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Rac2 in the zebrafish (*Danio rerio*) with a focus on neutrophils

by

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A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Immunobiology

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Ames, Iowa

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This dissertation is dedicated to my loving partner and family,
without whose support it would not have been possible to accomplish,
especially my grandpa,
G. Wayne Tell,
who didn't quite make it to the end,
but never doubted that I could.

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ABSTRACT

Rac2 is a small Rho GTPase known to have roles regulating actin cytoskeletal reorganization and gene expression in cells of hematopoietic origin. Loss of Rac2 activity in humans and mice results in neutrophil function deficits and severe bacterial infections. While *rac2* transcript had been confirmed in whole adult zebrafish preparations, little else was known about Rac2 in the zebrafish. This work substantially increases what is known about Rac2 in the zebrafish.

It was hypothesized that Rac2 function in zebrafish is similar to that in humans. In order to investigate this, a three-pronged approach was taken: 1) a descriptive study of the expression of Rac2 protein, using immunocytochemistry, and transcript, using qPCR, in the zebrafish neutrophil; 2) *in vitro* neutrophil function assays and Rac-specific small-molecule inhibition; 3) morpholino oligonucleotide gene knockdown of *rac2* in zebrafish embryos followed by exposure to one of two fish pathogens, *Aeromonas hydrophila* or *Edwardsiella ictaluri*, and assessment of embryo survival.

The presence of Rac2 protein is described for the first time in the adult zebrafish neutrophil. It was localized to the cytoplasm, as in human neutrophils. *rac2* transcript was found in whole embryo preparations by 12 hours post fertilization, earlier than previously described. Only *rac2*, and not *rac1* or *rac3*, was found in adult zebrafish neutrophils. Inhibition of Rac caused significant decreases in the key neutrophil functions of respiratory burst, phagocytosis, and NETs release. Following exposure to *Aeromonas hydrophila*, *rac2* morpholino-injected embryos exhibited significant increases in mortality as compared to uninjected or control morpholino-injected embryos.

Rac2 clearly plays a role in neutrophil function in the zebrafish. That role is similar to the one that Rac2 plays in human and mouse neutrophils.

CHAPTER 1. General Introduction

1.1 Introduction

As Amulic and colleagues so aptly put it their 2012 contribution to the Annual Review of Immunology, “Neutrophils were discovered at the dawn of the immunological sciences” by Paul Ehrlich. In 1880, he characterized cells that had a “polymorphous nucleus” and the tendency to retain neutral dyes, thus their name *neutrophils* (Amulic et al., 2012). Around the same time, Elie Metchnikoff stuck rose thorns into transparent starfish larvae to test his hypothesis that the mobile cells he had noticed might serve as host defense elements against invaders. Sure enough, after puncturing the skin, the rose thorns became surrounded by those mobile cells. Add to that his subsequent observation of *Monospora bicuspidata* being spontaneously ingested and destroyed by mobile blood cells in daphnia and Metchnikoff has been credited with developing “the full exposition and understanding of phagocytosis” (Gay, 1935; Rebeck and Crowley, 1955). So neutrophils have been studied for well over a century.

Despite a relatively long history of investigation, up until recently, neutrophils have been viewed as rather one-dimensional cells: short-lived, early-responding phagocytes that migrate into the tissues and then die by the time the rest of the immune system really gets involved in an immune response. This view has been challenged by evidence that neutrophil cytokine and chemokine secretion, as well as direct cell-to-cell contact, shapes the subsequent immune response (macrophage, dendritic cell, and T-cells) (for reviews of this shifting outlook, see (Amulic et al., 2012; Croker et al., 2012)). Neutrophils have now been shown, first *in vitro* and then in the zebrafish *in vivo*, to exhibit retrograde chemotaxis *away* from a site of inflammation (Buckley et al., 2006; Mathias et al., 2006). Neutrophils also cross-prime T-cells *in vivo* and traffic to lymph nodes (Beauvillain et al., 2007; Yang et al., 2010). Clearly, the traditional, simplistic view of the neutrophil has been complicated substantially.

Even though we have made great strides in our understanding of neutrophil biology and function over the past 100 years, there is still much that remains unanswered or merely inferred. Despite its origins with Elie Metchnikoff’s *in vivo* observations, the fact remains

that most of our knowledge of the immune system comes from examination of *in vitro* systems and histopathological samples, this makes teasing out the cellular dynamics difficult, especially in the very early stages of the immune response. The zebrafish, *Danio rerio*, offers some unique advantages as compared to more traditional model systems, such as mouse or *Caenorhabditis elegans*, perhaps the most powerful and notable in this context is the combination of high optical clarity through the first seven days of life, swift development, and the presence of both innate and adaptive arms of the immune system (Renshaw and Trede, 2012).

The zebrafish has already played a part in dissecting the role of the neutrophil in the immune response, as demonstrated by observations of retrograde chemotaxis, and the body of work from the labs of Drs. Stephen Renshaw, Ann-Marie Meijer, and Anna Huttenlocher. The more we discover about immune function in the zebrafish, the more powerful a model system it can be; but both its strengths and its weaknesses must be probed. To this end, we decided to harness zebrafish genetic tractability and external development to investigate the Rho small GTPase, Rac2, in embryos and neutrophils.

