THE ZEBRAFISH COELOMIC CAVITY: A MODEL TISSUE FOR STUDIES OF INNATE AND ADAPTIVE IMMUNITY

by

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ABSTRACT

The zebrafish (*Danio rerio*) is an emerging model in immunological and developmental studies. One of the practical limitations in this model is the ability to obtain immune cells in sufficient quantity. The zebrafish spleen contains about 100,000 leukocytes and the entire anterior and trunk kidney contains about 10^6 cells, 40% of which are red blood cells. A coelomic cellular exudate was observed following injections of adult zebrafish with LPS and fixed bacteria. Fish injected with *Edwardsiella ictaluri* also produced large exudate responses. Exudate cells were identified consisted of granulocytes, macrophages, lymphocytes, and nonspecific cytotoxic cells. Functional assays showed that the exudates contained cytotoxic activity against HL-60, YAC-1, and K562 targets. The granulocytes in these exudates phagocytosed Gram-positive and negative bacteria. We conclude that the zebrafish coelomic cavity is an immune responsive tissue appropriate for determination of host innate and adaptive immunity to infectious diseases.

INDEX WORDS: zebrafish, coelomic exudate cells, *Edwardsiella ictaluri*, cytotoxicity, phagocytosis

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DEDICATION

I want to dedicate this thesis to my parents, Wayne and Janie Moss, for their never-ending love and support. Their constant encouragement continuously motivates me to pursue my dreams and fully commit myself to everything I take on in life.

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CHAPTER 1

INTRODUCTION

The zebrafish (*Danio rerio*) has recently emerged as a relevant model in immunological and developmental studies. These small tropical fish, averaging only an inch or two in length, provide many advantages over the mouse or invertebrate models of infection. These advantages include high fecundity, inexpensive and easy maintenance, the availability of the entire genome sequence, transparent embryo through 3 weeks post fertilization, and the presence of both an innate and adaptive immune system. Disadvantages with the zebrafish model include the lack of available reagents such as antibodies, limited understanding of their immune cells, as well as the inability to obtain large numbers of cells. Dissecting out the kidney or spleen of zebrafish is a time consuming and difficult process yielding more red blood cells than leukocytes.

In this study, we proposed the use of the zebrafish coelomic cavity as an immune responsive organ and a source of obtaining leukocytes. It is known that in other species of fish that there are resident leukocytes present in the coelomic cavity. In this study we seek to determine the types and concentrations of cells that are consistently present in the coelomic cavity of the zebrafish. To accomplish this, LPS and fixed bacteria were injected into the coelomic cavity and zebrafish were exposed by immersion to *Edwardsiella ictaluri* in an attempt to recruit leukocytes to the coelomic cavity. Functional assays of these unfractionated exudate cells are also performed to establish the purpose they may play in the immune response to pathogens. This is the first study conducted on the zebrafish coelomic cavity and would provide

a valuable tool for future studies in this field. The need for a tissue source for cells of the immune system has become apparent as cell numbers from traditionally used teleost tissues such as head kidney, spleen, and blood are not practical to obtain for *ex vivo* experiments in such a small species.

CHAPTER 2

LITERATURE REVIEW

ZEBRAFISH AS AN INFECTIOUS DISEASE MODEL:

The zebrafish (*Danio rerio*), a small cyprinid teleost, has recently emerged as an ideal animal model to study developmental biology and immunology. There are many reasons that support the zebrafish as a model over other organisms. Low cost, easy maintenance, easy handling, and the requirement of minimal laboratory space are some of the advantages of working with zebrafish [1]. In addition, the entire zebrafish genome has been sequenced and it has been found to share a high degree of synteny to the human genome [2, 3]. The small size of the adult zebrafish permits the mounting of the whole animal for histological analysis [1]. Female zebrafish also exhibit high fecundity with one fish being able to produce up to 100-200 eggs per week [4]. Embryonic development occurs externally to the mother, and it is independent of maternal care allowing analysis of mutations that would be fatal during fetal development and not easily observed in mammalian models like the mouse system [4]. The transparency of the chorion and developing embryo in the first 3 weeks of life offers a significant advantage in the observation of internal structures in the developing fish [4]. Most tissues also form within 24 hours post-fertilization (hpf) and adult fish reach sexual maturity within 3 months [4]. The ease in which mutations can be developed and detected through different screens is also an advantage [2].

All the characteristics mentioned above have had a positive impact in the generation of specific zebrafish models to test individual biological pathways. Over 500 mutant phenotypes have been identified, many of which are relevant to aspects of vertebrate development or human disease states [5-7]. Forward genetic screening is used to establish most mutations in zebrafish, which is a unique method amongst vertebrates [8, 9]. The advantages of forward genetics include it unbiased nature. This method is driven by phenotypes where mutagens are employed to produce random changes in DNA and then phenotypic consequences are assayed [8]. The opposite approach is reverse genetics which is making progress in zebrafish but still faces limitations. Genes drive reverse genetics. It involves taking a gene of interest and finding a way to introduce an altered version or disrupt the endogenous gene and assay the phenotypic consequences [8]. The main criticism of reverse genetics is the reliance upon available knowledge of candidate genes [8]. As more knowledge becomes available in this area on zebrafish, more techniques of reverse genetics will be developed [8].

The zebrafish has a similar immune system to humans and other vertebrates and possess many developmental systems not found in invertebrate models such as *C. elegans* and *Drosophila* [1, 4]. These invertebrate models lack the components of an adaptive immune response while zebrafish have both innate and adaptive functions [1, 2].

The presence of the components of the natural and acquired immune responses has allowed the study of diseases that involve both of these systems. In several studies, zebrafish have been shown to be better models at mimicking human disease characteristics than the traditional mouse infectious disease model [1, 5, 10]. Studies in teleosts have demonstrated the presence of all major blood lineages [2]. Although these studies appear to emphasize the benefits of working with zebrafish as a model, there are also a few limitations. There are

currently few traditional reagents such as specific phenotyping antibodies available [2]. The functional properties of zebrafish immune cells are in the early stages of investigation [2] and considering the small size of the fish, these studies has shown to be difficult. The entire kidney only contains about 10^6 cells with 40% being mature red cells [2]. This restricts the number of cells obtained per fish of a particular hematopoietic lineage. This problem could be overcome by the ability to use large numbers of fish [2].

ZEBRAFISH IMMUNE SYSTEM:

The site of hematopoiesis in teleosts changes through the developmental process. In zebrafish this occurs in two waves termed primitive and definitive hematopoiesis [11]. Primitive hematopoiesis, occurs in the early embryo and it has been found to take place at two different sites: the intermediate cellular mass (ICM), which is functionally analogous to the yolk sac blood islands in mammals and birds; and in a less well-defined anterior mesodermal tissue [11-13]. Definitive hematopoiesis, beginning during embryogenesis and continuing through adulthood, takes place in the kidney which is considered to be the equivalent of the mammalian bone marrow [12]. The major lymphoid organs in teleosts are the thymus, kidney, and spleen [14].

The teleost kidney is composed of two segments, the pronephros or head kidney and the mesonephros or trunk kidney [14]. The head kidney contains predominantly hematopoietic tissue, sometimes referred to as “kidney marrow” in teleosts, and lacks renal activity [14]. The trunk kidney is important in excretion and dominated by renal tissue with some lymphohemopoietic tissue [14]. Macrophages, lymphocytes, granulocytes, and nonspecific cytotoxic cells are found in the kidney [14, 15]. In addition to the production of erythroid, myeloid, and lymphoid cells, the kidney plays an important role in the trapping of antigens and antibody production [14].

The teleost spleen, a red to black organ located ventral and caudal to the stomach, can be divided into red and white pulp. The marginal zone separating the red and white pulp is not fully developed in fish [2]. The white pulp consists of APCs, plasma cells, and lymphocytes and is intermingled with red pulp [2, 16]. The white pulp increases in size following antigen stimulation [2]. Germinal centers are not present in fish and class switching does not occur due to the absence of isotypes other than IgM and an IgD equivalent [2]. Most cells found in the spleen are erythrocytes, but small populations of lymphocytes and myeloid cells can be found [2].

The thymus is a paired organ found in the dorsolateral region of the gill chamber [14]. T cells are the major cellular component of this organ [14]. The thymus persists in older fish as thymic involution is not a general rule but has been known to occur under conditions of stress, aging, sexual maturity, season, and hormones [14].

NEUTROPHIL OR "HETEROPHIL" GRANULOCYTES:

There are two types of circulating granulocytes in an adult zebrafish, one of which is a neutrophil or "heterophil" and the other type (explained below) is an "eosinophil-like" granulocyte.

Heterophil granulocytes make up 95% of circulating granulocytes [17]. In the embryo at 18-30 hours post fertilization (hpf) these cells form in the yolk sac mesoderm and intermediate cell mass [12, 17, 18]. After 96hpf through adulthood the primary hematopoietic organ is the kidney although neutrophils can also be found in the spleen [12, 17, 18].

Neutrophils have distinct features distinguishing them from other cell types. They have a pale cytoplasm and a peripherally located multi-lobed (2-3 lobes) segmented nucleus [9, 17, 18]. In a cytoplasmic cross section, a typical cell contains roughly 110 electron-dense, elongated

granules about 0.42 ± 0.13 μm in length [17]. Upon staining there are two different types of granules. One type is comprised of small, azurophilic granules discernable by Wright-Giemsa staining and the other type consists of larger granules that fail to stain with Wright-Giemsa [18]. In electron microscopy, one granule type exhibits a round shape with a homogenous interior while the other has needlelike paracrystalline inclusions [18]. The histochemical phenotype of these cells includes being strongly myeloperoxidase positive, strongly reactive with acid phosphatase, and non-reactive with periodic acid-Schiff staining [18]. They also show weak nonspecific esterase activity [4].

The function of these cells has been studied in zebrafish embryos. A zebrafish peroxidase gene called myeloid-specific peroxidase (*mpx*) was isolated as a way to identify zebrafish granulocytes due to the nonspecificity of the histochemical myeloperoxidase stain [17]. The expression of *mpx* was examined through whole-mount in situ hybridization and the results indicated a greater specificity in this method of detecting leukocyte peroxidase gene expression [17]. An acute inflammation assay was designed by sectioning the tip of the tail from zebrafish embryos after which the aggregation of peroxidase activity to the trauma site was monitored [17]. By 8 hours post trauma, peroxidase activity had accumulated at the site suggesting these neutrophils migrated through tissues and played a role in inflammation [17].

Two different transgenic zebrafish models to study neutrophils have been developed. One type has neutrophils that express GFP under control of the myeloperoxidase promoter (zMPO:GFP) [19, 20]. The other transgenic zebrafish line (CLGY463) exhibits leukocyte-specific YFP expression in a subpopulation of embryonic neutrophils during late embryo and early larval development. These cells can be tracked in response to inflammation and infections

[21]. Colocalization between YFP and *mpx* showed these cells to be a subset of the neutrophil population.

Using the transgenic line expressing GFP, a similar experiment was conducted by clipping the fin of embryos 3dpf and monitoring the recruitment of neutrophils [19, 20]. Neutrophils migrating to the trauma site exhibited a highly polarized morphology consisting of a broad leading edge or pseudopod and a narrow trailing edge [19]. Also while migrating, pseudopod extensions and abrupt changes in direction were also observed [19]. The average speed of these migrating cells was calculated to be 9.94 ± 0.36 μm/min [19]. Once at the wound, some cells would continue moving in or around the area while others would stop and develop a rounded morphology with reduced pseudopod extensions [19]. To resolve the inflammation, it was found that the cells display reverse chemotaxis from the wound site back to the vasculature [19]. Apoptosis was not observed at the site even with a significant or prolonged response, but instead up to 80% of cells entering the wound were found to migrate back towards the vasculature [19]. A similar migration was found in a transgenic line expressing YFP. These cells also moved towards the trauma site. In addition to eliciting inflammation by tail clipping, bacteria were injected at 2-3dpf into the muscle tissue of embryos above the caudal vein plexus where many of the YFP positive cells accumulate. YFP positive cells were found to accumulate at the site of injury in response to injection of *E. coli*, *S. typhimurium*, or *M. marinum* [21]. Bacteria were ingested predominately by YFP-negative cells and only partly by YFP-positive cells. This negative population is believed to include macrophages or other neutrophils due to the colocalization of *Salmonella* with cells positive for macrophages and neutrophils [21]. These studies have shown that more than one leukocyte population, in addition to neutrophils, is responsible for phagocytosis.

EOSINOPHIL GRANULOCYTES:

The second type of circulating granulocyte in the zebrafish are referred to as "eosinophil/basophils" or eosinophils in previous literature based on their staining characteristics. They are less abundant than neutrophils with roughly a 5:1 neutrophil:eosinophil ratio occurring in the kidney and blood of adult fish [17]. It is not known at what developmental state they first originate and their functional role is unclear [9, 17, 22]. In the adult fish, all developmental stages are found in the kidney indicating the possible site of production [17]. The location for development in the embryo is currently unknown.

