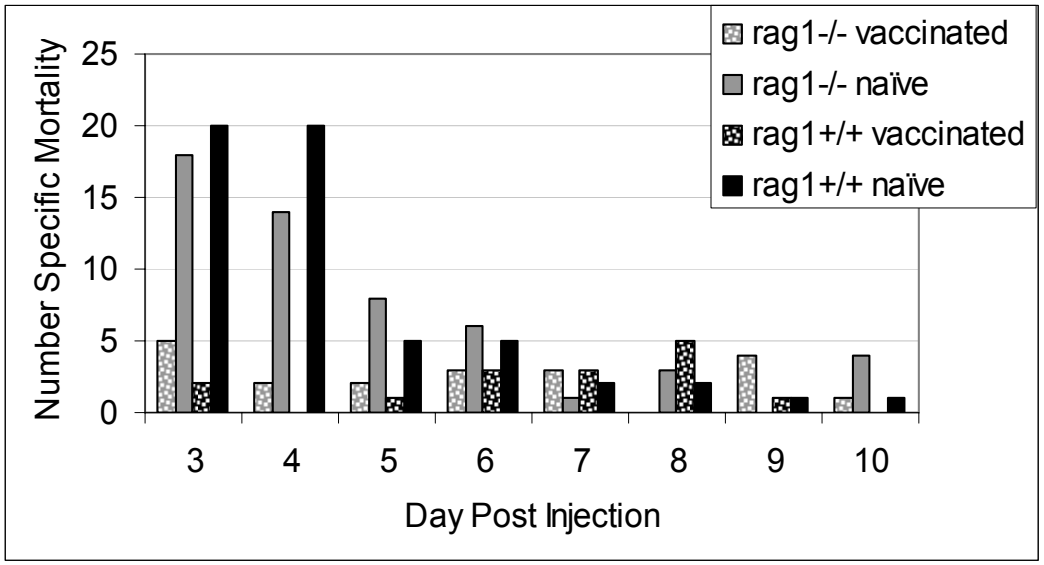

Figure 4.6 Number of specific mortality for *rag1-/-* MT and *rag1+/+* WT zebrafish over the course of 10 dpi. Vaccinated fish were injected with 10^2 CFU *E. ictaluri* 1 month prior a second injection with 10^4 CFU *E. ictaluri*. Naïve fish received 10^4 CFU *E. ictaluri* as a primary exposure.

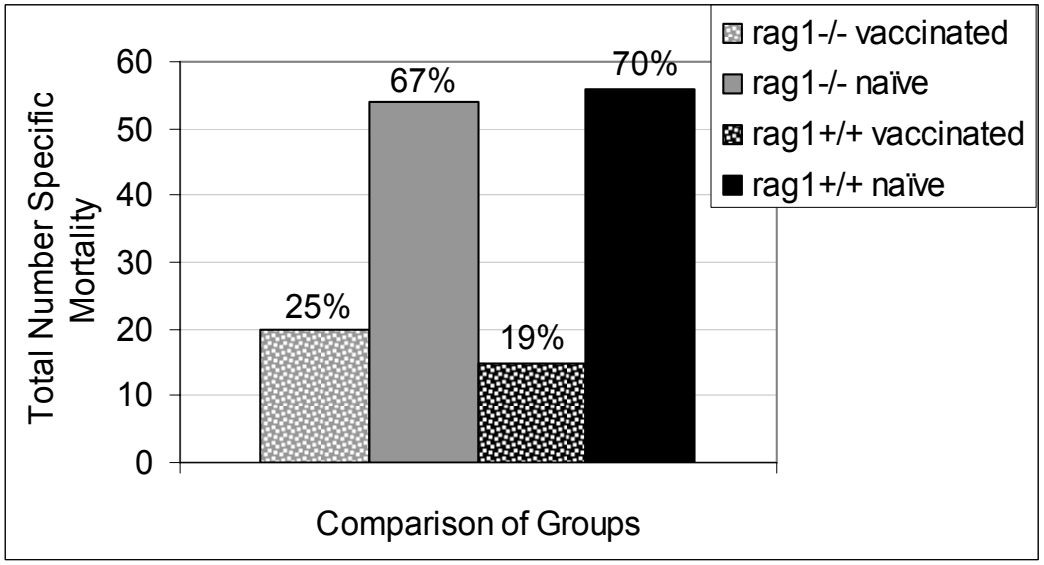

Figure 4.7 Specific mortality and corresponding mortality rate for *rag1-/-* MT and *rag1+/+* WT zebrafish following the protection exposure (10^4 CFU *E. ictaluri*/fish). Vaccinated fish were injected with 10^2 CFU *E. ictaluri* 1 month prior a second injection with 10^4 CFU *E. ictaluri*. Naïve fish received 10^4 CFU *E. ictaluri* as a primary exposure.

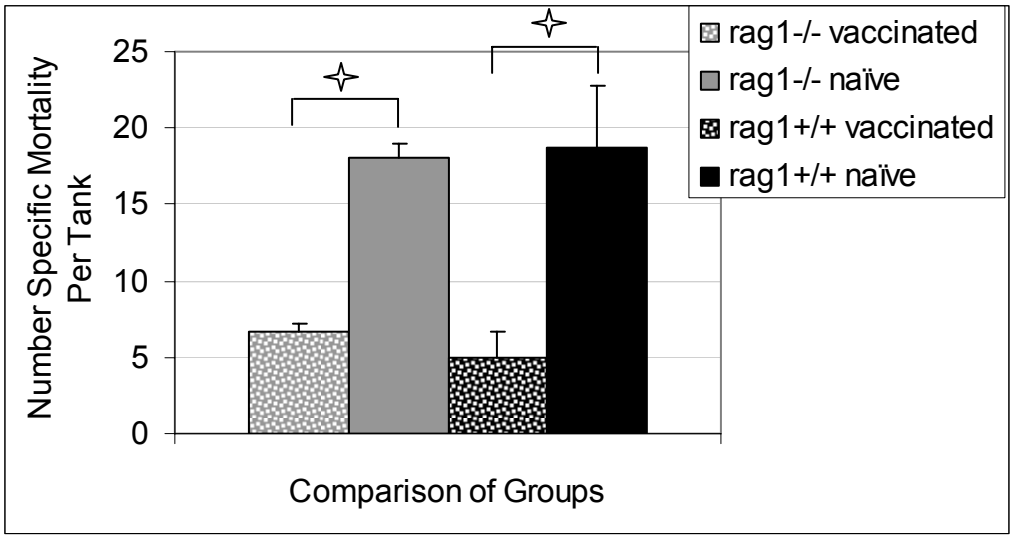

Figure 4.8 Specific mortality per tank for *rag1-/-* MT and *rag1+/+* WT zebrafish 10 dpi with 10^4 CFU *E. ictaluri*/fish. Vaccinated fish were injected with 10^2 CFU *E. ictaluri* 1 month prior a second injection with 10^4 CFU *E. ictaluri*. Naïve fish received 10^4 CFU *E. ictaluri* as a primary exposure. Mortalities were significantly reduced in vaccination exposed MT and WT fish. Error bars indicate standard deviation between tanks (n=3).

Trial 3: 1 month with antibiotic feed. Oxolinic acid feed was administered for 10 days to all fish used in this experiment. The rational was to allow vaccination exposed fish 10 days to mount a sufficient immune response against *E. ictaluri* then administer antibiotics in-feed for 10 days to eliminate persistant infection, and further allow a 10 day period for body clearance of the drug [113].

Figure 4.9 shows delayed mortality upon exposure to 10^4 CFU *E. ictaluri* in naïve and vaccination exposed fish that received oxolinic acid. Naïve MT fish show a decreased in overall mortality rate of 31 % when compared to trial 2 with greatest specific mortality at 9 dpi (Figures 4.7, 4.9, 4.10).

Naïve WT fish also showed a decrease in mortality from 70% in trial 2 to 41% in this trial (Figures 4.7, 4.9, 4.10). Highest specific mortality in naïve WT fish was recorded at 6 dpi and gradually decreased until 10 dpi (Figure 4.9). Vaccination exposed MT fish also had decreased mortality rates from 25% in trial 2 to 19% in this trial. On the contrary, mortality rates of vaccination exposed WT fish increase in this trial by 8% (Figures 4.7, 4.10). No significant differences in mortality rates between MT naïve and MT vaccination exposed fish, due to great variation between tanks within groups, was observed (Figure 4.11). The same was true for WT fish. Nevertheless, vaccination exposed MT and WT fish still showed numerical decrease in mortality rates, hence the same trend in protection was observed as in the previous trial, upon repeated encounter with *E. ictaluri* (Figures 4.10, 4.11).