The overall hypothesis of the dissertation was: Rac2 function in the neutrophils of zebrafish is similar to that in humans.

In order to investigate this hypothesis, four specific aims were developed:

Specific aim 1. Establish that Rac2 protein is present in adult zebrafish neutrophils and determine its expression pattern. Immunocytochemical staining was performed on zebrafish kidney marrow cells.

Specific aim 2. Describe expression of *rac2* in zebrafish embryos over time and in adult neutrophils, relative to the other two isoforms of Rac. Analysis of target gene expression was assessed via qPCR using whole embryos and adult neutrophil preparations.

Subhypothesis 1. Inhibition of Rac2 function will cause defects in neutrophil oxidative burst, NET release, and phagocytosis functions.

Specific aim 3. Quantify oxidative burst, NET release, and phagocytosis of opsonized bacteria in adult zebrafish neutrophils under Rac2 inhibitory conditions using NSC23766, a Rac-specific small molecule inhibitor. Neutrophil function was assessed using well-established neutrophil functional assays (Chuammitri et al., 2011; Jovanović et al., 2011; Palić et al., 2007).

Subhypothesis 2. Inhibition of Rac2 function will cause increased mortality of embryos exposed to bacterial infection.

Specific aim 4. Quantify mortality of embryos exposed to *Aeromonas hydrophila* or *Edwardsiella ictaluri* via the immersion route following Rac2-knockdown. Embryos were exposed to one of these species of bacteria at 48 hours post fertilization following Rac2-specific morpholino oligonucleotide knock-down.

1.2 Dissertation organization

This dissertation is organized in the alternative format, including a literature review and three manuscripts that have been modified from manuscripts submitted for publication or that we plan to submit for publication in the near future. Chapters 3 and 4 are modified from a single manuscript accepted for publication:

Tell, RM, Kimura, K, Palić, D. Rac2 expression and its role in neutrophil functions of zebrafish (*Danio rerio*). Accepted for publication by Fish and Shellfish Immunology.

Chapter 5 is prepared as a short communication for submission in the near future.

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CHAPTER 2. Literature Review

“Without the immune system, our body is nothing but an incubator for infectious diseases.” – Dr. Michael Cho, Seminar, April 20, 2011.

The immune system is a network of barriers, cells, and effector molecules that serves largely to protect animal hosts from external threats to their health. It acts as a warning system in cases of injury and infection. In order to do this, components of the immune system must be sensitive, so as to catch threats early, and specific, so as not to regularly react to host molecules leading to pathology. The specificity of the immune response increases over time, both during a single response to a pathogen and over the course of the lifetime of the host. In addition to warning of injury and infection, the immune system provides protection from and clearance of infectious agents. The components of the immune system are traditionally divided into two categories, innate and adaptive, though this division is somewhat arbitrary, as their actions are complexly intertwined.

The innate portions of the immune system are generally described as being present at birth/hatching, providing protection against broad categories of pathogens, and developing no “memory” of pathogens that have been previously seen in the host’s lifetime. While often not thought of as part of the immune system, intact skin and mucous membranes provide stern barriers to entry. These barriers are also associated with chemical means of defense against pathogens, some of which, such as lysozyme and antimicrobial peptides (AMPs), have direct microbicidal and cytolytic actions, as does the complement cascade. Many of these antimicrobial peptides and complement components act as chemical messengers to modulate the immune response by recruiting immune cells to the site of infection, enhancing phagocytosis, and other immune actions (Jenssen et al., 2006; Mandell et al., 2010). These innate immune peptides and proteins are able to essentially distinguish between the eukaryotic host cells and potential invaders by recognizing membrane differences in molecular composition and electrostatic charge (Mandell et al., 2010; Pasupuleti et al., 2012). Recognition of pathogen-associated molecular patterns (PAMPs) is a hallmark of what we

consider to be the innate portion of the immune system. These PAMPs are widely conserved amongst diverse pathogens. Cells involved in the innate immune response detect these PAMPs using pattern recognition receptors (PRRs), such as transmembrane Toll-like receptors (TLRs), cytosolic nucleotide-binding domain, leucine-rich repeat containing proteins (NLRs), and Dectin-1 (Kofoed and Vance, 2012). Expression of PRRs is not limited to immune cells, most notably, they are also present on various epithelial and endothelial cells (Grote et al., 2010; Marques and Boneca, 2011; Parker et al., 2007). Cells traditionally thought of as innate immune cells include: natural killer cells, neutrophils, macrophages, and dendritic cells. This last is often called a “bridge” between innate and adaptive immunity due to its role in antigen presentation.

The adaptive immune system is comprised of protections against specific pathogens which develop over the course of an animal’s lifetime and generate immunological memory of pathogens seen in the past, which allows for swifter more effective immune responses to those pathogens in the future. Adaptive immune cells include B and T lymphocytes. These cells recognize small parts of the molecular structure of antigenic molecules, known as *epitopes*. Each B or T cell recognizes only a single epitope of a single antigen, resulting in the exquisite specificity of the adaptive immune response. This specificity, as well as the great diversity of B and T cell receptors, is achieved by a combination of gene segment recombination, diversification of the sequence at the segment junctions, and point mutations throughout the gene. Upon recognition of its cognate antigen, a B or T cell will begin to replicate, resulting in a large number of that antigen-specific lymphocyte. B cells go on to produce a soluble form of their receptor, the well-known *antibodies* (Murphy et al., 2008). These antibodies bind to pathogens and soluble antigens, helping effector cells, such as neutrophils and macrophages, to better recognize, phagocytose, and destroy these invaders (Mandell et al., 2010).