On hematoxylin and eosin staining (H&E), these granulocytes exhibit eosinophilic cytoplasm and a peripherally located non-segmented nucleus [17, 18]. Compared to neutrophils, they have larger round or elliptical granules roughly 0.66±0.26μm in length with homogenous interiors lacking paracrystalline inclusions that do not stain with Wright-Giemsa [17, 18]. These cells are myeloperoxidase negative, acid phosphatase negative, and periodic acid-Schiff positive [18]. Other histochemical stains including Sudan Black, monocytic esterases, chloroacetate esterase, and toluidine blue have failed to reveal distinctive staining patterns to distinguish these cells from neutrophils [18].

LYMPHOCYTES:

In teleosts, the head kidney is the major source of antibody production [23] and the major source of B cell development based on functional, cellular, and molecular indices [24-26]. Rearrangement of B cell receptors has been found as early as day 4 of embryonic development and membrane Igμ expression begins at day 7, but lymphocytes are not found to be present in the head kidney until 3 weeks into development [12]. In one study, $rag1^{+}$ B cell precursor population was found in the developing zebrafish pancreas, indicating that this could be a site of

differentiation of B lymphocytes [27], although this finding has not been reported elsewhere. Later this population also expresses Igμ transcripts [27]. In the adult zebrafish, the kidney marrow is the main site of B cell development [28].

The thymus is the major site for T-cell maturation in teleosts as shown by the strong expression of many developmental markers (i.e. Ikaros, Rag, and TdT) [24]. T cell expression in the thymus is first found at four days post fertilization [29]. At this time TCRα transcripts are also present, but do not reach adult levels until 3 weeks post fertilization [29]. By day 9, TCRα expression can be found outside the thymus and in peripheral organs of the zebrafish with highest expression being found in the head kidney, trunk kidney, and intestine. Expression is also found in the spleen and it increases with the age of the fish [29]. The thymus is the only site of T cell development in the zebrafish [28].

It has been found that hematopoietic cells in the kidney marrow give rise to the T cell progenitors that migrate to the thymus throughout life and it is these progenitors that give rise to the mature T cells in the thymus [28]. Like in humans, mature T cells in zebrafish migrate to the medulla while immature cells migrate to the cortex of the thymus [30]. The histochemical phenotype of lymphocytes includes being acid phosphatase negative and toluidine blue positive [18, 31].

MACROPHAGES:

Macrophages are first seen in embryos 12-24hpf in the rostral lateral plate mesoderm, anterior to the heart [32]. From this location, they migrate over the yolk sac and differentiate. After the yolk sac, many invade the mesenchyme of the head or enter into blood circulation [32]. Some later macrophage production after 20hpf has been documented in the ventral venous plexus [12]. These early macrophages are actively phagocytic and highly mobile [4]. In

experiments where 30hpf embryos were challenged with low doses *E. coli* and *B. subtilis*, it only took a few hours for the circulation to be completely cleared of bacteria [32]. Embryos 2dpf have also been micro-injected with carbon particles which were cleared within one hour by macrophages [17]. After 2 weeks post-fertilization macrophages are found in the kidney [12].

In the adult zebrafish, macrophages are present in the kidney and spleen [17]. They are large cells containing numerous cytoplasmic phagosomes, a high cytoplasm to nuclear ratio, diffuse nuclear chromatin, and agranular cytoplasm [17, 18]. Many of these phagosomes have been seen to contain material similar to that of erythrocyte cytoplasm suggesting that hemophagocytosis is a normal occurrence in zebrafish [17]. Macrophages are myeloperoxidase negative and show weak nonspecific esterase activity [4, 18]. Two marker genes used for macrophages are *L-plastin* and *fms* [32, 33].

NONSPECIFIC CYTOTOXIC CELLS:

Nonspecific cytotoxic cells are the teleost equivalent to the mammalian natural killer (NK) cell [34-37]. These cells are found in the anterior kidney, liver, spleen, and peripheral blood of teleosts [34]. They play a number of important roles in immune responses by lysing tumor target cells, protozoan parasites, and virus infected cells [35-37]. They may also participate in anti-bacterial immunity through the elicitation and secretion of cytokines [36, 37].

In response to the protozoan parasite *Ichthyophthirius multifiliis*, catfish NCC redistribution occurs from the anterior kidney to the peripheral blood [38]. NCC have been characterized as being monocyte-like with a reniform nuclei, low nucleus/cytoplasm ratio, and moderate surface villi [39]. Treatment of NCC with retinolacetate and calcium ionophore A23187 increases cytotoxic activity towards target cells [40, 41]. NCC recognize protozoans and target cells through the NK target antigen (NKTag) which has been found to be a 48.17kDa

protein containing 422 amino acids with high percentages of tyrosine and serine residues [42]. Binding of multiple antigenic peptides (MAP) made from the cognate NKTag determinant increases NCC lysis of target cells [43, 44]. In another study, NCC were found to recognize bacterial nonmethylated DNA indicating a role in anti-bacterial immunity [45].

The recognition of antigens on target cells and protozoa is facilitated by a membrane protein called NCC receptor protein (NCCRP-1) [46]. NCCRP-1 has been sequenced and shares a high degree of identity in catfish, tilapia, and zebrafish and may serve a similar function in all 3 species [47]. The zebrafish NCCRP-1 protein is 66.2% identical to the homologues from catfish and tilapia [47]. This protein may be a type III membrane protein and consists of 235 amino acids with a derived molecular weight of 30,628 Da [46]. It is proline rich (9%), has two glycosylation sites, and 18% of all amino acids are potential phosphorylation sites (serine, threonine, tyrosine) [46].

The predicted structure and function of NCCRP-1 consists of an extracellular antigen binding domain, an N-terminus with BOX-1 motifs for cytokine activation, and a C terminal domain containing abundant phosphorylation sites (i.e., Y, S and T residues) [48, 49]. There is evidence that the N-terminus of NCCRP-1 initiates cytokine gene transcription through the JAK-STAT activation pathway [49, 50]. Therefore NCCRP-1 plays a dual role in the activation of NCC by first recognizing the target antigen and then initiating cytokine release [49]. A monoclonal antibody (5C6) has been developed against NCCRP-1 and effectively inhibits NCC cytotoxicity [51]. Activity of NCC appears to be regulated by a balance between kinases and protein phosphatases [52]. Sodium orthovanadate and sodium fluoride both enhance killing activity in NCC by acting as phosphate inhibitors [52].

There is evidence that granzymes and TNFα are present and play a role in NCC cytotoxicity [53-56]. While killer cells are able to recognize targets by a number of receptor mechanisms, the effector molecules used to facilitate target cell death are highly conserved [54]. Granule exocytosis and those mediated by members of the TNF superfamily are the most common pathways in which killer cells use to kill foreign targets [54]. Molecular evidence suggests the presence of granzyme-like proteases in NCC which indicates they could contain very small granules that are difficult to visualize using normal microscopic techniques [53].

The first non-mammalian granzyme cDNA sequence called channel catfish granzyme-1 (CFGR-1) was isolated from NCC and found to have a 50% similarity to granzymes A and K [53]. Fully active, soluble recombinant CFGR-1 was generated using *Pichia pastoris* as an expression system [56]. CFGR-1 was found to hydrolyze thiobenzyl ester therefore verifying its tryptase activity which had been predicted in prior observations by sequence comparison and molecular modeling [56]. Strong correlations have been found between cytotoxicity and residual tryptase activity in supernatants taken from NCC and target cells [56]. Other serine proteases have also been found in supernatants after NCC were incubated with target cells including Asp-ase, Met-ase, tryptase, and chymase [56]. Chymase was found to be highest followed by tryptase with little activities detected from the other two [56]. This suggested the presence of other granzymes with different substrate specificities which was further confirmed by searching the catfish EST database [56]. The presence of granule components such as perforin, granulysin, and serglycin were confirmed using RT-PCR providing further evidence of the granule exocytosis pathway in catfish NCC [56].

An additional serine protease with similarities to mammalian granzymes was found in NCC from tilapia (*Oreochromis niloticus*) and was named tilapia granzyme-1 (TLGR-1) [55]. In

recombinant form this protease demonstrated chymase-like activity [55]. All of these findings highly suggest the presence of granzymes in NCC which would play a major role in cell mediated cytotoxicity [55, 56].

In addition to the presence of granzymes, tumor necrosis factor-alpha has also been found on NCC of tilapia in membrane and secreted form [54]. In recombinant form, TNF was able to induce NCC cytotoxicity, protect NCC from activation-induced cell death, and up-regulate granule transcription ultimately suggesting the role it must play in their effector functions [54]. The functions of TNF in teleost NCC are also biologically similar to those of mammalian TNF [54].

In addition to the previously described effector molecules, NCC have also been found to constitutively express soluble FasL (sFasL) [57]. It has been shown that NCC release sFasL when NCCRP-1 binds to target cells and in response to single base oligodeoxyguanosine [57, 58]. The binding of guanosine 20-mers (dG20) up-regulates expression of sFasL in NCC [58]. Supernatants from these activated cells produces hypoploidy and annexin-V binding by FasR bearing target cells [58]. The release of sFasL by tumor cell activated NCC also produces DNA hypoploidy in *Tetrahymena* and the induction of hypoploidy can be neutralized by the addition of anti-FasL antibody [57]. Crosslinkage of the FasR on *Tetrahymena* also leads to DNA hypoploidy [57]. The presence of FasR on *Tetrahymena*, as confirmed by fluorescence microscopy and flow cytometry, further demonstrates the potential that the Fas ligand- Fas receptor system may be used in initiating anti-protozoa innate immunity as the induction of apoptosis may play a major mechanism of homeostatic control in protozoal infections [57].

Soluble cytokine like substances have been reported to be released in the peripheral blood of stressed tilapia in response to bacteria and environmental stressors [59, 60]. Serum from these

fish contains a “stress activated serum factor” (SASF) that increases NCC cytotoxicity possibly by stimulating the expression of anti-apoptotic proteins involved in the inhibition of programmed cell death [59, 60]. Following temperature stress, the serum from stressed fish was capable of amplifying cytotoxicity 3-4 fold in naïve NCC [59]. Neutralization of activating serum factors was confirmed by heat inactivation and freeze-thaw treatments that could partially remove amplification activity [59].

A similar amplification in NCC cytotoxicity was seen in tilapia injected with *Streptococcus iniae* [60]. Serum from these injected fish was also capable of amplifying cytotoxicity in naïve cells [60]. It was found that the stress serum increases expression of proteins involved in cell death including FasL, CAS, and FADD [60].

In addition to their role in innate immunity, NCC also may participate in the physiologic regulation of lipid metabolism due to their expression of the teleost orthologue of mammalian scavenger receptor [61].

NCC CATIONIC ANTIMICROBIAL PROTEIN (NCAMP-1):

A novel pattern recognition protein was isolated from nonspecific cytotoxic cells (NCC) in catfish. This 203 amino acid (22,064.63 Da molecular mass) highly conserved protein is referred to as NCC cationic anti-microbial protein-1/ncamp-1) [62]. Ncamp-1 characteristically has lysine rich regions and a sequence 51.2% similar to that of the zebrafish histone family 1-X [62]. Recombinant ncamp-1 prepared from *E. coli* lysates was found to bind GpC and CpG ODNs, and full length recombinants exhibited bactericidal activity against *E. coli* and *S. iniae* [62]. Although the sequence shows this is not a member of the TLR family, this protein may serve a novel ODN binding molecule and play an important effector function in immune responses against bacterial infections and play a necessary role in survival [62].

COELOMIC EXUDATE CELLS:

Coelomic exudate cells (CEC) have been studied in a number of fish species including tilapia (*Oreochromis niloticus*), carp (*Cyprinus carpio*), striped bass (*Morone saxatilis*), sea bream (*Sparus aurata*), and sea bass (*Dicentrarchus labrax*) [63-65]. In tilapia and carp it was found that CEC consist mostly of monocyte-macrophage cells, neutrophils, and eosinophils [63]. All three of these cell types were also found to have the ability to phagocytose foreign materials both *in vivo* and *in vitro*, however eosinophils were less actively phagocytic than neutrophils or macrophages [63]. Phagocytic rates of macrophages and neutrophils were also positively correlated with the doses of foreign materials [63]. Similar results were found in a study in CEC from striped bass. After injection with *Bacillus cereus*, the most common cells in the cavity were found to be macrophages, eosinophils, and neutrophils, all three of which were phagocytic [64].

In sea bream and sea bass, CEC consist primarily of granulocytes, lymphocytes, and macrophages [65]. Reported for the first time in these fish is the observation that these cells show conspicuous ultrastructural differences from the same cell types of blood and head kidney [65]. Cell type percentages were also found to differ between the fish in that macrophages were most abundant in sea bass while sea bream had more granulocytes [65]. Macrophages are the most abundant in sea bass, but after the intracoelomic injection of bacteria, the number of neutrophils increases quickly and are equally important in the phogocytosis of invading organisms [66].