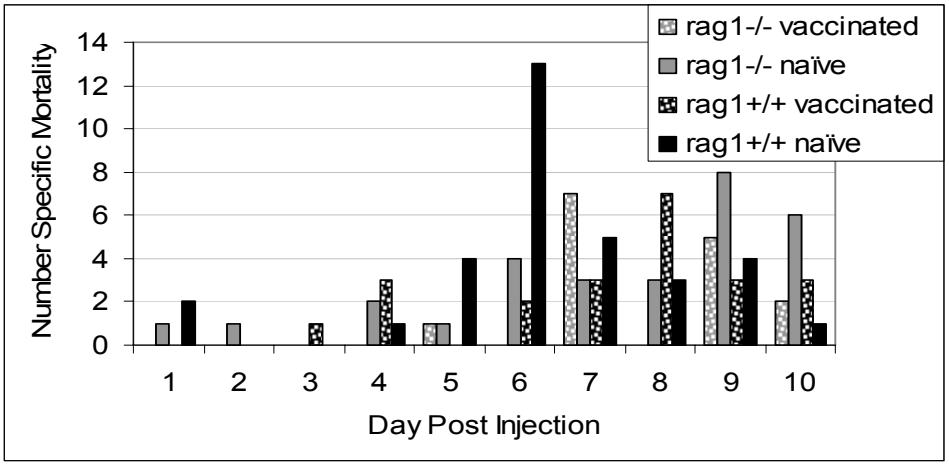

Figure 4.9 Specific mortality for *rag1-/-* MT and *rag1+/+* WT zebrafish over the course of 10 dpi. Vaccinated fish were injected with 10^2 CFU *E. ictaluri* 1 month prior a second injection with 10^4 CFU *E. ictaluri*. Naïve fish received 10^4 CFU *E. ictaluri* as a primary exposure. All fish received orally administered antibiotics for 10 days prior injection with 10^4 CFU *E. ictaluri*.

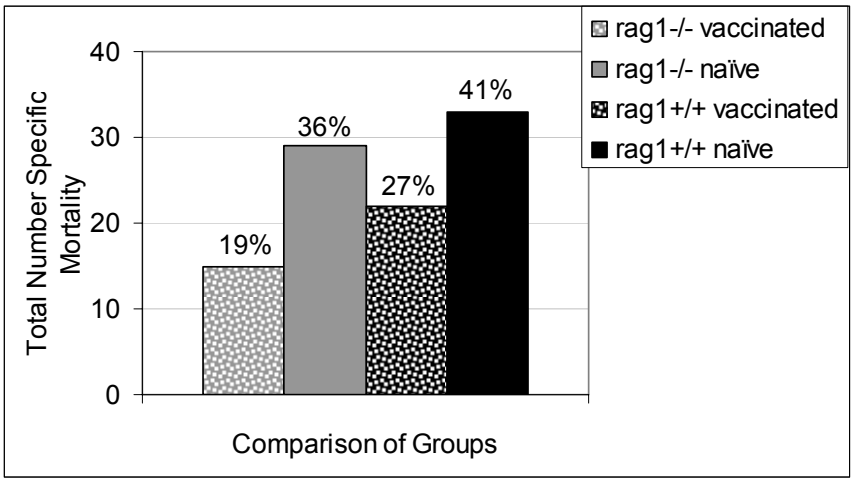

Figure 4.10 Specific mortality and corresponding mortality rate for *rag1-/-* MT and *rag1+/+* WT zebrafish 10 dpi with 10^4 CFU *E. ictaluri*/fish. Vaccinated fish were injected with 10^2 CFU *E. ictaluri* 1 month prior a second injection with 10^4 CFU *E. ictaluri*. Naïve fish received 10^4 CFU *E. ictaluri* as a primary exposure. All fish received orally administered antibiotics for 10 days prior injection with 10^4 CFU *E. ictaluri*.

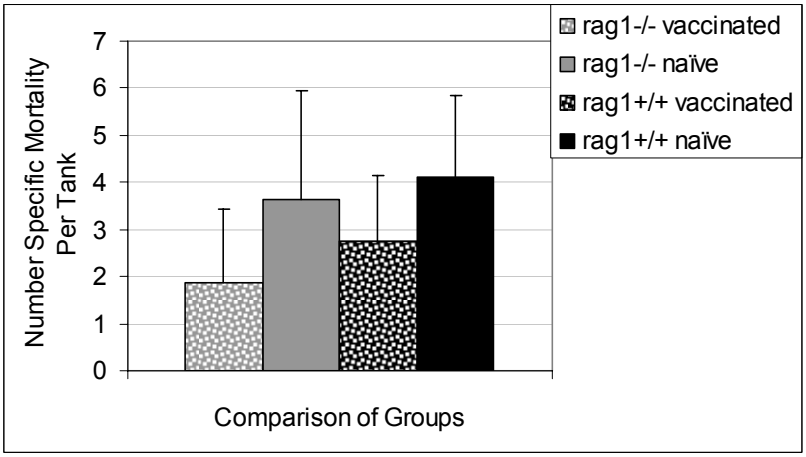

Figure 4.11 Specific mortality per tank for *rag1-/-* MT and *rag1+/+* WT zebrafish 10 dpi with 10^4 CFU *E. ictaluri*/fish. Vaccinated fish were injected with 10^2 CFU *E. ictaluri* 1 month prior a second injection with 10^4 CFU *E. ictaluri*. Naïve fish received 10^4 CFU *E. ictaluri* as a primary exposure. All fish received orally administered antibiotics for 10 days prior injection with 10^4 CFU *E. ictaluri*. Mortalities are numerically reduced in vaccination exposed MT and WT fish. Error bars indicate standard deviation between tanks (n=8).

Trial 4: 2 month. MT and WT zebrafish received 10^2 CFU *E. ictaluri* in a vaccination exposure. During the following 60 days moribund or dead fish were recorded and tested for *E. ictaluri* presence by reisolating bacteria from the brain.

Upon exposure to 10^4 CFU *E. ictaluri* naïve and vaccination exposed MT and WT fish mortalities were distributed over the 10 day observation period with highest specific mortality for MT fish at 4 dpi and for WT fish at 8 dpi (Figure 4.12). Cumulative mortality in these naïve MT and WT fish was 52% and 41% respectively (Figure 4.13). As expected, mortality rates of vaccination exposed WT fish decreased greatly to 26% after protection exposure. Vaccination exposed MT zebrafish showed an even more dramatic decrease from 52% to 17% in cumulative mortalities upon protection exposure (Figure 4.13). The decrease in mortality rate between naïve and vaccination exposed fish was significant for MT ($p< 0.001$) and WT fish ($p= 0.046$) (Figure 4. 14).

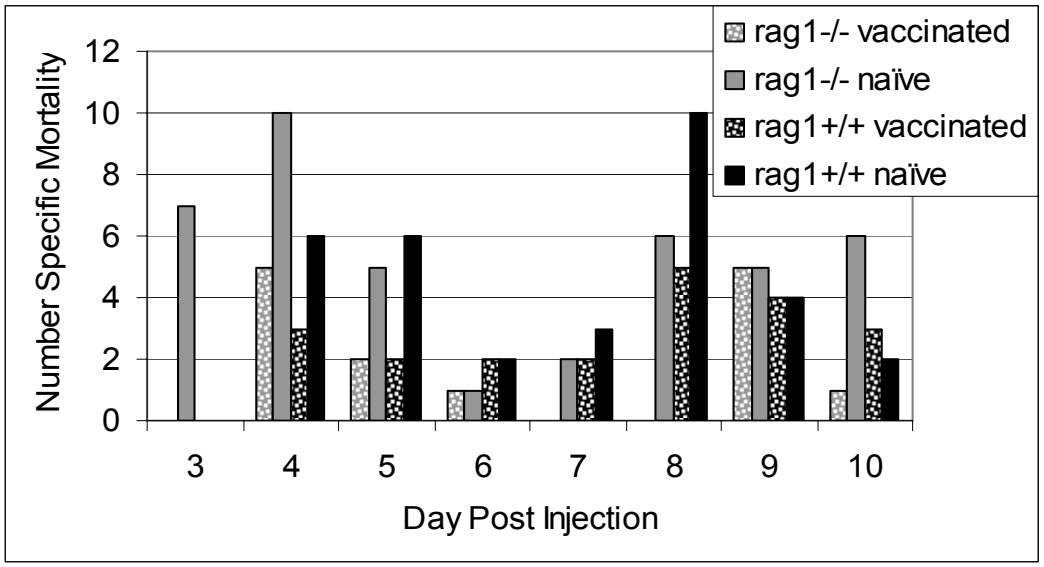

Figure 4.12 Specific mortality for *rag1-/-* MT and *rag1+/+* WT zebrafish over the course of 10 dpi. Vaccinated fish were injected with 10^2 CFU *E. ictaluri* 2 months prior a second injection with 10^4 CFU *E. ictaluri*. Naïve fish received 10^4 CFU *E. ictaluri* as a primary exposure.