The neutrophil is a professional phagocyte of the immune system and is the first line of recruited cellular defense upon infection or injury of the host animal. Neutrophils migrate from the bloodstream to the affected area via chemotaxis quickly and in large numbers (Colucci-Guyon et al., 2011; Deng et al., 2012; Rebuck and Crowley, 1955; Renshaw et al., 2007; Renshaw et al., 2006). Once there, they work to contain and destroy any invaders. This is initially done via phagocytosis and killing.

Phagocytosis a hallmark function of the neutrophil. It is an active process which requires cytoskeletal rearrangement. An intruder is recognized via PRRs or Fc γ Rs and subsequently internalized. Neutrophils are very efficient at this, internalizing an IgG-opsonized target in less than 20 seconds (Segal et al., 1980). For comparison, macrophages generally take several minutes to ingest similar types and amounts of targets (Henry et al., 2004). The granules that give neutrophils their name, are then called into action. Within 20 seconds after the formation of the phagosome, neutrophil granules of the azurophil and specific types have fused with this transient organelle, releasing their potent antimicrobial and proteolytic contents into that enclosed space, *degranulating* (Segal et al., 1980). The early stages of this phagosome-granule fusion are calcium-dependent, while fusion of primary granules with later-stage phagosomes proceed in a calcium-independent fashion (Nordenfelt et al., 2009).

Neutrophil granules are traditionally subdivided into two classes based on the presence or absence of myeloperoxidase (MPO): peroxidase-positive (also called primary or azurophilic) and peroxidase-negative granules. Further common subdivision of the peroxidase-negative granules is into specific (secondary) and gelatinase (tertiary) granules. This subdivision is based on density and a selection of marker proteins, which seem to be determined by when the granules were formed during the development of the neutrophil in which they reside (Borregaard et al., 2007). Additionally, the neutrophil contains secretory vesicles which may be of endocytic origin (Borregaard et al., 1992). Azurophilic granules are rich in antimicrobial proteins, such as MPO, defensins, and lysozyme, as well as proteases such as elastase and cathepsin G. Specific granules are rich in the antimicrobial proteins lactoferrin, pentraxin 3, and lysozyme, as well as containing proteases such as collagenase and some

gelatinase (though not as much as is found in gelatinase granules). Gelatinase granules are so-called because of their high content of the protease gelatinase, they also contain the antimicrobial protein lysozyme. Secretory vesicle release is important for firm adhesion of neutrophils during extravasation because of the β_2 integrins that they carry on their membranes (Faurischou and Borregaard, 2003; Häger et al., 2010; Sengeløv et al., 1993). Specific and gelatinase granules also carry β_2 integrins on their membranes, in addition, they carry 90% of the cytochrome *b* in a neutrophil (Borregaard et al., 1983). Cytochrome *b* is the portion of the NADPH oxidase that is membrane-bound when the neutrophil is inactive.

Another quintessential neutrophil function is the respiratory burst. The kinetics of oxygen consumption, used to measure respiratory burst function, in neutrophils are very similar to those for phagocytosis and degranulation. Within 10 seconds, oxygen consumption commences and reaches a linear asymptote 10 seconds after that (under the same conditions as those described to measure phagocytosis and degranulation, two paragraphs above) (Segal et al., 1980). The phagocyte NADPH oxidase is a multi-component electron-transfer complex. The catalytic, electron-transfer portion of the NADPH oxidase is a membrane-bound flavohemoprotein cytochrome *b*₅₅₈ and is a heterodimer made up of two subunits, p22^{phox} and gp91^{phox} (also known as NOX2) (Bylund et al., 2010). The rest of the NADPH oxidase complex resides in the cytoplasm until cellular activation, when the remaining components translocate to the granular membrane and associate with the cytochrome *b* to form a fully functional NADPH oxidase. These three subunits are found in a complex in the cytosol, p47^{phox}, p67^{phox}, and p40^{phox}. Additionally, Rac2, a cytosolic Rho small GTPase, translocates to the membrane simultaneously, but independently of the three-subunit complex (Diebold and Bokoch, 2005). The reactive oxygen species (ROS) that result from assembly and activation of the NADPH oxidase have traditionally been thought of as directly microbicidal (Babior, 1999). While it is likely that at least HOCl does have direct microbicidal action, it has become clear that the ROS also act to liberate phagosomal proteases and have a role in cellular signaling (Roos et al., 2003; Roos and Winterbourn, 2002; Segal, 2005). One of these roles is in the deployment of neutrophil extracellular traps.