In another study, coelomic exudate leukocytes from gilthead sea bream and sea bass were found to be cytotoxic towards HeLa and B16 melanoma tumor target cells by mechanisms of necrosis and apoptosis suggesting the recruitment of NCC-like cells [67]. Following

intracoelomic injection of gilthead sea bream with *Saccharomyces cerevisiae*, PEC were found to significantly increase with variations in proportions of cell types, in particularly acidophilic granulocytes which increase to a significant extent [68]. Respiratory burst activity, degranulation, and phagocytosis increased in PEC, but cytotoxic activity was slightly decreased [68]. Overall, leukocyte mobilization and innate immune responses were documented at the site of invasion [68].

While the previous studies have emphasized the importance of the coelomic exudate cell components in different teleost species, no reports have described similar studies in the zebrafish model.

TOLL LIKE RECEPTORS AND THE TELEOST RESPONSE TO LPS:

An important property of the immune system is the ability to recognize invading pathogens. This ability relies on the existence of small molecular motifs consistently found on these potentially pathogenic microorganisms known as pathogen-associated molecular patterns (PAMPs). PAMPs include bacterial lipopolysaccharide (LPS), bacterial flagellin, fungal beta-glucans, lipoteichoic acid from Gram positive bacteria, peptidoglycan, and double-stranded RNA (dsRNA) [69]. The initial recognition and response to PAMPs is mediated by genotypically encoded proteins expressed on cells of the immune system known as pathogen recognition receptors (PRRs) [69]. Vertebrates, invertebrates, and even plants share structurally and functionally similar PRRs [69]. Toll-like receptors (TLRs) are one type of PRR. Currently, 11 TLRs have been found in mammals, and to date many fish orthologs of mammalian TLRs have been described [70-73]. TLRs react with their specific PAMP to initiate a signaling cascade through five adaptor proteins which share a common Toll/interleukin-1 receptor (TIR) domain

with the TLRs [69]. This leads to a pathogen-specific cellular response through the release of pro-inflammatory cytokines and co-stimulatory molecules [69].

TLR4 is responsible for the recognition and response to LPS. LPS has a toxic component known as Lipid A which binds to LPS binding protein (LBP) [69]. LBP assists with binding LPS to CD14, a co-receptor with TLR4 and MD-2, for the detection of LPS [69]. The LPS-CD14 complex then interacts with TLR4 to initiate the signaling cascade and release of pro-inflammatory cytokines, chemokines, and antimicrobial proteins [69].

Currently a total of 24 putative variants of TLRs have been identified in zebrafish. At least one copy of orthologues of at least ten of the human TLR genes has also been found [74, 75]. In addition two fish specific TLRs (TLR21 and 22) have been described in zebrafish. One copy of each of the TIR-containing adapter proteins (MyD88, MAL (TIRAP), TRIF (TICAM), and SARM have been identified [74, 75]. The human adapter protein gene known as TRAM (TICAM-2) has not yet been identified in zebrafish.

One of the major differences in immune response between mammals and fish is the response to LPS. Fish are more resistant to the toxic effects of LPS than mammals and as a result, high concentrations of LPS have been used in experiments to induce immune activation responses [76-78]. Mammals are highly sensitive to LPS and relatively small amounts will induce a life threatening response. In one study, trout mononuclear phagocytes (rtMOCs) responded to stimulation with zymosan, MDP, particulate β-glucan, and poly(I:C) with the same sensitivity as mammalian cells [79]. However, with these same cells, the upregulation of TNF2 required about 1000-fold higher LPS concentrations than those needed for a similar response in mammalian cells [79].

TLR4 is present in non-mammalian vertebrates, but its function appears to be different from that of mammals and other vertebrates [79]. The accessory molecules CD14 and LY96 and adapter molecule TICAM2 which have not been found in fish may have evolved in higher vertebrates to initiate the robust response of TLR4 to endotoxin [79]. It was observed in one study that alternative signaling receptors such as beta-2 integrins may play a primary role in LPS recognition in fish [79]. When dechorionated zebrafish embryos are exposed by immersion to LPS, a relatively weak response occurs compared to exposing embryos to intact bacteria [77, 80]. The response to LPS in zebrafish embryos resulted in increased expression in the message of the pro-inflammatory cytokines tumor necrosis factor α (*tnfα*) and interleukin 1β (*il1b*) by about 2.2 fold [77]. These results can be compared to zebrafish immersion in bacterial suspension. In this study, up to 8 and 32-fold induction of *tnfα* and *il1b* transcripts occurred, which may be due to intact bacteria providing additional immune-stimulating components [80].

BACTERIAL INFECTION MODELS IN ZEBRAFISH:

Many bacterial infection models have been established in zebrafish. These models include zoonotic fish pathogens and other pathogens not known to infect fish. These models are important to understand the progression of many diseases that affect fish including research leading to the development of treatments or vaccines. In addition to studying fish pathogens, many human pathogens are capable of infecting zebrafish. Although not all diseases progress in an identical manner as in humans, zebrafish provide an easy way of studying the effects of these organisms *in vivo*. This allows for an understanding of how the bacteria function in a vertebrate model as well as understanding the roles the innate and adaptive response play in infection control. One of the most common bacterial models in zebrafish is infection with *Mycobacterium* spp [81-85]. A few other examples of the many bacterial models that have been developed

include *Streptococcus* spp. [86, 87], *Vibrio anguillarum* [88], *Salmonella typhimurium* [89], and *Edwardsiella* spp [80, 90].

The most common bacterial infection model studied in zebrafish is a gram positive bacterial infection caused by *Mycobacterium* spp. This pathogen is responsible for tuberculosis in humans. These bacteria are adapted macrophage pathogens. After being phagocytosed, bacteria survive in these macrophages which migrate deeper into tissues where they recruit other cells, including differentiated macrophages and lymphocytes and form granulomas to sequester the infection [81]. This model mimics the natural progression of tuberculosis in humans with the formation of granuloma- like lesions and the ability to establish acute or chronic infections based on inoculum [84]. Researchers have shown that intracoelomic injection of five organisms into the zebrafish embryo leads to a persistent granulomatous tuberculosis while the injection of 9,000 organisms leads to an acute sudden disease [85]. The granulomas undergo caseous necrosis like in humans, but in fish there are fewer lymphocytes surrounding the granuloma [85]. In mice, *rag1* mutants are hypersensitive to infection which demonstrates that adaptive immunity does play an important role in controlling the infection [85].

The natural route of *Mycobacterium* infection has been investigated and evidence suggests the bacteria are acquired through the intestinal tract [83]. In the zebrafish embryo model, infection with *Mycobacterium marinum* leads to high bacterial burdens in the absence of macrophages showing they are not an optimal growth niche [81]. Macrophages control the early growth of the bacteria and play a critical role in the dissemination of the infection into tissues [81]. Despite the fact that zebrafish embryos lack lymphocytes, infection with *M. marinum* leads to the formation of macrophage aggregates with the characteristics of granulomas [82].

The infection also leads to the activation of many granuloma-specific *Mycobacterium* genes and redirects embryonic macrophage migration [82].

Infection with different species of *Streptococci* has been widely used as a common gram positive bacterial model in the zebrafish [86, 87]. *Streptococcus iniae* is a pathogen in both fish and humans [87]. It is characterized by a focal necrotic lesion followed by invasion of all major organ systems including the brain [87]. The infection in zebrafish closely resembles human infection with *S. agalactiae* and *S. pneumoniae* [87]. Healthy zebrafish are unable to become infected with *S. iniae*. Abrasion of the dermis is necessary before immersion to establish an infection [87]. The disease is common in aquaculture due to stress elicited by wounds [87]. The human pathogen, *Streptoccus pyogenes*, is also capable of establishing an infection in zebrafish [87]. Compared to *S. iniae* in the zebrafish, *S. pyogenes* results does not elicit inflammation, but large numbers of extracellular streptococci invade the dorsal muscle, and myonecrosis occurrs prior to systemic infection [87]. With genetic systems available for streptococci, this model also allows for the identification of mutants that are attenuated for virulence in zebrafish [87]. Different virulence factors have been identified based on the local tissue environment and stage of infection which could be used to identify similar strategies shared by gram-positive pathogens [86].

Vibrio anguillarum is a gram-negative, flagellated, invasive pathogen of fish and causes a hemorrhagic septicemia called vibriosis [88]. This pathogen has been used to infect zebrafish, and the route of infection has been tracked by labeling the bacteria with GFP [88]. Following immersion, *V. anguillarum* is always found in the gastrointestinal tract [88]. At later time points it is found to be associated with the fish surface, indicating that the intestine and skin are the

main routes of infection [88]. These studies are important for probiotic research and vaccine development [88].

Salmonella typhimurium is a rod shaped, flagellated, gram-negative bacterium responsible for human gastroenteritis [89]. DS-Red labeled *S. typhimurium* has been used to visualize the course of infection in zebrafish embryos [89]. Low doses of a wild type strain causes a lethal infection in zebrafish with bacteria multiplying within macrophage-like cells and in the epithelium of blood vessels [89]. Mutations in lipopolysaccharide (LPS) synthesis were found to attenuate the bacteria for virulence [89]. An important finding using the zebrafish model was that at later time points only 20-35% of the bacteria remained in intracellular compartments while the majority of bacteria were extracellular in microcolonies [89]. These studies using zebrafish embryos allow for rapid and non-invasive real time analysis of the progression of infection with *S. typhimurium* in a vertebrate host.

Infection models have been established in zebrafish using *Edwardsiella tarda* and *Edwardsiella ictaluri* [80, 90]. Members of the Enterobacteriacae family, these gram negative rod-shaped intracellular pathogens are most commonly associated with channel catfish (*Ictalurus punctatus*) in which they cause enteric septicemia [91]. It is most commonly associated with stress and poor water quality. Bacteria typically enter susceptible fish through the gills, skin, or oral route [92]. In experiments using *E. tarda*, adult zebrafish were susceptible to infection by intracoelomic injection [80]. Adult fish were unable to be infected through static immersion unless the epithelial layer was scraped prior to immersion while embryos were susceptible to infection by immersion [80].

Most of the pathological effects of infection in injected fish were in the coelomic cavity. In contrast, naturally infected fish displayed pathology in the kidney and liver in the form of

abscesses and necrotic lesions [80]. Intracoelomic injection of bacteria led to a rapid (2hpi) induction of cytokines IL-1β and TNFα while fish that were scraped and then immersed showed a slower response to infection (12hpi) but launched a strong inflammatory response producing transcripts at much higher levels than intracoelomically injected fish [80].

In other studies, zebrafish were infected with *E. ictaluri* [90]. Adult fish were injected intramuscularly at different doses after which mortality occurred between 2 to 5 days post injection [90]. Histopathology performed on injected fish revealed increasing severity of splenic, cardiac, hepatic, and renal interstitial necrosis [90]. To analyze chronic infection, adult fish were injected with a low dosage of *E. ictaluri* and were held for one month [90]. Beginning at 12dpi and for another 2 weeks fish displayed abnormal spiraling and circling swimming behaviors [90]. Histopathology conducted on these fish revealed necrotizing encephalitis [90]. Non-scraped fish were infected by immersion at low (1.16×10^5), medium (1.16×10^6), and high doses (1.16×10^7) for 2 hours and mortality occurred between 5 and 9 days post exposure [90]. Mortality was low at 0% (low dose), 3.3% (medium dose), and 13.3% (high dose) and bacteria were isolated from dead fish [90]. Catfish exposed to the medium dose exhibited 100% mortality [90]. These studies have shown the use of zebrafish as a model of enteric septicemia of catfish.

The zebrafish models of infection play an important role in understanding the way pathogens cause disease. Mice have been the host model of choice for many years, but in some bacterial models zebrafish are better at mimicking human disease characteristics [1]. The zebrafish is an emerging model with relatively little information available regarding their immune system compared to other animal models. However, their small size, high fecundity, the presence of innate and adaptive immune systems, embryo transparency, low cost, and easy maintenance are some of the few advantages this organism has over other models [2]. This

research has shown that zebrafish is a successful model for studies of infectious diseases and will continue to grow in importance as more research is completed. The need for a tissue source for cells of the immune system has become apparent as cell numbers from traditionally used teleost tissues such as head kidney, spleen and blood are not practical in such a small species.

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CHAPTER 3

IDENTIFICATION, RECRUITMENT, AND FUNCTION OF ZEBRAFISH COELOMIC EXUDATE CELLS[1]

[1] Moss, L.D., L. Jaso-Friedmann, J.H. Leary III, S.T. Dougan and D.L. Evans. Submitted to the Journal of Immunology.