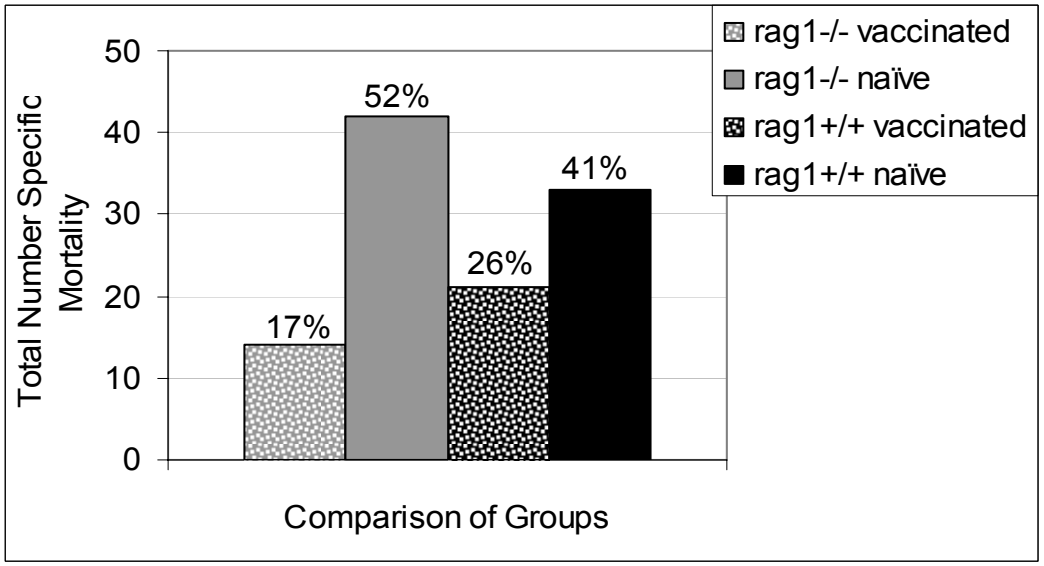

Figure 4.13 Specific mortality and corresponding mortality rate for *rag1-/-* MT and *rag1+/+* WT zebrafish 10 dpi with 10^4 CFU *E. ictaluri*/fish. Vaccinated fish were injected with 10^2 CFU *E. ictaluri* 2 month prior a second injection with 10^4 CFU *E. ictaluri*. Naïve fish received10^4 CFU *E. ictaluri* as a primary exposure.

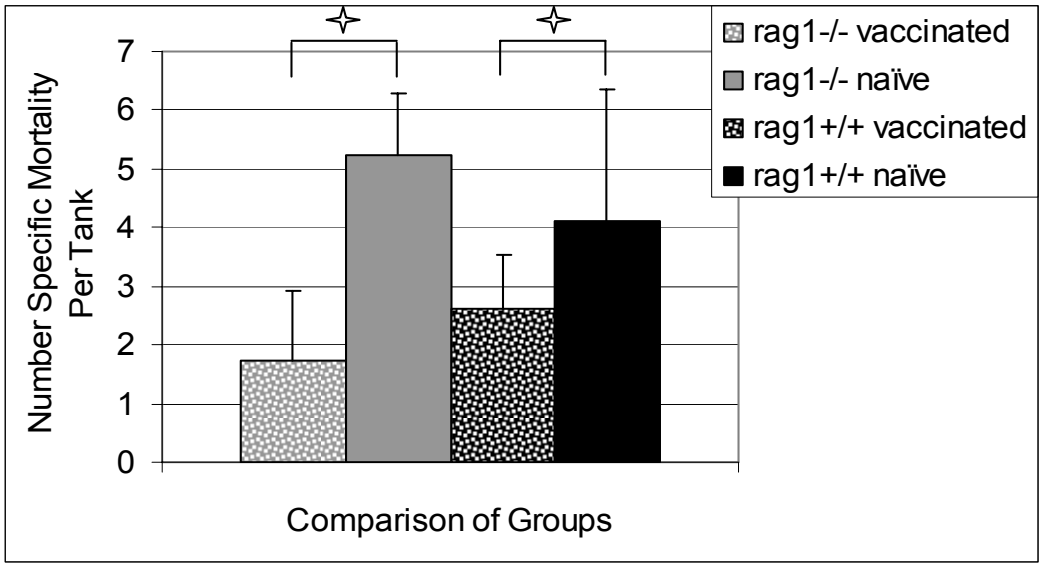

Figure 4.14 Specific mortality per tank for *rag1-/-* MT and *rag1+/+* WT zebrafish 10 dpi with 10^4 CFU *E. ictaluri*/fish. Vaccinated fish were injected with 10^2 CFU *E. ictaluri* 2 month prior a second injection with 10^4 CFU *E. ictaluri*. Naïve fish received 10^4 CFU *E. ictaluri* as a primary exposure. Mortalities were significantly reduced in vaccination exposed MT and WT fish. Error bars indicate standard deviation between tanks (n=8).

Trial 5: 2 month with antibiotic feed. Again, oxolinic acid feed was administered for 10 days to all fish utilized in this experiment. Since 2 months were in between vaccination exposure and protection exposure a 40 day period was allowed for body clearance of the drug.

Figure 4.15 shows delayed mortality upon exposure to 10^4 CFU *E. ictaluri* in WT vaccination exposed fish with highest specific mortalities between 7 to 9 dpi. WT naïve fish followed normal mortality patterns, peaking at 4 to 5 dpi and gradually decreasing until 10 dpi. Naïve MT fish specific mortalities gradually increased peaking at 9 dpi. Vaccination exposed fish showed highest specific mortalities of 8 fish at 4 dpi with much lower specific mortalities on the following

days. Decreased mortality rates of naïve MT fish and increased mortality rates in vaccination exposed MT and WT fish, fed oxolinic acid, compared to trial 4 was observed (Figure 4.13, 4.16). Although the nominal values suggest some protection the mortality differences between naïve and vaccination exposed fish were not significant in this trial. This was likely due to great variation between tanks within groups and decreased specific mortality in naïve fish (Figure 4.17).

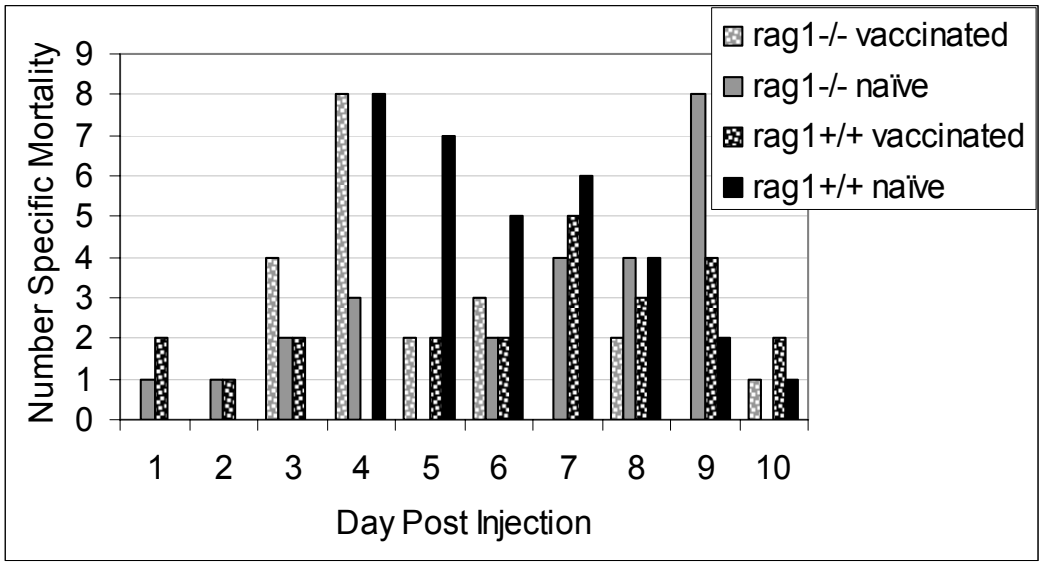

Figure 4.15 Specific mortality for *rag1-/-* MT and *rag1+/+* WT zebrafish over the course of 10 dpi. Vaccinated fish were injected with 10^2 CFU *E. ictaluri* 2 months prior a second injection with 10^4 CFU *E. ictaluri*. Naïve fish received 10^4 CFU *E. ictaluri* as a primary exposure. All fish received orally administered antibiotics for 40 days prior injection with 10^4 CFU *E. ictaluri*.

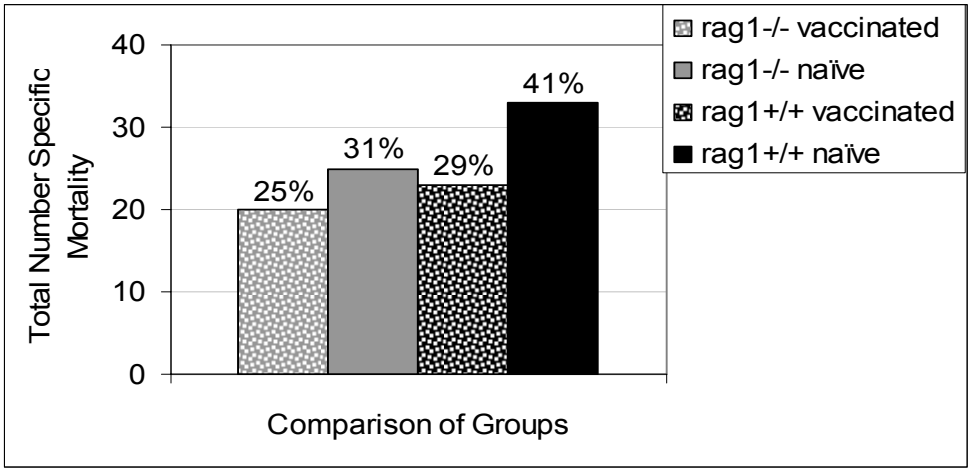

Figure 4.16 Specific mortality and corresponding mortality rate for *rag1-/-* MT and *rag1*+/+ WT zebrafish 10 dpi with 10^4 CFU *E. ictaluri*/fish. Vaccinated fish were injected with 10^2 CFU *E. ictaluri* 2 months prior a second injection with 10^4 CFU *E. ictaluri*. Naïve fish received 10^4 CFU *E. ictaluri* as a primary exposure. All fish received orally administered antibiotics for 10 days prior injection with 10^4 CFU *E. ictaluri*.