Neutrophil extracellular traps (NETs) are fibrous structures of decondensed chromatin decorated with histones as well as antimicrobial granule and cytoplasmic proteins (Brinkmann, 2004; Urban et al., 2009). NETs are released during what seems to be a specialized type of caspase-independent cell death (Fuchs et al., 2007). Their release is thought to be ROS-dependent, with MPO-derived ROS, such as singlet oxygen, gaining specific attention (Kirchner et al., 2012). There have been two published reports of ROS-independent NET release to date: the first by Marcos et al. (Marcos et al., 2010) was retracted; the second, by Gabriel et al. (Gabriel et al., 2010) indicates that in the case of *Leishmania donovani*, human neutrophils produce NETs even in the presence of catalase or the NADPH-oxidase inhibitor DPI. It is generally accepted that NETs entrap and kill various bacterial, fungal, and protozoal pathogens (Behrendt et al., 2010; Brinkmann, 2004; Ermer et al., 2009; Fuchs et al., 2007; Gabriel et al., 2010; Papayannopoulos and Zychlinsky, 2009; Remijns et al., 2011). NETs can serve to slow, immobilize, and inhibit the growth of various pathogens, as well as allowing for local concentrations of antimicrobial substances to remain high enough to continue their actions while limiting the consequences to surrounding host tissue (McCormick et al., 2010). NET release is phylogenetically conserved in gnathostomes (Chuammitri et al., 2009; Palić et al., 2007b).

Studying a system that is deficient in a component-of-interest is one way to learn the importance of that component and its function in the whole. Humans with genetic defects in their neutrophils have been instrumental to unlocking polymorphonuclear mysteries (Häger et al., 2010). The first severe congenital neutrophil function disorder to be recognized was chronic granulomatous disease (CGD, originally known as fatal granulomatous disease of childhood) (Windhorst et al., 1968). CGD patients have a genetic defect in one of the five subunits of the NADPH oxidase complex. The majority of cases, about two thirds, are caused by mutations in the gp91^{phox} gene (*CYBB*), which results in an X-linked recessive form of the disease (van den Berg et al., 2009). These patients suffer from recurrent fungal and catalase-positive bacterial infectious as well as granulomatous inflammation that often involves multiple organs (Kuijpers and Lutter, 2012). CGD patients and the animal models of this

disease that have been developed, have taught us a great deal about neutrophil function, in general, and the NADPH-oxidase, specifically (Häger et al., 2010).

Small GTPases hydrolyze GTP to GDP. Their endogenous GTPase activity is augmented and regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). GAPs enhance the intrinsic GTPase activity of the small GTPases and GEFs catalyze the exchange of GDP for GTP in the binding groove (Gasper et al., 2009). The Ras (**rat sarcoma**) superfamily of small GTPases contains numerous subfamilies, one of these is the Rho (**Ras homologs**) family.

Rho GTPases participate in and regulate actin cytoskeletal rearrangement and gene expression in many different types of cells (Hall, 1998; Van Aelst and D'Souza-Schorey, 1997). Rho small GTPases have an additional type of regulatory molecule, guanine nucleotide exchange inhibitors (RhoGDIs). RhoGDIs inhibit nucleotide dissociation from Rho GTPases, inhibiting GAP activity and preventing GEF-mediated nucleotide exchange, which essentially lock Rho molecules into their inactive, GDP-bound states. Most inactive Rho GTPase molecules are bound to RhoGDIs, which accounts for their localization in the cytosol (Garcia-Mata et al., 2011).

The Rac (**Ras-related C3 botulinum toxin substrate**) subfamily of Rho GTPases consists of four members in humans: RAC1, RAC2, RAC3, and RHOG (Wennerberg and Der, 2004). The three isoforms of Rac are $\geq 89\%$ homologous with one another at the amino acid level. There is some functional overlap between the three, but their expression patterns are different. While Rac1 is ubiquitously expressed and Rac3 expressed widely, Rac2 is found only in cells of hematopoietic origin. (Didsbury et al., 1989; Haataja et al., 1997; Williams et al., 2000). As a family, Rac GTPases regulate events downstream of membrane receptor activation, which includes regulation of actin cytoskeleton rearrangement, chemotaxis, gene transcription, and cell growth (Fenteany and Glogauer, 2004).

Rac2 has been shown to be involved in many important functions in cells of hematopoietic origin. In hematopoietic stem cells, Rac2 controls localization and retention in hematopoietic tissues (Cancelas et al., 2005; Deng et al., 2011; Gu et al., 2003). In B cells, Rac2 regulates adhesion and immunological synapse formation (Arana et al., 2008). In T cells, Rac2 plays a redundant (with Rac1) but critical role in T lymphopoiesis, as well as being essential to certain TCR signals and proper migration to and within secondary lymphoid organs (Faroudi et al., 2010; Guo et al., 2008). In mast cells, Rac2 regulates protease expression (Gu et al., 2002). In DCs, it is involved in phagosomal alkalization and antigen cross-presentation (Savina et al., 2009). Of course, Rac2 is also involved in many key functions of the neutrophil.