ABSTRACT:

The zebrafish (*Danio rerio*) (zf) is an emerging model in immunological and developmental studies. One of the practical limitations in this model is the ability to obtain immune cells in sufficient quantity to carry out functional studies. The zebrafish spleen has about 100,000 leukocytes and the entire anterior and trunk kidney contains about 10^6 cells, 40% of which are red blood cells. The coelomic cavity of both males and females 3-9 months of age constitutively contain 1.07×10^5 cells. In the present study, the cells in the coelomic cavity were identified as a potential read-out tissue for studies of innate and adaptive immunity. Coelomic cavity cells were characterized and their approximate composition consisted of granulocytes (60-80%), lymphocytes (10-20%), and nonspecific cytotoxic cells (NCC) (4-10%). Granulocytes were identified by their ability to phagocytose *E. coli* and *S. aureus*; the presence of large cytoplasmic granules in TEM; and presence of granulocyte specific transcripts. Lymphocytes expressed rearranged B- and T-cell transcripts. NCC were identified based on surface membrane NCCRP-1. Cell recruitment and exudate formation were observed in the coelomic cavity of adult zebrafish following injections with LPS and fixed bacteria with peak recruitment at 2×10^5cells/zf (2 hr post-injection/ 80% increase in cell numbers) and 2.5×10^5cells/zf (6hr post-injection/ 126% increase) respectively. Recruited cells were composed of increased numbers of granulocytes, lymphocytes, and NCC. In vivo responses to *Edwardsiella ictaluri* infection produced a peak cellular recruitment after 24hrs post-infection with 2×10^5cells/zf /54% increase). Functional assays found these cells to be cytotoxic towards conventional mouse (YAC-1) and human NK cell targets (HL-60 and K562) at 1:1, 2:1 and 4:1 effector:target cell ratios. Redirected lysis of zebrafish effector cells towards hybridoma cells expressing mab 9C9 demonstrated the protein identified by this antibody (i.e. Ncamp-1) is associated with activation

of the lytic cycle. These data demonstrated that the zebrafish coelomic cavity is an immune-responsive tissue that can be upregulated/activated and is appropriate for determination of host innate and adaptive immunity to infectious diseases.

INTRODUCTION:

The teleost immune system differs from mammals in that fish lack lymph nodes and bone marrow. The major lymphoid organs in teleosts are the thymus, kidney, and spleen [1]. There are four main types of leukocytes found in these organs: granulocytes, lymphocytes, macrophages and nonspecific cytotoxic cells. The two types of circulating granulocytes in an adult zebrafish are neutrophils and eosinophil/basophils [2-4]. Neutrophils are important in inflammatory responses and phagocytosis [2, 4-7]. The functions of eosinophil/basophil granulocytes in teleosts are currently unknown [4]. Lymphocytes are present in the zebrafish with B-cells predominately found in the bone marrow equivalent tissue (head kidney) of the adult fish while T cells are in the thymus [8-10]. Macrophages are present in the kidney and spleen and function in the phagocytosis of foreign materials [2, 4, 11]. Nonspecific cytotoxic cells (NCC) are the human equivalent of NK cells and are found in the head kidney, liver, spleen, and peripheral blood of teleosts [12]. NCC play a number of important roles in immune responses by lysing tumor target cells, protozoan parasites, and virus infected cells [13-15]. NCC also participate in anti-bacterial immunity by the elicitation and secretion of cytokines [16, 17].

The zebrafish (*Danio rerio*) has recently emerged as an ideal model for the study of developmental biology and immunology. Some of the many advantages of using zf as a model include its small size, low cost, easy maintenance and handling, high fecundity, and transparent developing embryos [18]. Currently critical limitations occur for use of zebrafish as a model of

immune function. These limitations include the lack of available reagents (i.e. antibodies for specific phenotyping) and the absence of research on functional properties of zebrafish immune cells [19]. Perhaps the most important limitation of this model involves the difficulty of obtaining sufficient numbers of cells for *ex vivo* study. The entire kidney contains only approximately 10^6 cells with 40% being mature red cells and the spleen contains fewer than 100,000 total leukocytes [19]. The difficulty of harvesting cells from the spleen and kidney in zebrafish provided the stimulus to determine whether the coelomic cavity could be used to obtain leukocytes for studies of innate and adaptive immune responses to infectious disease.

The coelomic cavity has been studied in a number of fish species including tilapia (*Oreochromis niloticus*), carp (*Cyprinus carpio*), striped bass (*Morone saxatilis*), sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) [20-22]. These studies have found the coelomic cavity to consist primarily of granulocytes, lymphocytes and macrophages with approximate distributions dependent on the species [20, 22]. Macrophages, neutrophils and eosinophils harvested from the coelomic cavity phagocytosed foreign materials [20, 21]; and coelomic exudates from sea bream and sea bass were cytotoxic towards HeLa and B16 melanoma tumor target cells by mechanisms of necrosis and apoptosis suggesting the recruitment of NCC-like cells [23]. Following intracoelomic injections in sea bream, leukocyte mobilization and innate immune responses have been documented in the coelomic cavity with granulocytes recruited in the highest number [24]. While the previous studies have emphasized the importance of the coelomic exudate cell components in different teleost species, reports have not described similar studies in the zebrafish model.

In this study, we report for the first time the use of the zebrafish coelomic cavity as a source for obtaining leukocytes and as a model tissue for the study of innate and adaptive

immunity. Leukocytes are actively recruited to the cavity in response to intraperitoneal injections with LPS and fixed *Edwardsiella ictaluri* as well as in response to live infection. Granulocytes from the zf coelomic cavity are phagocytic towards injected bacteria. Coelomic exudate cells were also cytotoxic towards conventional mouse and human NK cell targets. The recruitment of coelomic exudate cells in zebrafish provides a practical method of obtaining adequate cell numbers for future immunological studies using these fish.

MATERIALS AND METHODS:

Zebrafish. Adult WIK zebrafish [25] were maintained at 28°C in a UV filtered flow through system monitored for temperature, pH, and conductivity at the University of Georgia Zebrafish facility (UGA Animal Welfare Assurance #A3437-01). Fish were fed brine shrimp twice daily. Fish (3-8 months of age) were inbred by single pair brother-sister matings through the F2 generation. Only female fish were used in all experiments. All adult fish for infection studies were transferred into an isolated flow-through holding system and acclimated for at least 5 days prior to use.

Media. All cell cultures and coelomic cavity cells were maintained in RPMI 1640 (Media Tech, 50-020-PB) media supplemented with L-glutamine (200 mM), sodium pyruvate (100mM), MEM vitamin solution, MEM amino acid solution, MEM nonessential amino acid solution (Cellgro, Media Tech), 50mg/ml gentamicin (Schering-Plough Animal Helath Corp, Kenilwork, NJ), and 10% heat inactivated fetal bovine serum (FBS) (Atlanta Biologicals, 511150).

In vivo coelomic exudates cellular (CEC) responses to LPS and bacteria. Zebrafish were anesthetized using tricaine methanesulfonate (MS-222) (Argent Chem Lab). Fish were injected with 10ul of 0, 2.5, 5, 10, or 20ug/ml concentrations of LPS from *Escherichia coli*

055:B5 (Sigma, L6529) suspended in LPS-free sterile PBS (Sigma, 5493) into the coelomic cavity using a 0.3cc insulin syringe (Becton Dickinson, 309301) to find the best dosage for cellular recruitment. The optimal dosage was found to be 10ug/ml LPS and was used to inject the coelomic cavity over a longer time course. Control fish were injected with 10µl sterile LPS-free PBS. Cells were harvested at 0, .5, 1, 1.5, 2, 3 and 6 hours post injection and counted.

To determine the *in vivo* responses of zf to killed *Edwardsiella ictaluri* isolates (provided by A. Goodwin, UAPB, Aquaculture/Fisheries) from diseased catfish were grown 24 hours in 40 ml brain heart infusion broth (BHI, 27°C). Bacteria were washed (PBS) and fixed in 3.7% paraformaldehyde (in PBS) for 1.5-2 hours. Fixed bacteria were washed (3X in sterile PBS) and stored at 4°C. An optical density (OD) vs. CFU relationship was determined in our lab by growing bacteria and checking OD at various time points. Bacteria were plated at these times and corresponding numbers of CFU were determined. An OD of 1.0 is equal to 1.5×10^8 CFU/ml. Adult female zebrafish were each injected with 10^3 CFU (controls received 10µl sterile LPS-free PBS) suspended in 10µl sterile PBS. Coelomic exudates cells were harvested at 0, 1, 2, 3, and 6 hours post injection.

In vivo CEC responses to live bacteria were done using female zf as previously described except infection was accomplished by skin scarification. Female zebrafish were anesthetized in MS-222 and the skin lightly scarified (<5 mm) laterally behind the pectoral fin with a sterile razor. Recovery was 100%. An LD_{50} titration was done by immersing fish in 10^7, 10^6, 10^5, or 10^4 total bacteria in 250ml system water. As a control, fish were immersed in 250ml system water containing broth without bacteria. Fish were exposed for one hour after which they were housed in an isolated flow through system. The best dosage for infection was found to be 10^5 total bacteria in 250ml system water which was used for subsequent recruitment studies over a

multi-day time course. Cells were harvested every 24 hours through day 4 post-infection. Exudate cells from the coelomic cavity for all treatments were obtained by perfusion with 3ml media using a 10 ml syringe and a sterile 26 G polypropylene hub hypodermic needle (Kendall, 250321). Cells from each individual zf were enumerated by hemocytometer counting.

Flow Cytometry. Flow cytometry analysis was done using an EPICS XL-MCL four color SYSTEM II automated cell analysis system (Coulter Electronics Corp, Hileah, FL, U.S.A.) equipped with a 15mW air cooled argon-ion laser operating at 488nm wavelength. Monochromatic and polychromatic flow experiments were done using FITC, CFSE (Sigma, 21888) and PI (Sigma, 81845). Data collection analysis was performed using Coulter's System II software version 3.0.

Phenotype analysis of CEC from adult female zebrafish was carried out as previously described. Monoclonal antibody 5C6 [26] (IgM isotype) identifies NCC and mab 9C9 was prepared in house [27]. Monoclonal 9C9 is specific for the 29kDa protein referred to as nonspecific cytotoxic cell cationic antimicrobial protein (NCAMP-1). Isotype control antibodies were prepared in house and were used at equivalent concentrations. Cells were stained as previously described [28]. The conjugate was anti-Mouse IgM FITC (Sigma, F9259). Cells were washed and resuspended in PBS-azide and analyzed by flow on the same day. Cell sorting was done with Dako MoFlo (Flow Cytometry Facility, UGA). PI negative cells were identified and scatter gates were established. Cells were tentatively identified by morphology and staining with Wrights. Wrights stain photographs were taken with an Olympus Q-Color3 imaging system.

Scanning and Transmission Electron Microscopy. CEC were harvested from adult female zf, sorted (as previously described) and prepared [29] for analysis by transmission and

scanning electron microscopy (University of Georgia, College of Veterinary Medicine EM Facility).

PCR. Coelomic exudates cells were sorted as previously described. Total RNA from cells from each region was extracted using the RNeasy Mini Kit (Qiagen, 74104). RT-PCR analysis was done using total RNA from sorted CEC First strand cDNA synthesis with SuperScript III was done using the protocol by invitrogen. Thermal cycle settings were 94°C (30 seconds) for denaturation, 60°C (30 seconds) for annealing, and 72°C (1 minute) for extension at a total of 34 cycles. Table 1 shows chosen primer sequences.

Cytotoxicity. The ability of CEC to kill target cells was measured using a nonradiometric flow cytometric technique [30, 31]. Target cells were: human promyelocytic leukemia cells HL-60 (ATCC CCL 240); mouse lymphoma YAC-1 cells (ATCC TIB 160); and human myelogenous leukemia K562 cells (ATCC CCL 243). Briefly, each target cell was suspended in 1mL sterile PBS washed and stained with 5 mM CFSE (Sigma, 21888) for 10 minutes at 37°C. Cells were washed and resuspended in media at a concentration of 5×10^5 cells/ml. 5×10^4 CFSE labeled target cells were added to sterile 1.5 ml microtubes and effectors were added at Effector:Target cell ratios of 1:1, 2:1, and 4:1. Tubes were centrifuged briefly to concentrate cells at the bottom. Control tubes contained CFSE labeled target cells and media. Flow analysis was conducted after 1, 2, and 4 hours co-incubation at 37°C (5% CO_2). Target cell death was measured by counting remaining CFSE labeled cells (in the target cell gate) for 45s.

Redirected Lysis Experiments: Hybridomas expressing membrane surface IgM were produced in lab[27] and stained for surface expression of Ig protein. The hybridomas were positive for surface expression of 9C9 (anti-ncamp-1) and M2 (isotype control) at 96.3% and

Table 1: PCR primers chosen for specific zebrafish leukocytes and respective accession numbers. Primers were designed using Primer3 v.0.4.0 program.