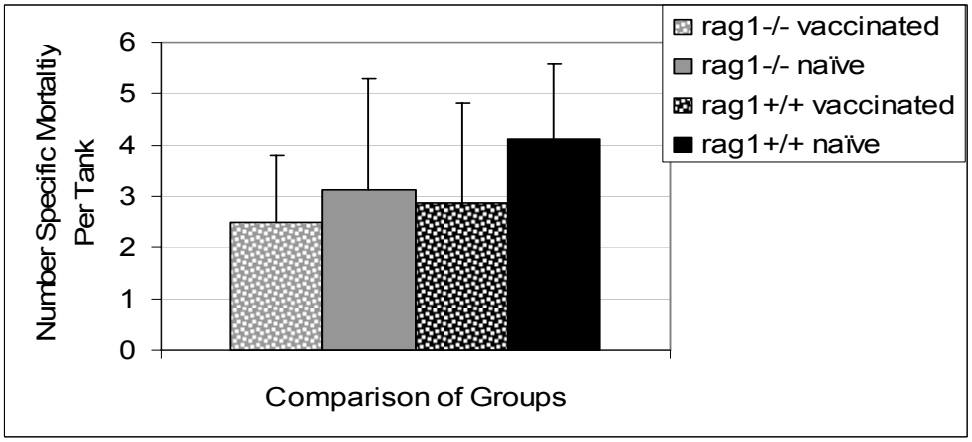

Figure 4.17 Specific mortality per tank for *rag1-/-* MT and *rag1*+/+ WT zebrafish 10 dpi with 10^4 CFU *E. ictaluri*/fish. Vaccinated fish were injected with 10^2 CFU *E. ictaluri* 2 months prior a second injection with 10^4 CFU *E. ictaluri*. Naïve fish received 10^4 CFU *E. ictaluri* as a primary exposure . All fish received orally administered antibiotics for 10 days prior injection with 10^4 CFU *E. ictaluri*. Mortalities are numerically reduced in vaccination exposed MT and WT fish. Error bars indicate standard deviation between tanks (n=8).

In summary, upon exposure to 10^4 CFU *E. ictaluri*, vaccination exposed MT fish showed decreased mortality rates, when compared to naïve MT fish. Differences in specific mortality of naïve and vaccination exposed fish was significant in trials where antibiotics were not administered. In trials where antibiotics were fed between vaccination and protection exposures, specific mortality decreased but were not significant between MT and WT fish.

Discussion

In this study, it was demonstrated that MT zebrafish, lacking an acquired immune system, show protection against a previously encountered pathogen. Evidence for memory in the innate immune system has been reported previously in invertebrates [45, 48, 51]. Innate immunity is still considered to lack specific memory, largely because there is no evidence of mechanisms that could provide such memory. Experiments by O'Leary *et al.* [47] on *rag1-/-* mice showed persistent memory mediated by natural killer (NK) cells. This is the first and only report of innate immune cells displaying memory in vertebrates, and has stimulated others to call for investigation of these provocative findings [114]. Immunocompetence in larval fish, as measured by humoral response to T-dependent and T–independent antigens, is not reached until 4 to 6 weeks post hatch depending on the species [29, 108, 115]. Therefore, a phenomenon previously experienced (Petrie-Hanson, personal communication) where larval catfish that had not yet developed acquired immunity when first exposed to *E. ictaluri*, demonstrated protection upon secondary exposure, called for further

investigation [42]. Since channel catfish only breed annually the window where larval fish are available for investigation is short and repeated experiments have to be conducted over several years. By utilizing MT zebrafish that lack B- and T cell driven immunity but posses intact cellular and humoral innate immune responses including NK cells, it was possible to investigate memory function of the innate immune system without the drawbacks associated with catfish. *E. ictaluri* was used because it was the pathogen associated with innate memory in channel catfish, and causes comparable ESC pathology in zebrafish [112].

Higher survival of MT fish during the onset of an infection can be attributed to increased cellular and humoral innate immune functions (Figures 4.2, 4.3). In chapter 3, a two-fold increase in neutrophils in MT zebrafish was discussed. Neutrophils play a major role in ESC pathogenesis in channel catfish, and increased numbers of these cells in MT fish could result in more rapid clearance of *E. ictaluri* during the initial stage of infection. However, this neutrophil response is short-lived. When observations were carried on for 18 dpi, WT fish mortalities subsided simultaneously with an activated acquired immune response whereas MT fish continued to die at a low rate (Figure 4.5). Specific antibodies to *E. ictaluri* have shown to increase phagocytic and bactericidal activity of neutrophils [116] and extracellular killing of *E. ictaluri* rather than intracellular killing appears to be more effective in channel catfish [109] and results in the lower mortality observed in the WT during the chronic phase of the infection. In channel catfish, macrophages are important in resistance against primary infection and live exposure to *E. ictaluri* results in an increase in macrophage-

mediated bacteriocidal activity [117]. Since in MT zebrafish the acquired immune system is non-functional the die-off phase is prolonged but mortalities at the onset of infection are decreased. Fish rely heavily on innate immune mechanisms to deal with pathogens [118], and the effectiveness of these mechanisms during initial infection is well demonstrated in the MT zebrafish model.

In trial 2, statistically significant differences in mortality upon protection exposure in MT zebrafish support previous findings of protection due to memory in innate immunity of larval channel catfish [42]. In additional studies (data not shown) investigating bacterial clearance, it was found that only 3 out of 10 MT zebrafish where able to clear *E. ictaluri* within 1 month. In the same study, *E. ictaluri* was not present in brain or kidney 30 dpi in WT fish. Therefore, an additional trial (trial 3) was conducted where all fish received orally administered antibiotics for 10 days. The rational was to eliminate *E. ictaluri* from the organism to prevent protection resulting from heightened innate immune responses due to continuous exposure to the pathogen. Oxolinic acid was the antibiotic of choice since recent studies in sea bass, *Dicentrarchus labrax*, found that pharmacokinetics of oxolinic acid was temperature dependent, and the elimination half-life of the drug was increased with higher temperatures and noted as 55 h at 22˚C [119]. In a follow up study, the depletion of oxolinic acid when treatment ceased reached a steady state in tissues from day 2 to the end of sampling on day 7 [113]. It was further stated that it can be assumed that warmwater fish are likely to process drugs faster than coldwater species and it is

well known that higher ambient temperatures increase xenobiotic metabolism and elimination rates [113]. Oxolinic acid belongs to the group of 4-quinolones and the mode of action on bacteria is by interference with the bacterial DNA gyrase and thus inhibits the supercoiling of DNA [120].

In trial 3, protection in vaccination exposed MT fish was observed. However, mortalities in naïve MT and WT fish were reduced in comparison and were not statistically significant. All fish received oxolinic acid, and it's persistence may have affected the immunological protection of zebrafish. It has been shown that oxolinic acid can persist at low levels (10–30 ng/g) for extended periods (4–8 weeks) in tissues of gilthead sea bream following multiple dosing *in situ* [113]. Antibiotics modulate immune responses in different ways, for example they may elevate non-specific defense mechanism while simultaneously depressing specific cellular defenses and protein synthesis [121, 122]. The effect of oxolinic acid metabolites can be more pronounced than that of the parent compound [120]. Therefore it might be that oxolinic acid or its metabolites, which have been shown to accumulate in macrophages and neutrophils and therefore have higher levels than measured in serum [123], have a bactericidal effect weeks after the antibiotic has been administered, explaining the decrease in mortalities in naïve MT fish upon first encounter with a lethal dosage of *E. ictaluri* (10^4 CFU/fish). This delayed protection effect of oxolinic acid has not been recorded elsewhere and might prove valuable in prophylactic treatment of catfish fry before release into ponds at 2 weeks post hatch when acquired immunity is not yet fully developed. Effects of quinolones on macrophages include

potentiated superoxide anion release which aids the host defense against microbial invaders [124]. Elsewhere it is stated that in *in situ* experiments quinolone activity was reduced intracellulary resulting in lesser effects on intracellular bacteria [125]. Research on effects of certain quinolones on neutrophils has shown that the phagocytosis of *Escherichia coli* in neutrophils was potentiated. Further it was found that adhesion of neutrophils was increased, and the production of superoxide anion and hydrogen peroxide was significantly potentiated [126]. The specific effects of oxolinic acid on macrophages and neutrophils have not been tested.

The general effects of oxolinic acid on fish, include decreased nonspecific and specific immune responses [122, 127]. Oxolinic acid also suppressed some immune parameters for weeks and months after elimination from the serum of rainbow trout [128]. The same study states that rainbow trout treated with oxolinic acid had a significantly lower antibody response to *V. anguillarum* at weeks 7 and 12 after vaccination. Further, after oral administration of oxolinic acid, the mitogenic response in head kidney cells was clearly suppressed causing leukopenia [120]. Immunity to ESC is dependent on an effective immune response; catfish that recover from infection are immune to the disease, emphasizing the importance of the relationship between the humoral and cellular immune responses in protection to ESC [109]. A decrease in lymphocytes and hence lower antibody levels could explain why vaccination exposed WT zebrafish that received in-feed oxolinic acid showed increased mortalities upon protection

exposure one and two months later (Figures 4.10, 4.16) in comparison to respective trials where no antibiotics were fed (Figures 4.7 and 4.13).