Rac2 is the star of another human primary immunodeficiency that has precipitated much study and discovery about how neutrophils work. A rare dominant negative mutation in Rac2, D57N, causes this immunodeficiency, which manifests as severe recurrent bacterial infections and delayed wound healing. Although a baseline neutrophilia is seen, many essential neutrophil functions are absent or drastically reduced including chemotaxis, respiratory burst, and secretion of granular proteins. *Rac2*^{-/-} mice reiterate those defects as well as exhibiting increased mortality in response to *Aspergillus* fungal infections (Abdel-Latif et al., 2004; Ambruso et al., 2000; Roberts et al., 1999; Williams et al., 2000). Rac2 is a key regulator of free barbed end formation during actin skeletal reorganization, which is necessary for neutrophil migration, chemotaxis, and phagocytosis (Sun et al., 2007). In its active, GTP-bound, form Rac2 goes on to interact with effector molecules downstream that are involved in actin cytoskeletal rearrangement and the assembly and function of the NADPH oxidase, all of which is important for proper neutrophil function in response to host threat (Diebold and Bokoch, 2005; Gu et al., 2003).

Rac2 is critical for degranulation of primary granules (Abdel-Latif et al., 2004). As mentioned earlier in this review, Rac2 is a cofactor required for proper NADPH oxidase subunit recruitment, assembly, and function (though Rac1 can fill in for Rac2 sometimes, it is not as efficient) (Bokoch and Zhao, 2006). Rac2 has also been found to be a critical

component of reactive oxygen and nitrogen species (RONS)-dependent NET formation in mice (Lim et al., 2011). Thus, there is evidence to support the importance of Rac2 in most major signaling pathways that regulate neutrophil function.

The zebrafish, *Danio rerio* (Hamilton 1822), is a teleost, or bony fish. A member of the family Cyprinidae, along with carp and fathead minnows, this freshwater fish hails originally from South and Southeastern Asia. Commonly found in stagnant and standing water, including ditches and rice patties, zebrafish have also been found in streams and rivers, including the Ganges. They are a warm water species, preferring water temperatures of 28°C. These small fish (~3 cm long as adults), are omnivorous and easily tolerate home fish tank and laboratory aquaculture setups (Lawrence, 2007; Spence et al., 2008).

The zebrafish was introduced as a vertebrate model system around 30 years ago by developmental biologists, such as George Streisinger, and ever since then has been swimming its way into the hearts and minds of scientists in many varied disciplines such as genetics, toxicology, and immunology (Lawrence, 2007; van der Sar et al., 2004). It offers many advantages to scientists seeking a model organism. In addition to its small size, which allows for high density culture in a relatively small laboratory footprint, zebrafish are highly fecund, have short generation length (3 to 6 months) and develop quickly. By 24 hours post fertilization (hpf), the developing heart tube can be seen beating in the developing embryo. Hatching from the chorion occurs during the third day of development (48-72 hpf) (Sprague et al., 2006). Because of their external development, all of this can be observed easily with a microscope. The high optical clarity of embryos through 7 days post fertilization (dpf) is a unique feature amongst vertebrate laboratory model animals. The ability to observe cellular dynamics in live embryos combines with the genetic tractability of the zebrafish in transgenic reporter lines of zebrafish (Hall et al., 2009; Renshaw et al., 2006; Renshaw and Trede, 2012). In these fish, cellular dynamics, interactions, and reactions can be observed in real time. Add fluorescently tagged bacteria and you have a recipe for decoding much about host pathogen interactions in the immune response.

The zebrafish has gained much traction as an immunological model over the past decade. As a gnathostome, it has both innate and adaptive immune systems with remarkable similarity to those in humans and other mammals, but a delay in the development of functional adaptive immunity until 4-6 weeks post fertilization allows for study of innate immune function in the absence of adaptive immunity (Ellett and Lieschke, 2010). All major immunological cell types have been described, including neutrophils, monocytes/macrophages, dendritic cells, NK cells, T and B cells (Renshaw and Trede, 2012). When it comes to infecting a developing zebrafish embryo, multiple infection routes are available: immersion, which is believed to more closely mimic primary infection routes for many natural fish pathogens, or injection (IM, IV, or into embryonic spaces such as the hindbrain ventricle), which allows for close control of infectious dose and assurance of successful introduction of bacteria into each embryo, are commonly used methods (Harriff et al., 2007; Milligan-Myhre et al., 2011; Pressley et al., 2005).

As with any model system, there are also potential disadvantages to using the zebrafish as an immunological model. The primary hematopoietic organ of the zebrafish (and most other fish) is the kidney, more specifically, the anterior or head kidney (Ellett and Lieschke, 2010). In contrast to terrestrial animals, fish live in an aquatic environment. This places them in constant, intimate contact with that water, and anything in it, including potential pathogens. This difference is thought to be part of the evolutionary reasoning behind another set of potential drawbacks to the zebrafish as an immunological model organism. Zebrafish (and other fish species) are known to be very tolerant to lipopolysaccharide (LPS) exposure (Novoa et al., 2009). LPS is a major component of the Gram negative bacterial outer membrane and is a commonly used laboratory immune stimulant and inflammation inducer. In mammals, TLR4 is the primary PRR for LPS. Zebrafish TLR4, however, does not respond to LPS (Sepulcre et al., 2009). Interesting work out of Dr. Carol Kim's lab at the University of Maine showed that the intracellular signaling portion of TLR4, as well as downstream signaling pathways, are still intact in zebrafish cells and react as expected, using a chimeric TLR4 molecule fusing the intracellular portion of zebrafish TLR4 to the extracellular portion of mouse TLR4 (Sullivan et al., 2009).