PCR Primer	Accession #	Forward Primer	Reverse Primer
Actin	AF057040	CTCTTCCAGCCTTCC TTCCT	CTTCTGCATACGGTCA GCAA
Ncamp-1	BC066386	GGCTGCTTCTCCAGC TAAAA	TCCGTTCTTCTGGTCA AACC
Rearranged TCR alpha constant domain	AY476728	ACCAAGTGGGAAAC TCATGC	CGCTGGATGATGAAC AAATG
NCCRP-1	NM_130921	TGTTGTGATCTGCCA GCTTC	AGCACTCCAGGTCCT CTTCA
Lymphocyte cytosolic plastin-1 (*lcp1*)	BC062381	GCCAAGGTGGTTCAT GACTT	CCTTCTCCAGAGCCTT GTTG
Myloid Specific Peroxidase (*mpx*)	BC056287	GAGAGGCTGTTTGCC TTCAC	AGGCTCAGCAACACC TCCTA
Colony stimulating factor 1 receptor (*csf1r*)	NM_131672	CAGAAACGTTCTGCT CACCA	AATCTCCCACAGCAT GATCC
Rearranged Ig mu heavy chain	AY646245	TGGCACTGGCACTAT TTTTG	TGCACTGAGACAAAC CGAAG

99.8% respectively. Both 9C9 and M2 expressing hybridomas were used as target cells and labeled with CFSE. Effectors were added at Effector:Target cell ratios of 1:1, 2:1, and 4:1, briefly centrifuged, and co-incubated at 37°C (5% CO_2) for 4 hours. Target cell death was measured by counting remaining CFSE labeled cells (in the target cell gate) for 45s.

(Total Labeled Control Targets) – (Total Labeled Targets After Co-incubation with Effectors)/ (Total Labeled Control Targets) x 100 = % Target Cell Death

Phagocytosis. Alexa Fluor 488 conjugated *Escherichia coli* (Invitrogen, E13231) or *Staphylococcus aureus* (Invitrogen, S23371) were washed in sterile PBS and opsonized (30 minutes at 37°C) with 1:10 dilution of heat inactivated catfish serum. Zebrafish were anesthetized in MS-222 and injected into the coelomic cavity with 10µl sterile PBS containing 10^7, 10^6, or 10^5 bacteria. At 24, 48, and 72 hours post injection, CEC were harvested as previously described, washed with media and analyzed for fluorescence by flow cytometry. Extracellular fluorescence of unphagocytosed bacteria stuck to the membrane surface of cells was quenched with 0.2% trypan blue (Sigma T-6146).

RESULTS:

Cell Sort and Identification: Zebrafish coelomic exudate cells obtained by perfusing the coelomic cavity with 3mL of media were analyzed based on size (FSC) and granularity (SSC) by flow cytometry and sorted into individual populations. Four cell populations are discernable and classified as high, intermediate or low on FSC and SSC characteristics. To characterize each cell population, Wrights stain, transmission electron microscopy (TEM), scanning electron microscopy (SEM), PCR analysis, and phenotyping was performed on each sorted group of cells.

Sorted cells in population 1 (Figure 1A) were approximately 10μm in size and classified as a $FSC^{hi}SSC^{hi}$ fraction. With Wrights stain, cells in this population were observed to contain basophilic granules and a peripherally located non-segmented nucleus (Figure 1B). In SEM they are shown to have a microvillous surface (Figure 1C). One group of cells present in this population contain spherical granules and a peripherally located non-segmented electron dense nucleus (Figure 1D). Other cells within this population were found to have smaller more elongated granules and a multi-lobed nucleus (Figure 1E). Through PCR analysis population 1 expresses actin mRNA which was chosen as a positive control (Figure 2A). This population was confirmed to consist of leukocytes through expression of lymphocyte cytosolic plastin-1, an actin binding protein expressed only on leukocytes. Primers were designed to detect expression of Ncamp-1, a potentially novel ODN binding molecule that may play a role in anti-bacterial immune defenses. Specific cell types expressing this protein are currently unknown. Population 1 expresses Ncamp-1 mRNA. Other primers chosen include colony stimulating factor-1 receptor found only on macrophages and myeloid specific peroxidase expressed predominately by neutrophils. Population 1 expresses both of these markers but is negative for T and B cell markers. Monoclonal antibodies and primers were made to detect expression of NCCRP-1, a receptor protein located on nonspecific cytotoxic cells used in the recognition of antigens on target cells and protozoan parasites. A monoclonal antibody was also made to detect surface expression of Ncamp-1. Cells from population 1 express NCCRP-1 mRNA and are positive for surface expression of both Ncamp-1 (22.9%) and NCCRP-1 (25.2%) (Figure 2B).

Sorted cells in populations 2 were approximately 5μm in size and are classified as being $FSC^{int}SSC^{lo}$ (Figure 3A). With Wrights stain, these cells have a high nuclear:cytoplasm ratio with a dark staining nucleus and a small rim of non-staining cytoplasm (Figure 3B). Scanning

EM shows cells with a microvillous surface (Figure 3C). In transmission EM these cells were found to have a large electron dense nucleus with a small rim of cytoplasm (Figure 3D). PCR analysis shows this population to express both actin and lymphocyte cytosolic plastin-1. In addition, primers were designed for TCR alpha constant expressed only by T cells, and Ig mu heavy chain expressed only by B cells. This population was positive for both T- and B-cell markers (Figure 4). This population is negative for membrane expression of Ncamp-1 and NCCRP-1.

Sorted cells from population 3 (Figure 5A) were approximately 3-4μm in size and stain completely with Wrights stain (Figure 5B). These cells are considered $FSC^{lo}SSC^{lo}$. Scanning EM shows cells with a smooth ruffled outer membrane surface (Figure 5C). These cells contain small cytoplasmic granules as evident in TEM (Figure 5D). PCR analysis shows this population to express both actin and lymphocyte cytosolic plastin-1. Population 3 expressed both actin and lymphocyte cytosolic plastin-1. In addition to Ncamp-1, population 3 also expresses NCCRP-1 but has little or no expression of other markers (Figure 6A). This population is positive for Ncamp-1 and NCCRP-1 on its outer membrane surface at 38% and 27% respectively (Figure 6B).

Infection Model: The coelomic cavity of a zebrafish following perfusion with 3mL of media regardless of size (27-35mm), age (3-9 months), or sex contains on average 1.07×10^5 cells (range 4.2×10^4 to 1.86×10^5 n=49 fish). Harvesting cells from the coelomic cavity of male fish flushes millions of spermatids from the cavity. These spermatids are approximately 3μm in size (Figure 7A), are electron dense and can be difficult to distinguish from other smaller cells in microscopy (Figure 7B). Therefore, female fish were used in all models to avoid any complications with this issue. In order to characterize the infection model using *E. ictaluri*, three

different approaches were taken. First, ZF were injected in the coelomic cavity with different concentrations of LPS and CEC counts were done at one hour (Figure 8A). A concentration of 10μg/ml showed the best response. ZF were injected with 10μg/ml over a time course and CEC counts were done. Peak recruitment of cells into the coelomic cavity (2 x 10^5/zf) occurred two hours post injection with 10μg/ml LPS (80% increase over control) $p<0.05$ (Figure 8B). Cell numbers in the exudates returned to pre-injection levels by 6 hours post-injection. To determine the CEC responses to killed *Edwardsiella*, ZF were injected with 10^3, 10^5, and 10^7 killed bacteria/fish and CEC counts conducted (Figure 8C). Figure 8D shows the peak cellular response (2.5 x 10^5/zf) occurred at 6 hours following injection with 10^3 bacteria (126% increase over control) $p<0.0083$. Cell counts returned to pre-injection levels by 24 hours post injection. Another component to establish the CEC model consisted of the determination of the LD_{50} of *Edwardsiella* infection and associated changes in CEC numbers. Figure 8E shows an LD_{50} of approximately 10^5 bacteria in 250ml water. Bacteria isolated from dead fish were confirmed to be *E. ictaluri* and were capable of infecting ZF and produce disease to satisfy Koch's postulate. Peak recruitment of cells into the coelomic cavity (2 x 10^5cells/zf) occurred 24 hours post infection (54% increase over control) $p<0.014$ (Figure 8F).

Cytotoxicity: Target cells were labeled with CFSE and were distinguishable from zebrafish cells using flow cytometry (Figure 9A). Histograms were established to count the number of CFSE labeled target cells remaining at each time point to determine the number of targets remaining after incubation with non-labeled zebrafish effector cells. The percent target cell death was then calculated as previously described. The optimum co-incubation time was first determined by adding effector cells to HL-60 targets at a 1:1, 2:1, or 4:1 ratio for 1, 2, and 4 hours (Figure 9B). Four hours co-incubation showed the best cytotoxicity and was chosen as the

Figure 1

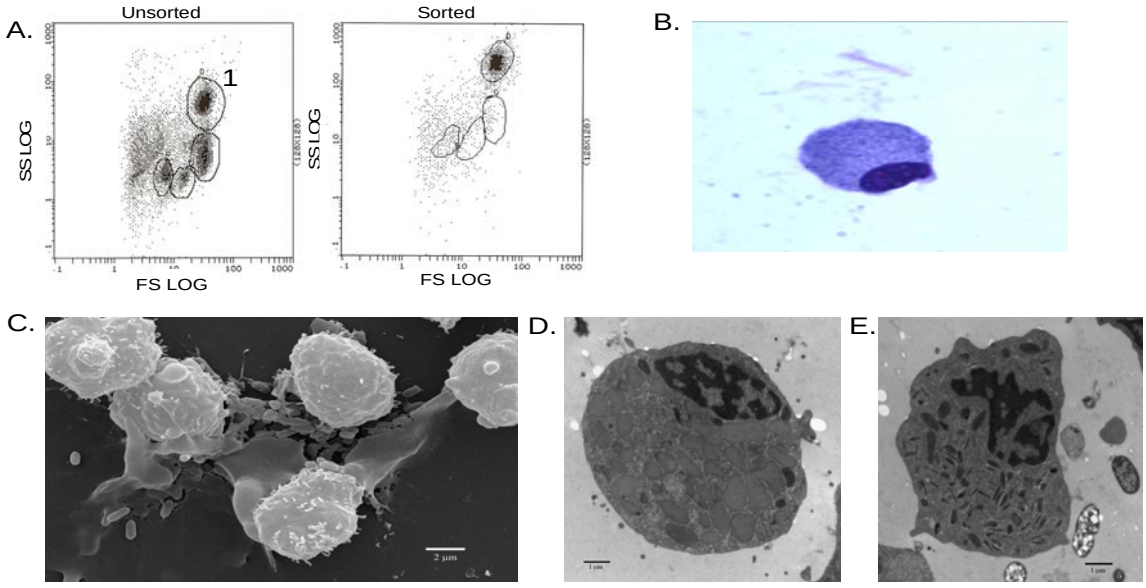


Figure 1: Zebrafish coelomic exudate cells were sorted into individual populations A) Cells were sorted into population 1 based on FSc (size) vs SSc (granularity) B) Wrights stain showing basophilic granules and a peripherally located non-segmented nucleus C) Scanning EM image shows a highly microvillous surface D)Transmission EM showing cells in population 1 containing spherical granules and a peripherally located non-segmented nucleus E) Transmission EM of a second cell type present in population 1 containing smaller elongated granules and multi-lobed nucleus

Figure 2

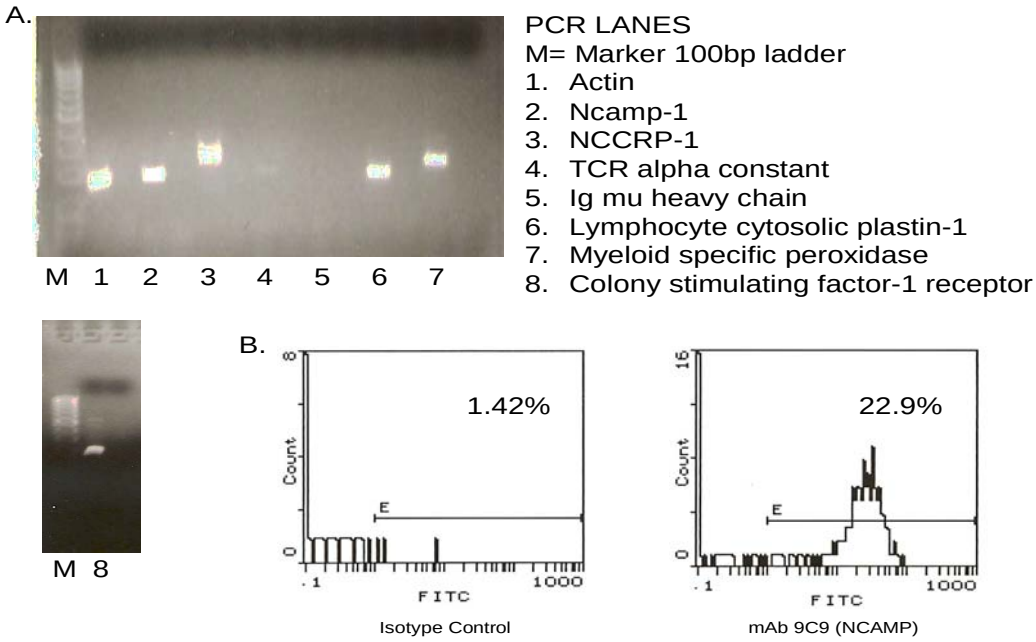


Figure 2: A) PCR analysis of population 1 and corresponding list of primers used B) Phenotype of population 1: Cells were stained with monoclonal antibodies to show surface expression of Ncamp-1 (mab 9C9) as compared to the isotype control (M2).