Trial 2-5 show either statistical or numerical decrease in cumulative mortalities in MT zebrafish upon secondary exposure to *E. ictaluri* when compared to MT fish that received a primary exposure. The basis of protection due to persistently heightened innate immune system components was eliminated by orally administered oxolinic acid treatment in trials 3 and 5. These results show that MT zebrafish, lacking an acquired immune system, are able to mount a protective immune response to *E. ictaluri* following a primary exposure, and this response provides protection similar to that of WT zebrafish following repeated encounter to the same pathogen. No previous record of memory function in the innate immune system in fish has been documented. O'Leary *et al*. [47] found that SCID mice devoid of T cells and B cells demonstrated substantial contact hypersensitivity responses to haptens that persisted for at least 4 weeks and were elicited only by haptens to which mice were previously sensitized. No contact hypersensitivity was induced in mice lacking all lymphocytes, including NK cells. These observations indicate that NK cells can mediate long-lived, antigen-specific adaptive recall responses independent of B cells and T cells [47]. Based on PCR results (chapter 2) MT zebrafish posses NK cells and further experiments will investigate the role of NK cells in MT zebrafish innate memory responses.

Innate immune system memory has been documented in invertebrates. In *Drosophila melanogaster* it has been shown that priming with a sublethal dose of

Streptococcus pneumoniae protects against an otherwise-lethal second challenge of *S. pneumoniae*. This protective effect exhibits coarse specificity for *S. pneumoniae* and persists for the life of the fly [43]. In the same study, fly phagocytes were the essential effector of the primed response and after thorough investigation it was conclude, that a priming dose of *S. pneumoniae* alters the fly immune system in a persistent manner that specifically allows phagocytes to recognize and kill *S. pneumoniae* more efficiently [43].

Endocytosis studies on MT zebrafish (chapter 5) showed strong activation of phagocytes after incubation with *E. ictaluri*. Macrophage populations have been found to play an important role in ESC infections of catfish. Resistant populations of channel catfish have been reported to have more macrophage aggregates in spleen and head kidney than in susceptible populations [129], and macrophages from resistant catfish more effectively killed bacteria between 1 and 3 h after infection [117]. Also, macrophages from fish surviving exposure to live *E. ictaluri* were capable of killing large numbers of bacteria per macrophage at 2.5 hpi [117]. Microarray analysis of gene expression changes in blue catfish liver after infection with *E. ictaluri* indicated the strong upregulation of several pathways involved in the inflammatory immune response and potentially in innate disease resistance. A multifaceted response to infection with *E. ictaluri* was observed, encompassing the complement cascade, iron regulation, inflammatory cell signaling, and antigen processing and presentation. The induction of several components of the MHC class I related pathway following infection with an intracellular bacterium is also reported for the first time in fish [130]. These

findings emphasize the importance of the innate immune response of fish for survival and could explain why MT zebrafish show similar good survival rates as WT fish do. Microarray analysis focusing on gene expression of the innate immune system in MT zebrafish after first and repeated exposure to a pathogen should give valuable insight into which components are capable to provide a specific primed immune response.

In conclusion, the data presented here support initial observations of innate immunity memory responses in channel catfish fry. MT zebrafish have proven to be an ideal animal model to study innate immune responses without the overshadowing effects of acquired immunity. Further, MT zebrafish are a unique model for characterizing the molecular basis underlying specific phagocyte activation, NK cell priming and other adaptive aspects of innate immunity. Future research utilizing these MT fish will help to elucidate the full potential of the innate immune system.

CHAPTER V

COMPARISON OF ENDOCYTIC ACTIVITY IN PHAGOCYTES OF *RAG1-/-* MUTANT AND *RAG1+/+* WILD-TYPE ZEBRAFISH

Abstract

The innate immune response constitutes the first line of defense against invading pathogens and consists of a variety of immune defense mechanisms including active endocytosis by macrophages and granulocytes. Endocytosis can be used as a reliable measure of selective and non-selective mechanisms of antigen uptake in the early phase of an immune response. Numerous assays have been developed to measure this response in a variety of mammalian and fish species. The small size of the zebrafish has prevented the large-scale extraction of monocytes/macrophages and granulocytes for endocytic assays. Described is a new procedure to overcome this limitation. Pooled zebrafish kidney hematopoietic tissues were used as a source of phagocytic cells for flow-cytometry based endocytic assays. *Rag1-/-* mutant zebrafish are known to lack an acquired immune system and this study investigates endocytic functions of phagocytes from these fish and compares it to wild-type fish. FITC-Dextran, Lucifer Yellow and FITC-*Edwardsiella ictaluri* were used to evaluate selective and non-selective mechanisms of uptake in zebrafish phagocytes.

For the first time it was shown that zebrafish phagocytes utilize macropinocytosis and Ca^{2+} dependant endocytosis mechanisms. No significant differences in the endocytic uptake of phagocytes were observed between *rag1-/-* mutant and *rag1*+/+ wild-type zebrafish.

Introduction

The zebrafish, one of the most popular animals of developmental biologists, is rapidly gaining ground as an infection and immunology model [3, 5, 95]. The ease of producing specific zebrafish mutants make this fish an excellent model for experimental immunology [29]. Recently *rag1-/-* mutant zebrafish have been produced by a reverse genetic approach [1]. The first ongoing *rag1-/-* zebrafish breeding colony has been established at the College of Veterinary Medicine at Mississippi State University (CVM-MSU), and in characterization studies, these *rag1-/-* zebrafish have been found to lack functional B and T cells and therefore rely solely on their innate immune system [105]. To further characterize *rag1-/-* zebrafish, the focus of this study was to compare the endocytic ability of *rag1-/-* mutant (MT) and *rag1*+/+ wild-type (WT) zebrafish. This information will prove valuable in further epidemiological and immunological experiments utilizing this new MT zebrafish strain.

Fish possess a well-developed, non-specific innate immune system, and phagocytes play an important role in the fish defense against microorganisms [6, 39, 101, 131-136]. Phagocytic function has been used as an immunological parameter to evaluate the health status and immune function of different fish

species under diverse biotic and abiotic factors such as pollutants [137], diets [138], temperature [139], pathogens [131] and genetic variation [140]. Cells of the monocyte/macrophage and granulocyte lineage are important elements of the immune defense system. These cells take up and destroy foreign material, and macrophages present antigens to immunocompetent cells and produce cytokines. Multiple mechanisms of endocytosis are used by different cell types [141]. In seabream the capture of antigens by surface receptors, such as the mannose receptor (MR) and glucan receptor was reported [142, 143]. In mammals these receptors allow efficient delivery of antigen to the processing compartment via receptor-mediated endocytosis [144]. MR-dependent endocytosis can be assessed by fluorescein isothiocyanate-labeled dextran (FITC-DX) uptake and inhibited by EDTA, anti-mannose receptor mAbs or mannan, a natural ligand of the MR in mammals [145]. Antigens that fail to bind to cell surface receptors can still be taken up by fluid phase endocytosis but with a lower efficiency [146]. Fluid phase uptake can occur via distinct mechanisms: micropinocytosis and macropinocytosis [146]. In mammals, macropinocytosis is a potent non-selective mechanism of antigen uptake limited to immature dendritic cells and their myeloid progenitors, and monocytes/macrophages activated by exogenous stimuli [147, 148]. The internalization of solutes by macropinocytosis is much more effective than other fluid-phase uptake mechanisms, particularly micropinocytosis mediated by clathrin-coated vesicles [148]. Lucifer Yellow (LY) is traditionally used to assess macropinocytosis [149-151]. Cytochalasin D (CCD) is a cell permeable mycotoxin that inhibits macropinocytosis by blocking the

formation of microfilaments and microtubules, but has no significant effect on receptor-mediated endocytosis [146]. Little is known about the selective and non-selective mechanisms of antigen uptake in fish. Zebrafish have been established as a model for the infectious disease Enteric Septicemia of Catfish caused by the intracellular pathogen *Edwardsiella ictaluri* [112]. The described endocytic assay was used to investigate the mechanisms of uptake of *E. ictaluri*.

The small size of the zebrafish (3–5 cm) and its kidney are drawbacks, and preclude large-scale extraction of homogenous suspensions of monocytes or neutrophils. The inability of researchers to extract sufficient numbers of phagocytes from the kidney and the lack of phagocytic cell lines have delayed the development of phagocytic assays for the zebrafish model system [37].

The aim of this study was to develop methods to measure the phagocytic capabilities of zebrafish phagocytes and to compare the cellular components of the innate immune system of MT and WT zebrafish, focusing on the endocytic abilities of monocytes/macrophages and granulocytes.

Materials and Methods

Zebrafish care. Zebrafish were housed in the CVM-MSU specific pathogen free fish hatchery [2]. Maintenance and propagation of fish were performed according to modified standard protocols [53] and are posted at : http://www.cvm.msstate.edu/zebrafish/index.html.

All experiments were approved by the Institutional Animal Care and Use Committee at Mississippi State University.