Zebrafish have true neutrophils, polymorphonuclear granulocytes of myelocytic lineage that express high levels of *mpx*, myeloid-specific peroxidase, a homologue of myeloperoxidase (Ellett and Lieschke, 2010; Lieschke et al., 2001; Renshaw et al., 2006). These cells have been shown to exhibit all major neutrophil functions: migrating actively, exhibiting chemotaxis to sites of injury or infection, phagocytosing invading bacteria and particles, performing respiratory burst, and releasing NETs (Colucci-Guyon et al., 2011; Deng et al., 2012; Deng et al., 2011; Lieschke et al., 2001; Palić et al., 2007a; Renshaw et al., 2007; Renshaw et al., 2006). In fact, zebrafish neutrophils have been observed to phagocytose over 250 bacteria per hour in the tissues! (For comparison, in the same situation, macrophages were observed to phagocytose fewer than 150 bacteria per hour (Colucci-Guyon et al., 2011)).

When the research for this dissertation began, almost nothing had been described about Rac2 in zebrafish. Salas-Vidal and colleagues had performed a genomic screen showing that zebrafish transcripts for *rac1*, *rac2*, and *rac3* were present in zebrafish (Salas-Vidal et al., 2005). Additionally, *rac2* had been included in a high throughput whole mount in situ hybridization project that was submitted directly to ZFIN.org (Thisse and Thisse, 2004). This data showed *rac2* transcript in a subpopulation of blood cells, the thymus, the pharynx and pectoral fins of developing embryos.

Preliminary results from our lab showed the presence of Rac2 protein in zebrafish neutrophils (Figure 2.1). In order to explore the potential use of the whole zebrafish kidney degranulation and NETs release assay in investigation of the neutrophil activation pathways, whole zebrafish kidneys were exposed to a specific inhibitor of small Rho GTPases, *Clostridium difficile* Toxin B (ToxB), and stimulated with calcium ionophore (CaI). After pre-treatment of kidneys with ToxB, a significant reduction was observed in degranulation (75% of non-inhibited control, $p < 0.05$) and NETs release (82% of non-inhibited control, $p < 0.1$) (Figure 2.2) when compared to non-inhibited kidneys. This functional data demonstrated the potential for use of whole zebrafish kidney assays to detect differences in neutrophil function from kidneys exposed to specific signaling pathway inhibitors *in vitro*, showing

potential for use in investigation of neutrophil activation pathways. Furthermore, small Rho GTPases, and possibly Rac2, appeared to have a role in zebrafish neutrophil degranulation and NETs release.

Since embarking upon the research for this dissertation, there has been contribution to the field of Rac GTPases in zebrafish out of the lab of Dr. Anna Huttenlocher at the University of Wisconsin-Madison. In 2011, they developed a Rac2D57N line of zebrafish that expresses the dominant negative human Rac2 that is responsible for the human primary immunodeficiency in addition to the native zebrafish Rac2 (Deng et al., 2011). They showed that the Rac2D57N line recapitulates the human primary immunodeficiency and exhibits higher mortality to *Pseudomonas aeruginosa* infection, despite normal macrophage response. With this line, as well as a gene knockdown approach using a *rac2* translation-blocking morpholino oligonucleotide, they demonstrated an essential role for Rac2 in the regulation of 3D motility and the polarization of F-actin dynamics and PI(3)K signaling *in vivo*. They also observed increased mobilization of Rac2-deficient neutrophils from hematopoietic tissue into circulation, which implies that mobilization of neutrophils does not require traditionally defined cell motility.