Figure 3

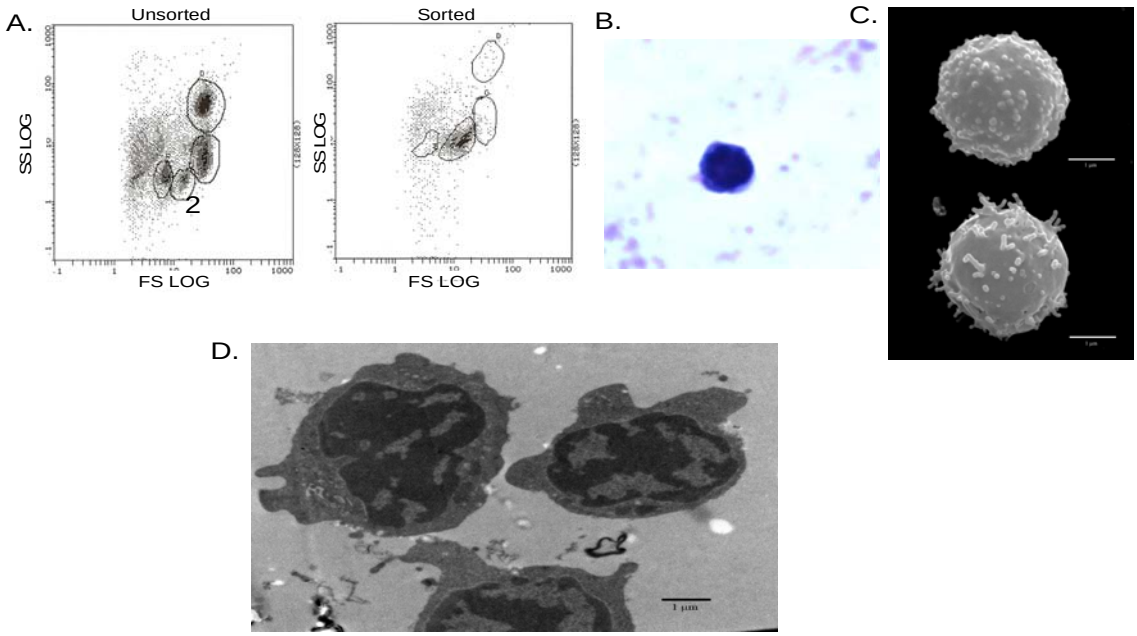
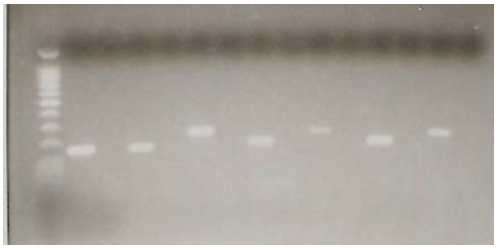


Figure 3: Zebrafish coelomic exudate cells were sorted A) Unsorted cells sorted into population 2 based on FSc (size) vs SSc (granularity) B) Wrights stain shows a dark staining cell with a small rim of non-staining cytoplasm C) Scanning EM of cells about 5µm in length with a microvillous surface D) Tranmission EM of cells with a large electron dense nucleus and a small rim of cytoplasm

Figure 4



M 1 2 3 4 5 6 7

M 8

PCR LANES
M= Marker 100bp ladder
1. Actin
2. Ncamp-1
3. NCCRP-1
4. TCR alpha constant
5. Ig mu heavy chain
6. Lymphocyte cytosolic plastin-1
7. Myeloid specific peroxidase
8. Colony stimulating factor-1 receptor

Figure 4: PCR analysis of population 2

Figure 5

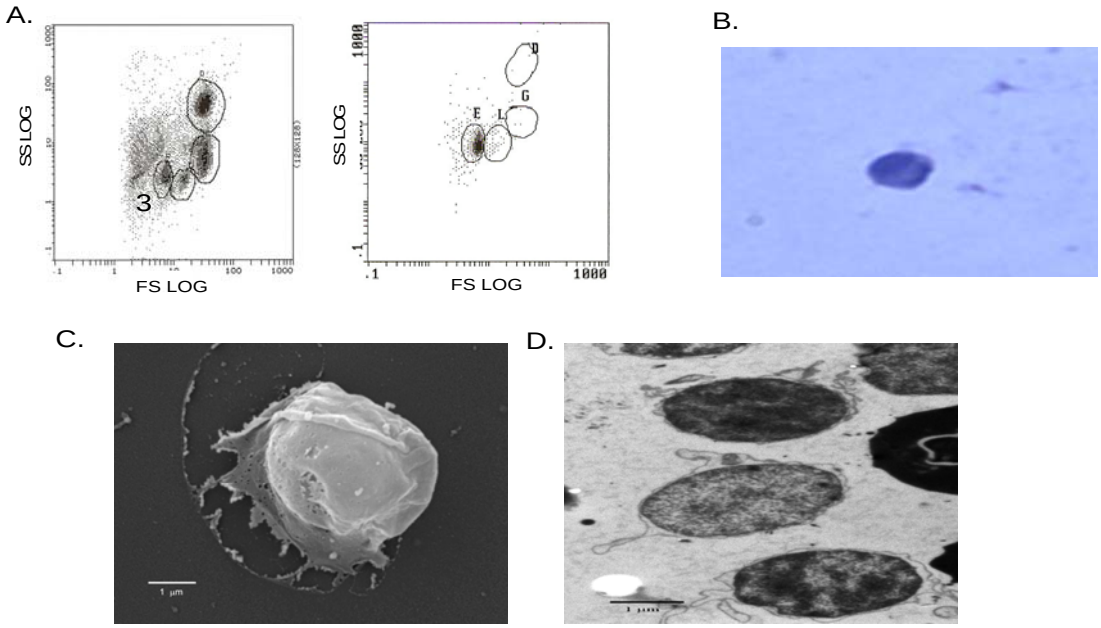


Figure 5: Zebrafish coelomic exudate cells were sorted A) Unsorted cells sorted into population 3 based on FSc (size) vs. SSc (granularity) B) Wrights stain image C) Scanning EM showing a cell approximately 3-4µm in size with a smooth ruffled membrane D) Transmission EM showing cells with small cytoplasmic granules

Figure 6

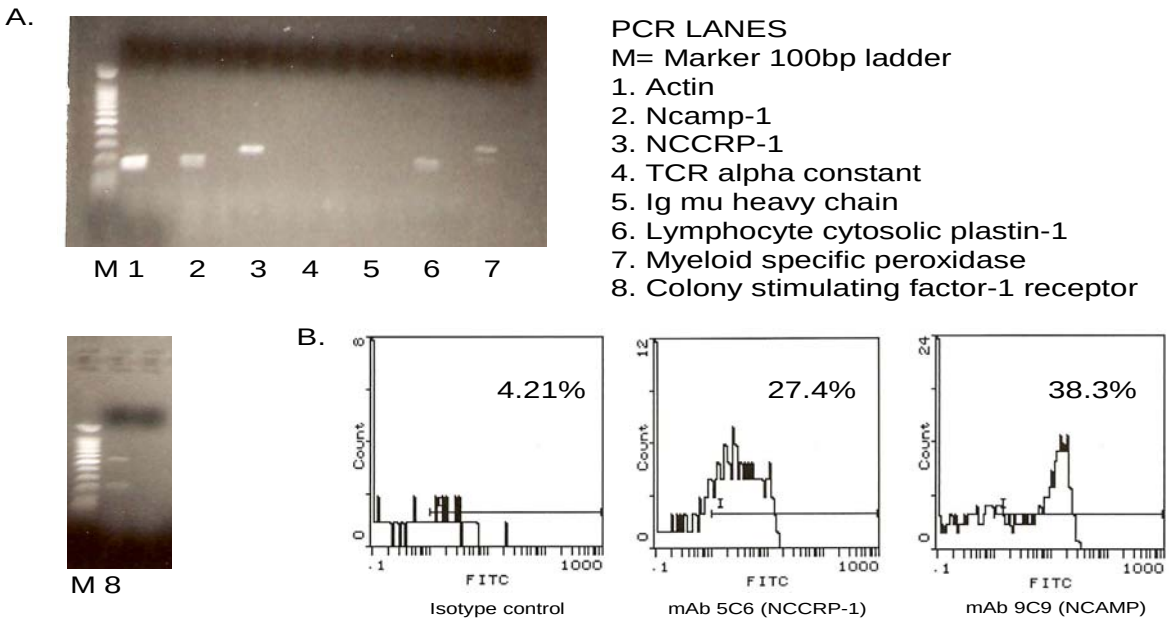


Figure 6: A) PCR analysis of population 3 and corresponding list of primers used B) Phenotype of population 3: Cells were stained with monoclonal antibodies to show surface expression of NCCRP-1 (mab 5C6), Ncamp-1 (mab 9C9), or isotype control (M2).

Figure 7

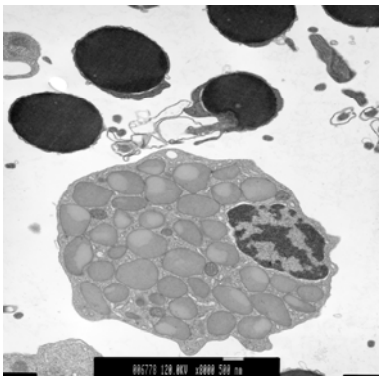
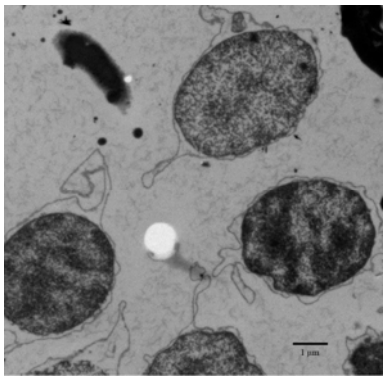
A.  B. 

Figure 7: A) Flushing male zebrafish coelomic cavities results in several million spermatids characterized as being entirely electron dense and approximately 2-3μm in size. Image is of spermatids next to a granulocyte. B) NCC are similar in size to spermatids at approximately 3-4μm in size.

time point for subsequent experiments. Zebrafish coelomic exudates cells were added to HL-60, YAC-1, and K562 targets at a 1:1, 2:1, or 4:1 ratio for four hours. Target cell death was 5-10% with effectors added at a 1:1 ratio. Target cell death was 10-15% death at a 2:1 ratio and 15-20% at a 4:1 E:T ratio (Figure 9C).

The necessity of membrane fluidity was demonstrated in ZF effector mediated target cell death by co-incubating effector cells at 4°C with K562, EL-40, and HL-60 target cells. Cytotoxicity was inhibited by 100% with K562 and YAC-1 targets and 64% in HL-60 cells at a 4:1 ratio after four hours co-incubation (Figure 9D).

The relationship between membrane expression of Ncamp-1 and activation of the lytic cycle was analyzed by performing a redirected lysis using hybridomas expressing surface M2 (isotype control) or 9C9 (anti-ncamp-1). M2 hybridomas are killed by effector cells at 1:1(8.4%), 2:1(12.4%) and 4:1(26.8%) E:T ratios after 4 hours of co-incubation. In comparison, co-incubating effectors with 9C9 hybridomas resulted in higher killing at all ratios with 25.3% (1:1), 37.3% (2:1), and 50% (4:1) (Figure 10).

Phagocytosis: Fish were injected with 10^5, 10^6, or 10^7 labeled bacteria and cells were harvested and analyzed using flow cytometry at different hours post injection. Population 1 was the only group to exhibit fluorescence after injecting fish with labeled *E. coli* or *S. aureus*. Peak fluorescence occurred 6 hours post injection with 10^7 *E. coli* and a maximum of 20% positive cells. Peak fluorescence occurred 2 hours post injection with 10^7 *S. aureus* with up to 40% positive cells (Figure 11). Trypan blue (0.2%) was used to quench any extracellular bacterial fluorescence. Fluorescence was titrated out by injecting 10^6 and 10^5 bacteria. By 48 hours post injection with either bacteria, the coelomic cavity contained fewer than 5% positive cells in all populations.