Cell preparation. Zebrafish were anesthetized in MS-222 [53], a stock solution of anesthetic was prepared by combining 400 mg of Finquel® (Argent, Redmond, WA), 97.9 ml double distilled water and 2.1 ml TRIS base (1M). The pH of the stock solution was adjusted to ~7. Just before use 4.2 ml of the stock solution was mixed with 100 ml system water. Kidneys where surgically remove with the aid of an Olympus SZ40 stereoscope and dissecting procedures were followed as described by Dr. David Stachura (University of California at San Diego, personal communication). First, a midline incision from the posterior end to the gills was made. Most of the internal organs were removed and kidney was scraped from the body cavity ventral to the spine (Figure 5.1). To obtain single kidney cells, published kidney cell suspension protocols were modified [152]. Ten whole kidneys per fish strain (MT or WT) were pooled in 1 ml tissue culture media (RPMI-1640 supplemented with 10% fetal bovine serum, 1% Glutamax-1). Cells were disrupted from the whole kidney tissue by pipetting up and down. Cell suspensions were passed through a 40 µm cell strainer, collected in a 50 ml conical tube and rinsed with 1 ml tissue culture media. This procedure yielded approximately $7x10^6$ mixed kidney cells per ml.

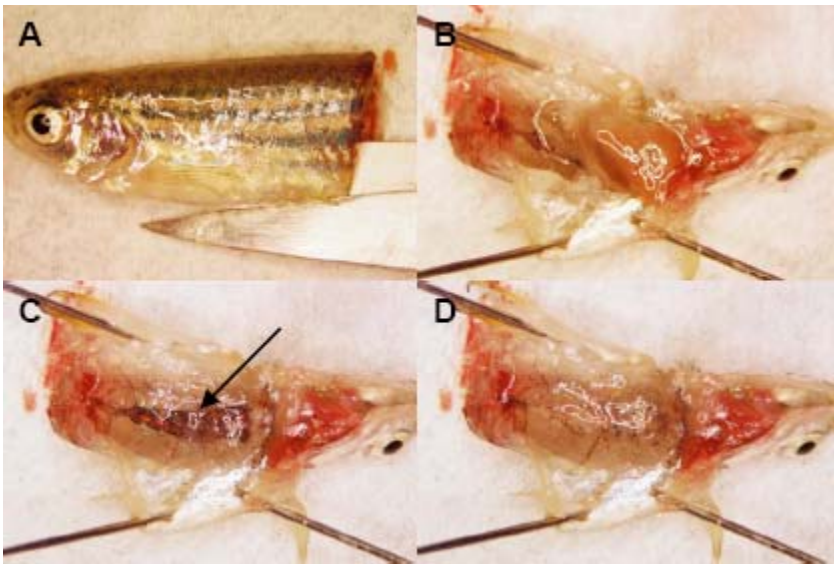

Figure 5.1 Kidney removal in zebrafish. A) midline incision B) body cavity with internal organs C) internal organs removed and black arrow pointing at kidney tissue D) after removal of kidney.

Endocytosis assay. The ability of kidney macrophages/monocytes and granulocytes to endocytose FITC-DX 70 (Sigma-Aldrich Inc., St. Louis, MO), LY (Invitrogen Corporation, Carlsbad, CA) or FITC-*Edwardsiella ictaluri* (FITC-*E. ictaluri*) was measured following published procedures [149, 150]. Briefly, 100 µl of kidney hematopoietic cell suspension per sample was incubated for 30 min at 30˚C representing fish holding temperature and at 37˚C to measure active endocytosis or at 4˚C to determine background levels of endocytosis (negative control). The cell suspension was washed three times by centrifugation in cold PBS and analyzed using a FACS Calibur (described below). To determine the mechanism of endocytosis, three different inhibitors were used. Cells were incubated for 5 min in the presence of inhibitor prior to 30 min incubation with FITC-DX 70, LY, or FITC-*E. ictaluri*. To inhibit macropinocytosis and

phagocytosis, 500 µg/ml Cytochalasin D (CCD) (Sigma-Aldrich Inc., St. Louis, MO) was used (with ethanol controls). To inhibit Ca^{2+}-dependent endocytosis that is usually receptor-mediated, samples were incubated with 0.6 µL/ml EDTA. Ten mg/ml mannan (Sigma-Aldrich Inc., St. Louis, MO) was added to inhibit specific uptake by the MR. Each endocytic assay was carried out in triplicate.

Labeling of *E. ictaluri* with FITC. *E. ictaluri* (93146 WT#19) was labeled with FITC following a *Vibrio anguillarum* labeling protocol by Chavez-Pozo [153]. Bacteria were grown over-night in brain heart infusion (Becton Dickinson, Franklin Lakes, NJ) supplemented with 50 µg/ml FITC (Sigma-Aldrich Inc., St. Louis, MO) at 30˚C in a light-protected environment. Bacteria were washed three times in PBS by centrifugation for 10 min at 1000 g and inactivated by heating at 60˚C for 20 min. After an additional washing step, optical density was measured and bacterial concentrations were adjusted to 1.8 $x10^8$ colony forming units (CFU)/ml. Use of the labeled bacteria in endocytosis assays was done as described above.

Flow cytometry. Samples were mixed gently, acquired and analyzed by FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). First, the instrument settings were adjusted to obtain optimal separation of the different cell populations present in zebrafish kidney leukocytes [12, 22]. A total of 200,000 cells per sample were collected. Data were analyzed as dot plots using Side scatter (granularity) (SSC) and Forward scatter (size) (FSC) parameters (Figure 5.2A). After setting a gate on macrophages/monocytes and granulocytes,

incorporation of FITC-DX, LY or FITC-*E. ictaluri* was measured as green fluorescence (FL1) at 530 nm (Figure 5.2B), expressed as Mean Fluorescent Intensity (MFI), and analyzed using CellQuest™ Pro software (Becton Dickinson, Franklin Lakes, NJ).

Statistical analysis. All endocytosis assays were performed in triplicate. Data were expressed as mean and standard deviation (SD). One-way analysis of variance (ANOVA) with LSD correction (least statistical significance) for multiple comparisons with a level of significance at $p \leq 0.05$ was used to calculate differences among treatment groups. Statistical analyses were performed using SPSS for Windows 15.0 (SPSS Inc., Chicago, IL).

Results

Characterization of zebrafish leukocytes. When zebrafish phagocytes were characterized by flow cytometry, three main cell populations distributed according to their size (FSC) and granularity (SSC) (Figure 5.1A). Gate A represents the cells of interest, monocytes/macrophages and granulocytes. Gate C shows the characteristic decreased numbers of lymphocytes in MT fish when compared to WT fish [105].

Fluid-phase uptake and receptor-mediated endocytosis in zebrafish phagocytes.

Non-selective uptake. Lucifer Yellow was actively taken up by phagocytes at 30˚C. This uptake was numerically increased at 37˚C. No significant differences were observed between zebrafish MT and WT for the 30˚C and 37˚C treatments. The addition of 0.5 µl CCD (500 µg/ml in ethanol) significantly inhibited LY uptake in zebrafish cells (Figure 5.3). When increased CCD concentrations were compared to ethanol controls, effects of inhibition due to ethanol toxicity on the cells was observed. Therefore, in further experiments only 0.5 µl CCD per 100 µl of sample volume was used.

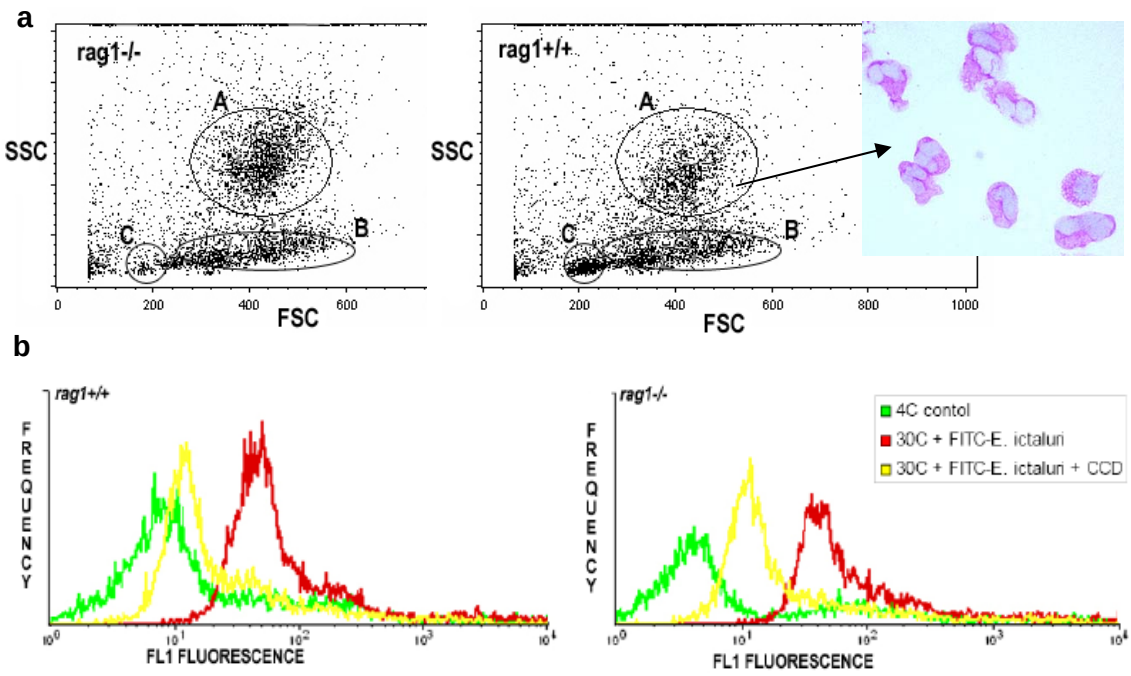

Figure 5.2 a) Forward Scatter (FSC) and Side Scatter (SSC) characteristics of kidney cell suspensions differentiate 3 distinct cell populations in *rag1-/-* MT and *rag1+/+* WT fish. A: macrophage/monocytes and granulocytes B: hematopoietic precursors C: lymphocytes and lymphocyte-like cells. Note the reduction of around 10% to 4% in $SSC^{low}FSC^{int}$ cells (gate C), characteristic of small lymphocytes, in MT fish. Kidney phagocytes in gate A were analyzed for endocytosis. b) Endocytosis was assessed by measuring green fluorescent intensity (FL1) in the gated macrophage/monocytes and granulocytes by flow cytometry. The fluorescent peaks in this example indicate macropinocytosis of FITC-*E. ictaluri* and inhibition by CCD.