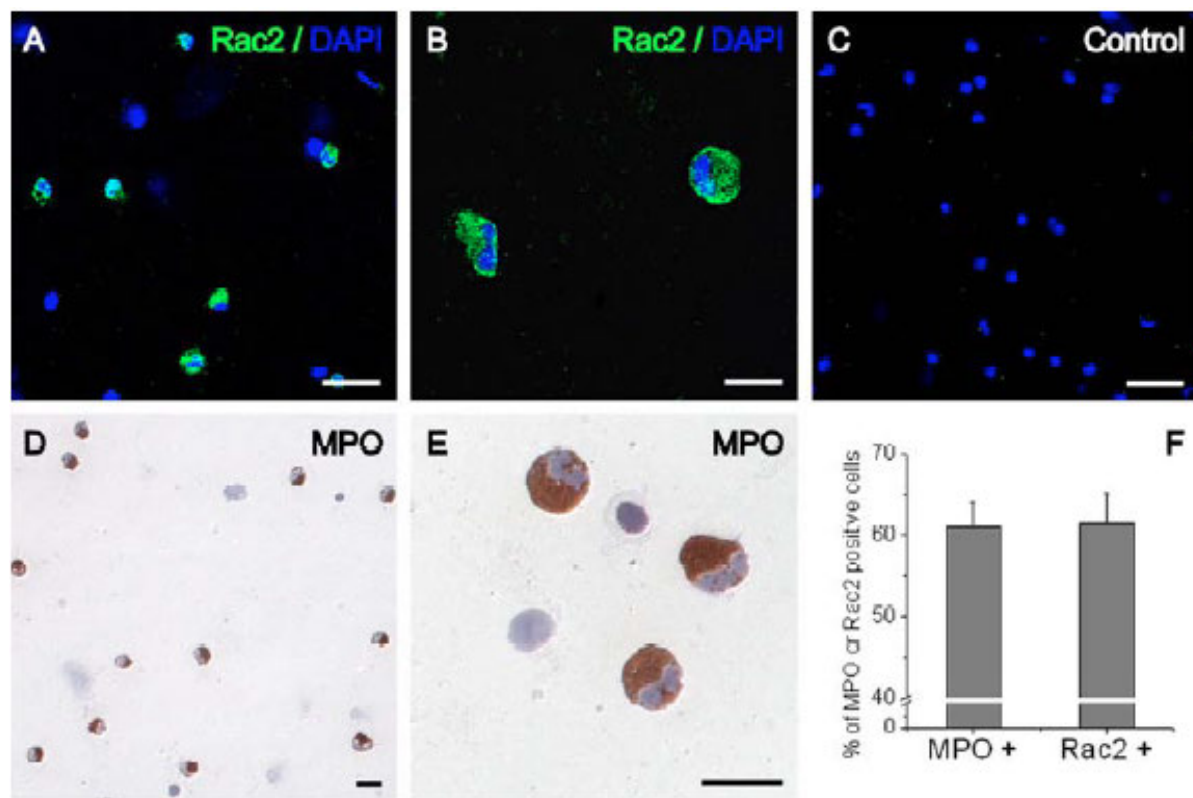


Figure 2.1. Rac2 is present in myelopoietic zebrafish kidney cells. A: Not all zebrafish kidney cells are stained positive for Rac2 (DAPI nuclear staining blue, anti-Rac2 staining green). B: Cytoplasmic localization of Rac2 visualized with anti-Rac2 specific antibody (green). C: Control slides do not show non-specific immunoreactivity. D: MPO specific staining of zebrafish whole kidney cell suspensions (dark red-brown). E: Characteristic neutrophil morphology and MPO positive reaction in zebrafish whole kidney cell suspensions. F: MPO positive to MPO negative ratio, and anti-Rac2 positive to anti-Rac2 negative ratio are not significantly different ($P > 0.8$, Mean \pm SEM, $n = 3$ separate slides, 5 visual fields each, >200 counted cells; MPO slides from 10 individual fish were analyzed)

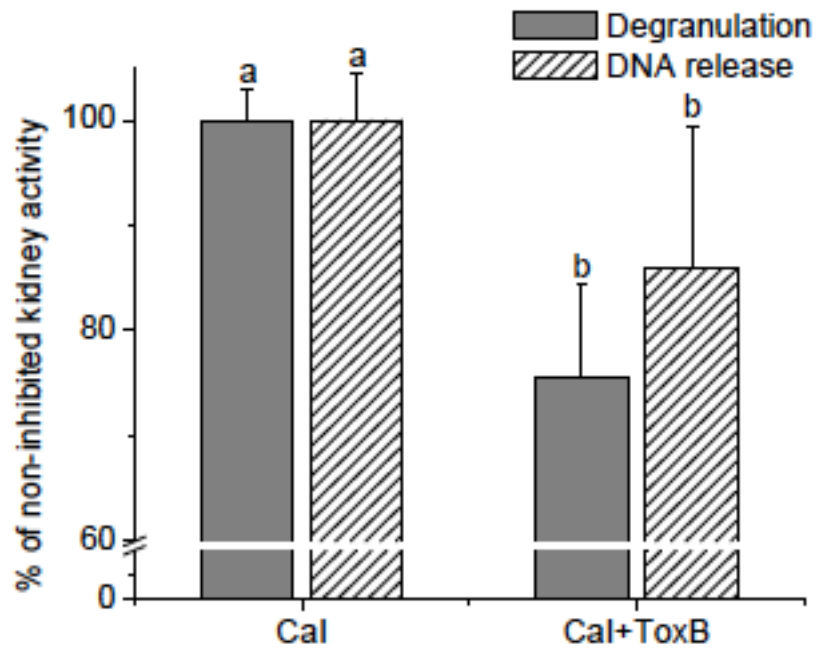


Figure 2.2. Pre-treatment of whole zebrafish kidneys with Rho GTPase specific inhibitor, *Clostridium difficile* toxin B (ToxB), decreased MPO exocytosis and NETs release. Different letters (a-b) represent statistically significant difference ($P < 0.1$, Mean \pm SEM, n = 4)

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CHAPTER 3. Rac2 expression in the zebrafish (*Danio rerio*)

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Abstract

Rac2 is a member of the diverse family of Rho small GTPases which play key roles in the regulation of many cellular processes. Loss of Rac2 activity results in severe bacterial infections and neutrophil function deficits. We describe *rac1*, *2*, and *3* expression over the first three days of developments, as well as the presence and localization of Rac2 protein in adult zebrafish neutrophils. The mRNA for each Rac isoform was detected in zebrafish embryos as early as 12 hours post fertilization. Immunocytochemistry and confocal microscopy of adult zebrafish neutrophils confirmed diffuse Rac2 protein within the cytoplasm. Only *rac2* was found in sorted neutrophil samples. Zebrafish expression of Rac2 during early embryonic development as well as in adult neutrophils allows for comparative studies of innate immune responses in this animal model.