Figure 8

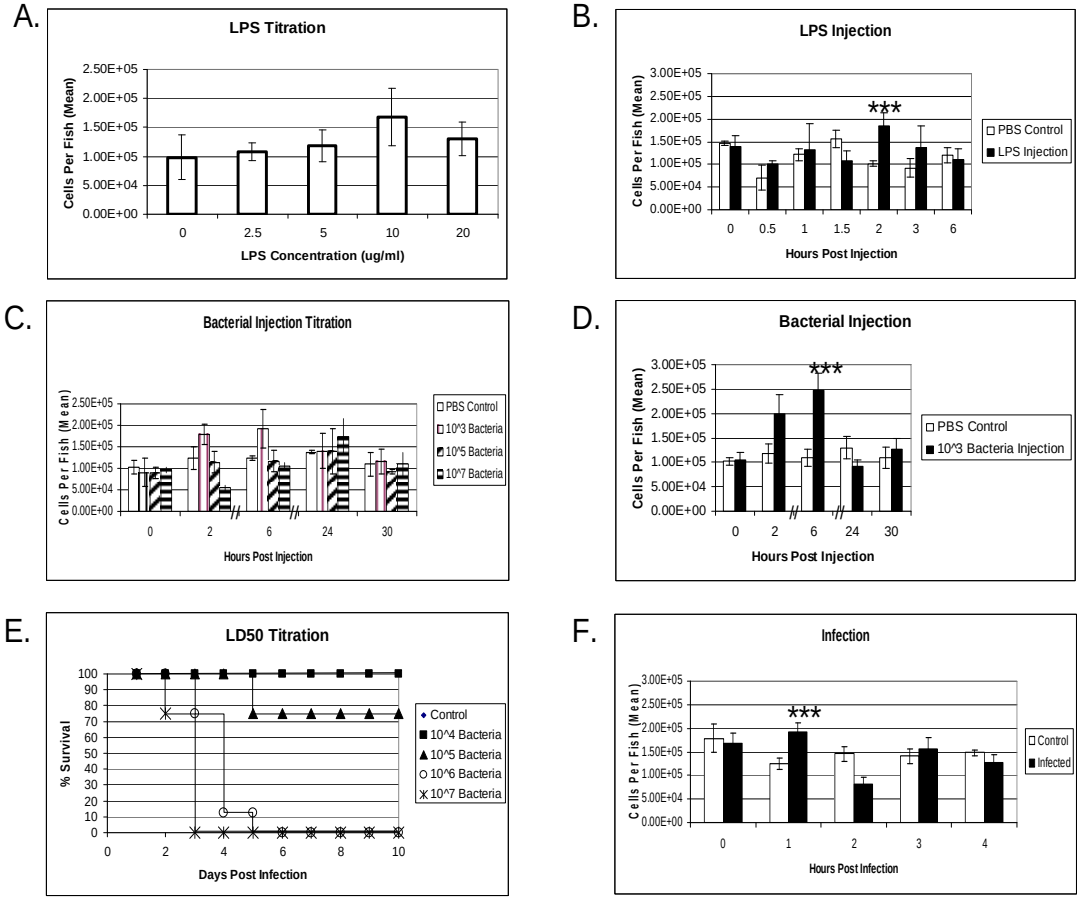


Figure 8: A) LPS injection titration. Fish were injected with 0, 2.5, 5, 10, or 20ug/ml concentrations of LPS and cells were harvested on hour post injected and counted. The best response occurred with 10µg/ml LPS. B) LPS injection time course. Fish were injected with PBS or 10µg/ml LPS and cells were harvested and counted at 0, 0.5, 1, 1.5, 2, 3 and 6 hours post injection. Peak recruitment occurred 2 hours post injection with 80% increase over control ($p<0.051$). C) Fixed bacteria injection titration. Fish were injected with PBS, 10^3, 10^5, 10^7 bacteria and cells were harvested and counted at 0, 2, 6, 24, and 30 hours post injection. Best recruitment occurred with 10^3 bacteria injected per fish. D) Fixed bacteria injection time course. Fish were injected with PBS or 10^3 bacteria and cells were harvested and counted 0, 2, 6, 24, and 30 hours post injection. Peak recruitment occurred 6 hours post inject with 126% increase over control ($p<0.0083$). E) LD50 curve after immersion of scraped fish exposed to *E. ictaluri* for one hour and monitored for 10 days F) Fish were scraped and immersed in *E. ictaluri* for one hour. CEC were harvested and counted 0, 1, 2, 3, and 4 days post infection. Peak recruitment occurred one day post infection with 54% increase over control ($p<0.014$).

Figure 9

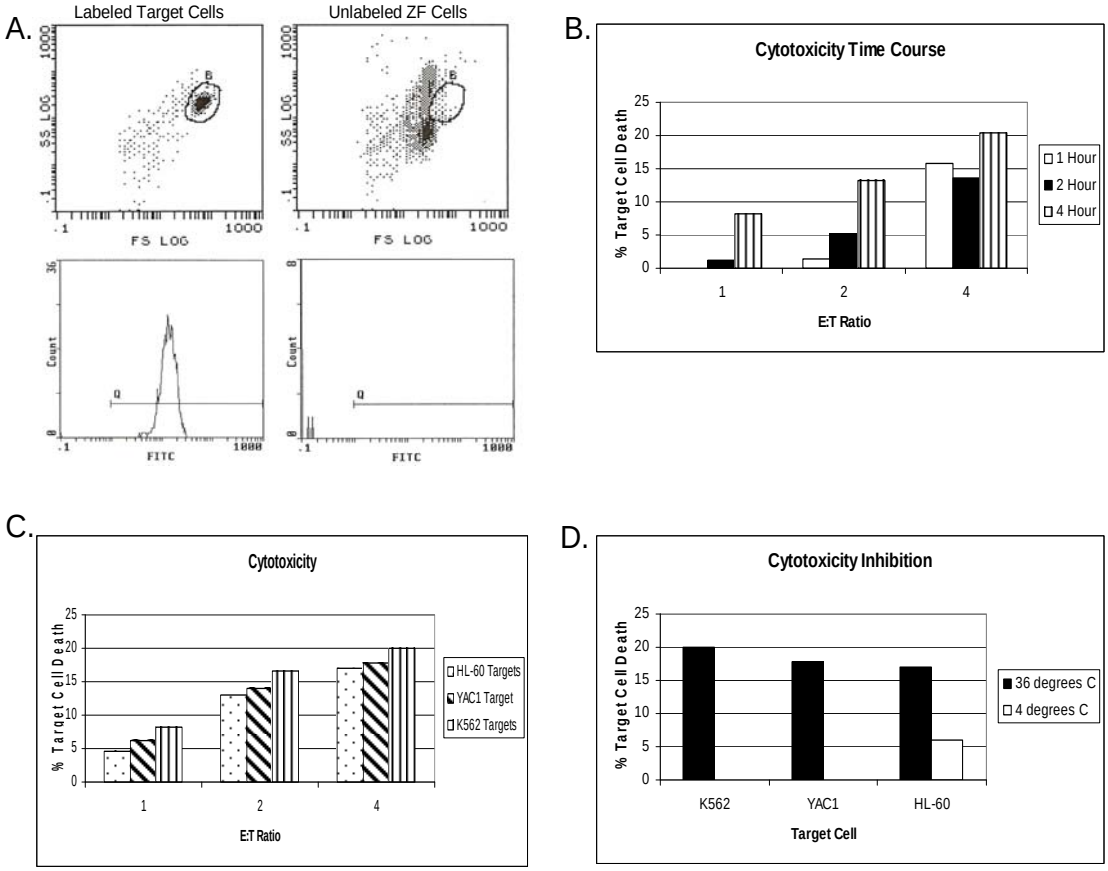


Figure 9: Cytotoxicity of zebrafish coelomic exudate cells A) CFSE labeled target cells are distinguishable from unlabeled zebrafish coelomic exudate cells using flow cytometry B) Cytotoxicity against HL-60 targets at E:T ratios of 1:1, 2:1, and 4:1 for 1, 2, and 4 hours co-incubation. Formula for cytotoxicity: (Total Labeled Control Targets) – (Total Labeled Targets After Co-incubation with Effectors)/ (Total Labeled Control Targets) x 100 = % Target Cell Death C) Cytotoxicity against HL-60, YAC-1, and K562 target cells with effectors added at a 1:1, 2:1, and 4:1 E:T ratio for 4 hours D) Cytotoxicity can be in inhibited by co-incubating effector cells at 4°C with HL-60, YAC-1, and K562 targets.

Figure 10

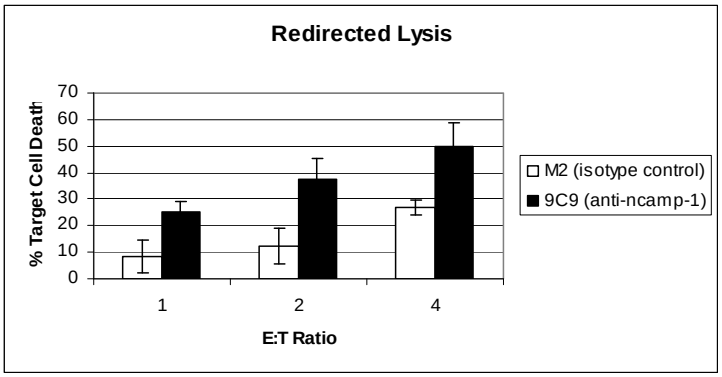


Figure 10: Redirected lysis was performed using CFSE labeled hybridomas expressing surface IgM. Hybridomas expressed either M2 (isotype control) or 9C9 (anti-ncamp-1).
(Total Labeled Control Targets) – (Total Labeled Targets After Co-incubation with Effectors)/ (Total Labeled Control Targets) x 100 = % Target Cell Death

Figure 11

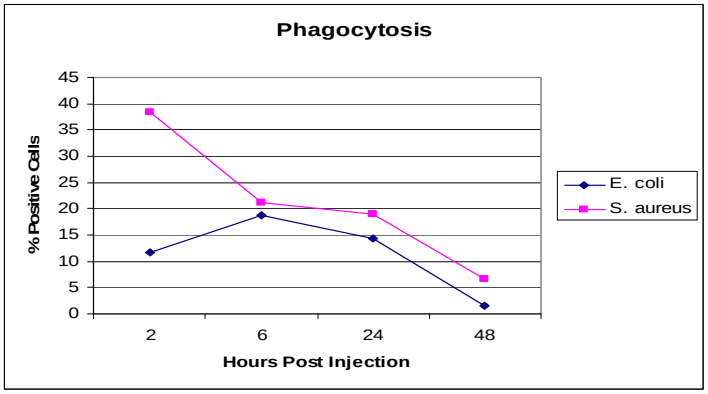


Figure 11: Phagocytosis of zebrafish coelomic exudate cells after injecting fish with 10^5, 10^6, or 10^7 labeled *E. coli* or *S. aureus* for 2, 6, 24, and 48 hours. Cells were harvested and analyzed using flow cytometry. Population 1 was the only one to exhibit fluorescence. All other populations were negative. Fluorescence titrated out with decreasing amounts of bacteria injecting into the cavity.

DISCUSSION:

The composition of teleost peripheral blood is variable dependent on species and consists of lymphocytes, monocytes and granulocytes (neutrophils and eosinophils). In most teleosts, unlike in mammals with granulocytes being most abundant, lymphocytes are found in largest numbers (60-85%) [32]. Granulocytes are the next most abundant population, making up 20-30% of leukocytes in peripheral blood [32]. Among these granulocytes, neutrophils are most predominant with eosinophils found in low concentrations (0-3%) [32]. Monocytes are also present in low numbers (<5% of the leukocyte differential) [32]. The peripheral blood composition of adult zebrafish was determined through smears and found to consist of the same trend with lymphocytes being most prevalent (82.95%), followed by granulocytes (~10%) and monocytes (~8%) [33, 34]. In addition to blood, differential counts of the zebrafish spleen and kidney have been documented in previous studies. Manual counts of only granulocytes, macrophages and lymphocytes yield a distribution in the spleen of approximately 25% granulocytes (11% neutrophils and 14% eosinophils), 16% macrophages, and 59% lymphocytes [34]. Distribution in the kidney is approximately 44% granulocytes (24% neutrophils and 20% eosinophils), 14% macrophages, and 42% lymphocytes [34]. Nonspecific cytotoxic cells (NCC), the teleost equivalent of the mammalian natural killer (NK) cell, was recently discovered [35] and have been reported in the anterior kidney, liver, spleen, and peripheral blood of species including the channel catfish (*Ictalurus punctatus*) and tilapia (*Oreochromis niloticus*). Percent composition of NCC related to the differential count has not yet been documented.