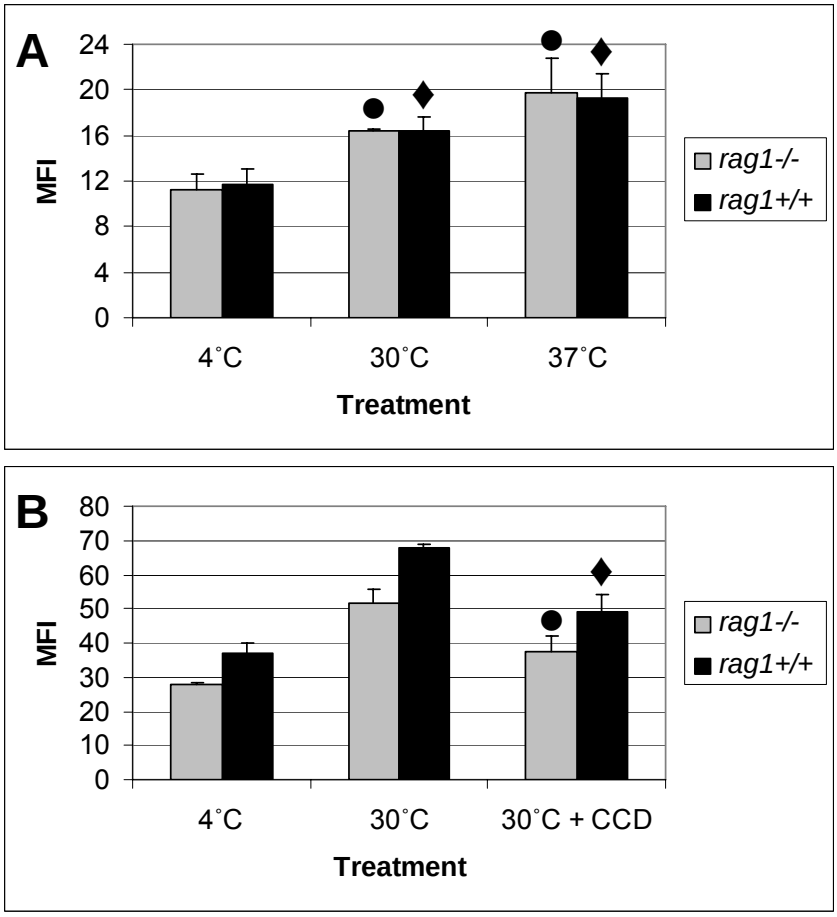

Figure 5.3 Macropinocytosis of LY in *rag1-/-* MT and *rag1+/+* WT zebrafish. A) Significant uptake of LY at 30˚C and 37˚C in MT (●) and WT (♦) phagocytes was observed. No significant difference in mean fluorescent intensity (MFI) between MT and WT phagocytes was observed. B) Significant inhibition of LY at 30˚C with CCD was observed in phagocytes from both strains (●♦).

Mannose receptor-mediated antigen uptake. *In vitro* incubation of zebrafish phagocytes with FITC-DX 70 showed no significant increase in antigen uptake measured as MFI at 30˚C or 37˚C when compared to uptake at 4˚C control in both MT and WT fish, indicating that no active uptake of FITC-DX 70 occurred (Figure 5.4).

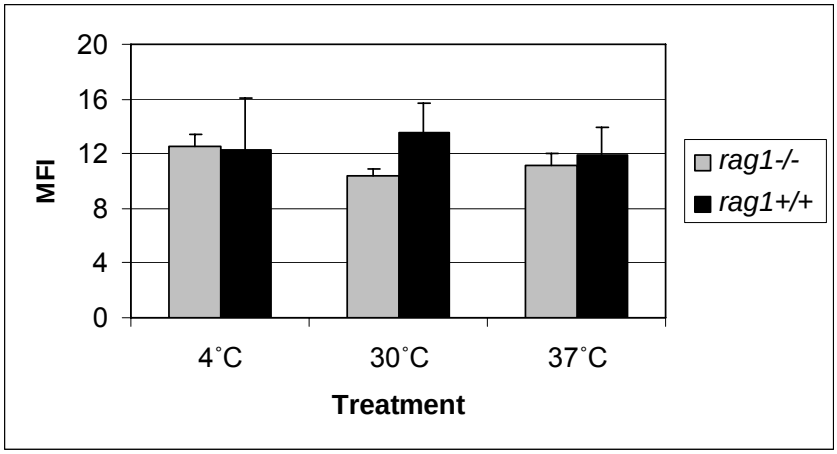

Figure 5.4 Endocytosis of FITC-DX in *rag1-/-* MT and *rag1+/+* WT zebrafish. No significant differences in the uptake of FITC-DX 70 at 30˚C and 37˚C in MT and WT phagocytes were observed.

Mechanisms of *E. ictaluri* uptake in zebrafish phagocytes. Renal phagocytic cells were incubated with FITC- *E. ictaluri* in the presence of CCD or EDTA inhibitors (Figure 5.5). Phagocytic cells of both MT and WT fish demonstrated significant active uptake of *E. ictaluri* at 30˚C. A further significant increase in antigen uptake was observed at 37˚C when compared to the 4˚C control cells. No significant differences were observed between MT and WT phagocytosis within the same temperature except when CCD or EDTA was included at 30˚C. When cells were incubated with CCD prior to the addition of FITC-*E. ictaluri,* a significant reduction of actin-dependent uptake was measured in MT and WT fish cells, indicating that macropinocytosis plays an important role in the internalization of this pathogen. EDTA also showed a numerical, but not a significant inhibitory effect in the MT fish, but significant effect in WT fish indicated that receptor-mediated endocytosis was also involved in the uptake of *E. ictaluri* in the WT.

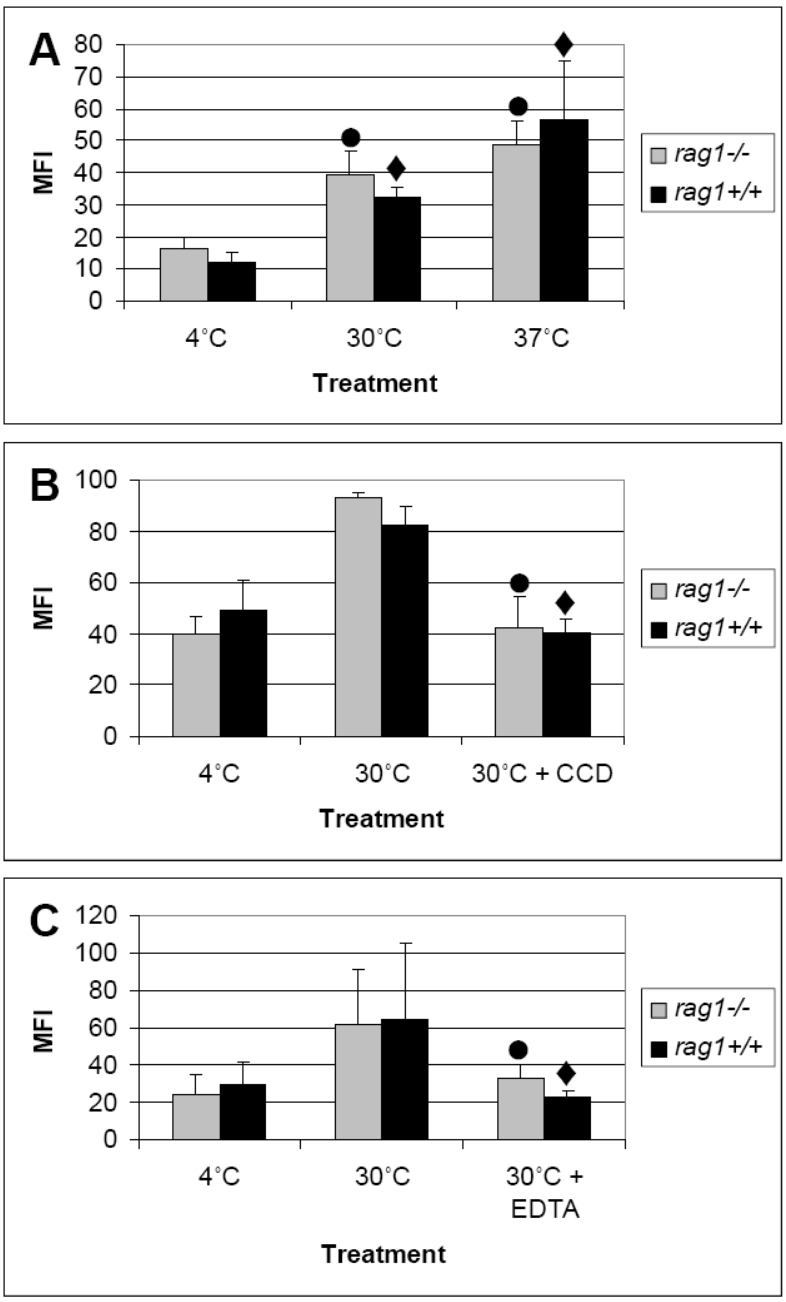

Figure 5.5 Macropinocytosis and receptor-mediated endocytosis of FITC-*E. ictaluri* in *rag1-/-* MT and *rag1+/+* WT zebrafish. A) Significant uptake of FITC-*E. ictaluri* at 30˚C and 37˚C was demonstrated when compared to 4˚C control in MT (●) and WT (♦) phagocytes. B) Significant inhibition of FITC-*E. ictaluri* uptake at 30˚C with CCD, and C) significant inhibition with EDTA were observed, when compared to 30˚C treatment without inhibitors. No significant differences in mean fluorescent intensity (MFI) were found between MT and WT fish in any of the treatments.