The coelomic cavity leukocyte composition has been investigated in many fish species including tilapia, carp (*Cyprinus carpio*), striped bass (*Morone saxatilis*), sea bream (*Sparus aurata*), and sea bass (*Dicentrarchus labrax*) [20-24, 36]. In these species, constitutive

expression of leukocytes in the coelomic cavity consists of granulocytes, lymphocytes and macrophages [22, 24, 36]. The most abundant cell type present in sea bass was macrophages while sea bream had more granulocytes [22]. This is a finding different from other organs in fish where lymphocytes are most prevalent [34]. Ultrastructural differences have been documented between coelomic exudate cells and the same cell types located in the blood and kidney [22]. These differences include granulocytes that are larger in size, cytoplasmic granules with new ultrastructural features, and the presence of a potentially new granule population [22]. These differences may represent different levels of activation amongst cells in the same population. Differences in lymphocytes have also been reported in the coelomic cavity with small dense cytoplasmic granules [22]. The coelomic cavity of zebrafish has not previously been explored before this study; however studies have been conducted on leukocytes in the blood, kidney and spleen.

Flow analysis and sorts on zebrafish whole kidney marrow, blood and spleen has been reported in a previous study [34]. Here it was reported that the major blood lineages of zebrafish could be isolated and sorted by light scatter characteristics in flow cytometry. In flow cytometry, forward scatter (FSC) is directly proportional to cell size and side scatter (SSC) is proportional to cellular granularity. Erythrocytes were found in exclusively a FSC^{lo} fraction [34]. Myelomonocytic cells, which include neutrophils, eosinophil/basophils, and macrophages were all found in one population in a FSC^{hi}/SSC^{hi} fraction [34]. Lymphocytes were found in a $FSC^{int}SSC^{lo}$ fraction [34]. Immature precursors were located in a FSC^{hi}/SSC^{int} population, but were only found in the kidney and not in any other organ due to the kidney being the main hematopoietic site in adult zebrafish [34]. In the zebrafish coelomic exudate cells, similar characteristics were found regarding isolating cells based on light scatter characteristics. The

largest population, also FSC^{hi}/SSC^{hi}, was found to consist of the granulocytes. Lymphocytes were also found in an $FSC^{int}SSC^{lo}$ fraction. The only difference was erythrocytes were found to be in an FSC^{hi}/SSC^{int} location, as they were approximately the same size as granulocytes with less internal complexity. Another population of cells, identified as NCC, is also present in the coelomic cavity and is found in an FSC^{lo}/SSC^{lo} fraction. The approximate constitutive composition of these cells in the coelomic cavity based on flow cytometry is granulocytes (60-80%), lymphocytes (10-20%) and NCC (4-10%). The presence of lymphocytes in the coelomic cavity indicates this tissue is not only important in innate immune responses but for adaptive responses as well. Female zebrafish were used for all experiments in the present study to avoid confusion due to the presence of large numbers of spermatids in the male cavity [37] that are approximately the same size as NCC. Males can be used for future immune-read out experiments as these spermatids do not appear to interfere with leukocyte function. As done in the previously reported study [34], cells from the coelomic cavity were sorted based on these light scatter characteristics to properly characterize individual populations based on Wrights stain, phenotype, and electron microscopy images.

Each sorted cell population from the coelomic cavity has a distinctive staining characteristic using Wrights stain. Eosinophil/basophils contain a non-segmented peripherally located nucleus. Neutrophils contain a multi-lobed nucleus and pale cytoplasm. Lymphocytes contain a large dark staining nucleus with a small rim of non-staining cytoplasm. Erythrocytes are characterized by an oval shaped nucleus and large rim of cytoplasm. The same staining characteristics have been reported in previous literature [2, 34, 38]. In the current study, cell sizes of leukocytes from the coelomic cavity were made based on measurements taken in electron microscopy images. Granulocytes were found to be approximately 10μm in diameter,

lymphocytes are 5μm, and NCC are 3-4μm. Erythrocytes are approximately the same size as granulocytes at 10μm. These sizes slightly differ from leukocyte sizes previously reported in other tissues of zebrafish. Based on 2μm and 10μm latex beads in flow cytometry, these sizes had previously been reported as myeloid cells (15.2μm), lymphoid cells (8.3μm), and erythroid cells (6.2μm). Transmission electron microscopy images of the granulocyte population from the zebrafish coelomic cavity shows two different populations of granulocytes. One type contains large spherical granules with a peripherally located non-segmented nucleus. The other contains a multi-lobed nucleus and more elongated granules. This is consistent with previous findings in zebrafish granulocytes. Eosinophil/basophil granulocytes have been reported as having round or elliptical granules approximately 0.66 ± 0.26μm in length with marbled variation in electron density [2]. Neutrophil granulocytes contain distinctive elongated granules approximately 0.42 ± 0.13μm in length [2]. Lymphocytes from the zebrafish coelomic cavity have a characteristically large nucleus and scant cytoplasm (high nuclear:cytoplasm ratio). This is also consistent with previous literature on fish lymphocytes [39]. NCC were found to contain tiny cytoplasmic granules but no readily visible nucleus. Previous reports have suggested the presence of these microscopic granules [40]. NCC have not been previously described in the zebrafish.

Studies have been conducted on the recruitment of leukocytes to the coelomic cavity in several species of fish as a result of intra-coelomic injections. Upon injection of sea bass and sea bream with bacteria or yeast into the coelomic cavity, granulocyte numbers were found to quickly increase (<5 hours post-injection) and as much as double in number [24, 36]. The zebrafish coelomic cavity responds in the same manner to bacterial injection with peak responses 6 hours post injection and a 126% increase in leukocyte numbers over control fish. Intra-

coelomic injections of LPS also lead to an increase in leukocytes (80% increase over control) by 2 hours post-injection.

Edwardsiella ictaluri has previously been established as an effective infection model in zebrafish [41]. This gram negative bacteria is responsible for enteric septicemia in channel catfish (*Ictalurus punctatus*) [42]. In the present study, *E. ictaluri* was found to be a suitable pathogen to elicit an immune response in the coelomic cavity. Only dermally abraised fish were susceptible to infection and mortality resulted 3-5 days post infection by immersion. *Edwardsiella tarda* has been used in previous studies on adult zebrafish and the same outcomes regarding susceptibility and mortality were found [43]. Peak cellular recruitment to the coelomic cavity of dermally abraised zebrafish following immersion in *E. icaturi* was found 24 hours post infection (54% increase over control). This suggests the coelomic cavity is an important tissue for combating infection. No previous studies have been reported on coelomic cavity recruitment following bacterial immersion.

Macrophages, eosinophils and neutrophils are the only cells types found in previous studies responsible for phagocytosis of pathogens [20, 21, 36]. The same is true in the zebrafish coelomic cavity. Granulocytes are the only actively phagocytic population following bacterial injection with as many as 40% of cells in that population being positive for phagocytosed bacteria as soon as 2 hours post-injection. Coelomic exudate cells from sea bream and sea bass have also been documented to be cytotoxic towards HeLa and B16 melanoma tumor target cells by mechanisms of necrosis and apoptosis suggesting the presence of NCC-like cells in the coelomic cavity [23]. However, NCC have not yet been documented in the coelomic cavity of fish. NCC have been found to be constitutively present in the blood, kidney, liver and spleen of many fish species prior to enrichment [44], and they have been found to mobilize during

infection [45]. In zebrafish, a population of cells resembling the characteristics of NCC in electron microscopy and that express surface NCCRP-1 are constitutively present in the coelomic cavity. Sorted cell populations of zebrafish coelomic exudates could not be used in cytotoxicity assays due to the loss of large numbers of cells during the sort process. Unsorted coelomic exudate cells from zebrafish are cytotoxic (approximately 20% target cell death) towards both human (HL-60 and K562) and mouse (YAC-1) target cells after 4 hours co-incubation at a 4:1 E:T ratio. Target cell death occurred at a 4:1 E:T ratio as soon as after 1 hour co-incubation. This level of cellular cytotoxicity is interesting, as leukocytes from other species of fish have required higher E:T ratios to achieve this level of target cell death. Previous studies using coelomic exudate cells in sea bream used greater E:T ratios (50:1) for 2 hours and only achieved 20% cytotoxicity. The same was found in studies using catfish NCC where E:T ratios of 20:1 after 4 hours did not exceed 20% target cell death [46]. This finding indicates that zebrafish have a potent innate immune response capable of quickly and efficiently killing invading pathogens via mechanisms of cellular cytotoxicity.

After determining that coelomic exudates are cytotoxic towards human and mouse NK targets, the role of a protein known as NCC cationic antimicrobial protein-1 (Ncamp-1) in relation to activation of the cell's lytic cycle was examined. Ncamp-1 may potentially be a new pattern recognition receptor (PRR). In previous studies, Ncamp-1 has been found to bind GpC and CpG ODNs, and full length recombinants exhibit bactericidal activity against *E. coli* and *S. iniae* [28]. In zebrafish, Ncamp-1 is expressed on the surface of granulocytes and NCC. Other PRRs have been linked to the cell's lytic cycle and activation of pathways leading ultimately to membrane permeability and destruction of the invading pathogen. A few examples of PRRs found to be associated with inducing cytotoxicity include TLRs 3, 4, 7, 8 and 9[47-50]. The role

of Ncamp-1 was examined by performing a redirected lysis using hybridomas expressing 9C9 (anti-Ncamp-1) or M2 (isotype control). Certain levels of M2 cytotoxicity is expected due to it being a foreign cell type. Healthy cells are protected from NK-cell mediated lysis due to the expression of major histocompatibility complex (MHC I) class I ligands which are important for NK-cell inhibitory receptors [51]. Without these ligands, NK-cells or their equivalent (NCC) are activated to kill these foreign cells. Many surface receptor proteins in previous studies have been discovered to function in activation of NK cells through redirected lysis. The engagement of non-activating surface proteins such as CD38 and 2B4 with antibodies has been found to lead to the enhancement of cell mediated cytotoxicity [52, 53]. The same was found with engaging Ncamp-1 with 9C9 expressing hybridomas. Cell mediated cytotoxicity was greatly increased (>100% over isotype control) by crosslinking Ncamp-1. Therefore Ncamp-1 appears to not only play a role in antigen recognition, but also functions in leukocyte activation. Cells harvested from the coelomic cavity of zebrafish that have not been subject to infection are considered to be rested cells. Resting NK cells in previous literature have been reported as being less lytic against target cells than IL-2 activated NK cells [54]. These resting cells are not less responsive IL-2 activated cell, but their activation is more stringent [54]. However, rested cells have been shown to kill via redirected lysis by using antibodies to engage specific receptors responsible for activating cytotoxicity [54]. This further provides evidence of the association of Ncamp-1 with activation of cellular cytotoxicity.

For the first time in this study, we have demonstrated the ability to use the zebrafish coelomic cavity as an immune read-out organ. These methods will allow for the collection of greater numbers of leukocytes in addition to the ability to study individual cell types found in the coelomic cavity. The small size of the zebrafish has previously inhibited the harvest of large

numbers of cells due to the difficulty of dissecting out the kidney and spleen. Harvesting these organs is a time consuming process and the majority of cells from the organs are erythrocytes. Cells from the coelomic cavity take under 30 seconds to harvest and produce as many, if not more than either the kidney or spleen. This finding will be an asset to future studies in immunological and developmental studies using the zebrafish.

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CHAPTER 4

CONCLUSION

The zebrafish coelomic cavity was found to be an immune responsive organ appropriate for determination of host innate and adaptive immune responses to infectious diseases. The coelomic cavity is an excellent and easy source of leukocytes for future studies using zebrafish. This method of obtaining immune cells is substantially faster than prior techniques with a maximum of 30 seconds required to adequately perfuse the cavity with media to obtain cells. In comparison, harvesting the kidney and spleen in zebrafish is a time consuming and difficult process yielding more red blood cells than leukocytes in the end.

The coelomic cavity was found to contain resident leukocytes consisting of granulocytes, macrophages, lymphocytes, and nonspecific cytotoxic cells. An average healthy zebrafish contains approximately 10^5 leukocytes in the coelomic cavity. Additional leukocytes were found to be recruited to the cavity as a result of injections with fixed *E. ictaluri* or LPS. Scraped fish exposed to infection with *E. ictaluri* through immersion were also found to recruit leukocytes to the coelomic cavity. Injection of fixed bacteria into the coelomic cavity results in the best recruitment compared to LPS injection or live infection.

Functional assays performed on coelomic exudate cells demonstrated these leukocytes to be cytotoxic towards HL-60, YAC-1, and K562 target cells. When injected with labeled *E. coli* or *S. aureus*, only the granulocyte population exhibited phagocytic capabilities as demonstrated by intracellular fluorescence detected in flow cytometry.

Both innate and adaptive immune cells were found to be recruited to the coelomic cavity. This finding will be an asset to future studies in immunological and developmental studies using the zebrafish.