Discussion

With an average length of 3–5 cm and blood yield of 10 µl per zebrafish, commercially available phagocytosis test kits are non-practical for use in zebrafish. Traditional tests using fluorescent or light microscopy to calculate phagocytic index or phagocytic capacity are labor intensive and can be biased [135, 154-156]. Studies in which microscopic assessment of phagocytosis in head kidney granulocytes of fresh water fish were compared to the flow cytometry method reported good correlations [157, 158]. Our findings also support flow cytometry as a suitable method for endocytic studies in fish. A well known difficulty of zebrafish immunological research is the paucity of mAbs against zebrafish blood cells, which excludes use of fluorescent activation cell sorting, a technique sometimes used in other fish species to sort for cells of interest [159]. With this in mind, our method utilizes flow-cytometry to gate the cells of interest, and avoids the need to separate cell populations by gradients, an extremely tedious task in animals like the zebrafish that are extremely small in size. Utilizing flow-cytometry greatly reduces the use of animals but at the same time allows many replicates to be analyzed quickly in order to obtain reproducible and reliable data. Whole blood assays to test phagocytosis in mammalian macrophages and neutrophils have been described [160-163]. Since fish erythrocytes are nucleated, lysis is more difficult compared to mammals, and could negatively affect leukocyte populations [164]. In addition, after lysis large nuclei are still present in the sample and could falsely increase leukocyte counts. Further, separation of whole blood zebrafish cells using flow cytometry was

difficult due to the overshadowing effect of red blood cells. Separation of whole blood cell populations, utilizing the specific stains $DiOC_6$ and $DiOC_5$, has been demonstrated in common carp [165] and other species with nucleated erythrocytes [166]. This technique was unsuccessful in zebrafish whole blood (data not shown). Since the kidney serves as hematopoietic tissue in fish and delineation of cell populations can be done using forward and side scatter properties [22, 29] (Figure 5.1A), our method overcomes all of the above mentioned difficulties.

The problem of superficial adherence of antigen to the cell surface and/or background non-specific antigen uptake was overcome by simply comparing the results in the experimental sample against those obtained in a control sample incubated at 4°C. The fluorescence detected in the control sample corresponds to adherence and/or non-specific antigen uptake, which is not affected at low temperatures, while active cellular functions, including phagocytosis, are inhibited [162].

Macropinocytosis is a major endocytic pathway involved in non-selective bulk fluid-phase uptake [141]. LY was chosen as a model antigen to demonstrate macropinocytosis [167]. Published studies using LY to demonstrate macropinocytosis in teleost fish are not available. In our study, active uptake of LY and FITC-*E. ictaluri* at 30˚C as well as 37˚C by MT and WT zebrafish renal phagocytes was demonstrated. Zebrafish are increasingly utilized as a model for human pathogens [99, 100, 168, 169] as well as economically important fish pathogens [101, 102, 112], therefore, two incubation temperatures for phagocytic

cells were evaluated. Since tested compounds where successfully taken up at 30˚C, the normal zebrafish holding temperature, and 37˚C, the optimal temperature for mammalian pathogens, the data presented here underlines the usefulness of zebrafish in epidemiological studies. Importantly, for the first time it is shown that zebrafish phagocytes were able to capture LY by macropinocytosis as was previously demonstrated for humans and cattle immature dendritic cells [150, 170]. The data indicate that, like cattle [149] and human dendritic cells, zebrafish phagocytes use macropinocytosis for a bulk-fluid uptake of soluble antigens.

In a previous study zebrafish was evaluated as a model for Enteric Septicemia of Catfish caused by *E. ictaluri* [112]. Therefore, FITC-*E. ictaluri* antigen was included to further investigate the mechanism of bacterial uptake in zebrafish phagocytic cells. Skirpstunas *et al*. reported that chemical inhibitors were used to demonstrate the importance of cellular microfilaments and receptor-mediated endocytosis in uptake of *E. ictaluri* using mammalian epithelial cells [171]. The inhibitor CCD specifically binds to actin and causes microfilament depolymerization, which results in altered cell morphology and interference with bacterial adherence and entry [172]. EDTA blocks receptor mediated endocytosis which involves phagocytosis and micropinocytosis. The data presented here demonstrate that intracellular invasion by bacteria requiring surface alterations to facilitate either adherence and/or internalization was substantially reduced after incubation with CCD and EDTA. These findings lead to the conclusion that a complex system of interactions for internalization is utilized by this intracellular

pathogen. Further studies are needed to fully understand the effects of *E. ictaluri* on mechanisms of endocytosis in cells of the innate immune system of fish.

FITC-DX is used to demonstrate MR-mediated endocytosis, and is accepted as a classical model antigen for mammalian antigen presenting cells [149]. In fish the uptake of FITC-DX by renal macrophages and granulocytes has not been reported. All organisms possess abundant mannose, glucose and other sugars on cell surfaces. Specific receptors recognize these sugars, and are well-characterized in mammals. The MR is the most studied of the lectin-like receptors [173, 174]. These receptors constitute an essential part of the host defense system because they are involved in phagocytosis of infectious agents and in the internalization of parasites that manage to survive and replicate inside phagocytes, mainly macrophages [175]. Fish possess the putative MR proteins that exhibit structural similarity to other vertebrate MR proteins, suggesting that the putative receptors are present in all vertebrates [143]. FITC-DX is used as a classical antigen to demonstrate MR dependent endocytosis in mammals [145]. In zebrafish the more specific mannose-6-phosphate receptor has been biochemically characterized [176, 177]. When phagocytes were incubated with FITC-DX 70 no uptake occurred. Esteban *et al*. reported an involvement of glucan receptor but not MR in the phagocytosis of pathogens by seabream blood leukocytes [142]. Combining the result from this study and Esteban *et al*. [142] it could be speculated that perhaps the MR binds different ligands in fish than in mammals or that the MR is not expressed in phagocytic zebrafish cells.

In conclusion, flow cytometry is a quick, reproducible, and objective method to evaluate the endocytic capacity of zebrafish renal phagocytes. The proposed method is easy to implement and should prove especially useful in immunological, toxicological and epidemiological research. Additionally, this method is particularly useful for simultaneous multi-parameter analyses of small sample volumes. No significant differences in endocytosis between kidney phagocytes from MT and WT fish were observed. Mutant zebrafish can be utilized to further investigate mechanisms of the innate immune system without the influence of adaptive immune responses. This provides a simple and rapid assay for studying innate immunity in zebrafish. This zebrafish endocytosis assay will enable researchers to study the effects of pathogens, environmental toxins, and stress on fish immune health and provides an immunological method that makes zebrafish a more attractive model system for addressing immunology-related questions.

CHAPTER VI

CONCLUSION

Rag1-/- mutant (MT) zebrafish breeding colonies have been established and it was shown that this immunodeficient fish survive to adulthood and are fertile. With optimized and standardized rearing protocols progeny of these MT fish are readily available for immunological research.

Further, molecular studies revealed that MT fish lack functional B and T lymphocytes yet retain NK cell and NCC populations concluding that acquired immunity is absent. Therefore, MT zebrafish provide the platform to investigate the roles of NK cells, NCCs, macrophages, neutrophils and eosinophils in immune responses.

The newly developed, flow cytometry based, endocytosis assay is quick, reproducible, objective, and useful for simultaneous multi-parameter analyses of small sample volumes. No significant differences in endocytosis between kidney phagocytes from MT and *rag1+/+* wild-type (WT) fish were observed concluding that cellular innate immunity is fully functional in MT fish.

Innate memory function, observed in invertebrate models, larval channel catfish, and with limitations in SCID mice, has been confirmed utilizing MT zebrafish. Specifically, in the present work it has been shown that MT zebrafish were able to mount a protective immune response to the pathogen *Edwardsiella*

ictaluri and generate protection similar to WT zebrafish upon repeated encounter to the same pathogen.

These results suggest that *rag1-/-* MT zebrafish provide a unique model for investigating innate immune responses, especially memory function, because fully functional innate defenses are present without the influence of lymphocytes and lymphocyte associated acquired immune responses.

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