3.1. Introduction

Molecules of the small Rho GTPase superfamily participate in the regulation of actin cytoskeletal rearrangement and gene expression in many different cell types (Hall, 1998; Van Aelst and D'Souza-Schorey, 1997). The Rac subfamily of Rho GTPases consists of four members: Rac1, Rac2, Rac3, and RhoG (Wennerberg and Der, 2004). The three isoforms of Rac have similar sequences and partial overlap in function, but their pattern of expression differs: Rac1 is ubiquitously expressed; Rac2 is found only in cells of hematopoietic origin; and Rac3 is widely expressed, but concentrated in neural tissue (Didsbury et al., 1989; Haataja et al., 1997; Williams et al., 2000).

Rho GTPases, including Rac2 and the other in the Rac subfamily, act as bi-molecular switches: when GDP-bound they are inactive, when GTP-bound they are active. These Rho

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molecules have some intrinsic GTPase activity, but are aided and regulated by GTPase activating proteins (GAPs), guanine nucleotide exchange factors (GEFs), and guanine nucleotide exchange inhibitors (RhoGDIs) (Vetter and Wittinghofer, 2001). GAPs enhance the intrinsic GTPase activity of Rho small GTPases. GEFs catalyze the exchange of GDP for GTP. RhoGDIs inhibit nucleotide dissociation from Rho GTPases, inhibiting GAP activity and preventing GEF-mediated nucleotide exchange, essentially locking Rho molecules into their inactive, GDP-bound states. The RhoGDI-bound state of most inactive Rho GTPase molecules accounts for their cytosolic localization (Garcia-Mata et al., 2011). Once GTP-bound, active Rac2 interacts with effector molecules downstream that are involved in actin cytoskeletal rearrangement and the assembly and function of the NADPH oxidase, all of which is important for proper neutrophil function in response to host threat (Diebold and Bokoch, 2001; Gu et al., 2003).

The neutrophil represents the first line of recruited cellular defenses during invasion of pathogens or loss of tissue integrity. These professional phagocytes are classically described as having three important immune functions: chemotaxis, phagocytosis (and subsequent oxidative burst) and degranulation resulting in release of potent antimicrobial substances (Papayannopoulos and Zychlinsky, 2009; Witko-Sarsat et al., 2000). A fourth function has been described relatively recently: the release of neutrophil extracellular traps, or NETs (Brinkmann et al., 2004; Witko-Sarsat et al., 2000). More than ten primary immunodeficiencies that cause abnormal neutrophil function in humans have been described, including multiple leukocyte adhesion deficiencies and chronic granulomatous disease (Geha et al., 2007).

Rac2 deficiency is a rare primary immunodeficiency in humans which leads to severe recurrent bacterial infections. Though exhibiting a baseline neutrophilia, essential neutrophil functions are absent or drastically reduced in these individuals, including chemotaxis, oxidative burst, and secretion of granular proteins. Rac2^{-/-} mice reiterate the defects seen in humans and also exhibit increased mortality to *Aspergillus* fungal infections (Abdel-Latif et al., 2004; Ambruso et al., 2000; Roberts et al., 1999). Recently, Rac2 was also found to be a

critical component of reactive oxygen and nitrogen species (RONS)-dependent NET formation in mice (Lim et al., 2011). Therefore, recent studies suggest the involvement of the Rac2 molecule in most major signaling pathways that regulate neutrophil function.

The zebrafish (*Danio rerio*) is a popular laboratory model system that is gaining popularity as an immunological model organism (Meeker and Trede, 2008). As a gnathostome, both adaptive and innate immune systems are present. Considering its high optical clarity for the first several days of development, relatively small size (0.8 mm as an egg to 3cm as adults), and genetic tractability, the zebrafish offers many advantages for whole animal experimentation. While the genes for Rac1, 2, and 3 have been identified in the zebrafish genome, the early (prior to 3 days post fertilization [dpf]) expression during embryonic development was not studied (Salas-Vidal et al., 2005). Recently, the transgenic zebrafish line, Tg(zMPO:GFP)^{uw}, was used to investigate the role of Rac molecules in embryonic zebrafish neutrophil function (Deng et al., 2011), but the presence and distribution of Rac subtypes in adult zebrafish neutrophils remains unclear.

We describe Rac1, 2, and 3 gene expression over the first three days of development, as well as the presence and localization of the Rac2 protein in adult zebrafish neutrophils for the first time. The mRNA for each Rac isoform was detected in zebrafish embryos as early as 12 hours post fertilization (hpf). We were unable to detect transcript for *rac1* or *rac3* in isolated adult neutrophil suspensions. The zebrafish expresses Rac2 during early embryonic development as well as in adult neutrophils, allowing for comparative studies of innate immune responses in this animal model.

3.2. Materials and Methods

Animals

Zebrafish adults and embryos were bred, raised, and maintained according to standard procedures (Westerfield, 2000) and their use was approved by Iowa State University Committee of Animal Care. Fish were housed in Aquatic Habitats[®] recirculation systems (Apopka, FL, USA) with 10% daily water exchange using dechlorinated tap water at 25°C.

Whole blood and kidney cell suspensions that were used for cell sorting were collected from $Tg(mpx:GFP)^{i113, i114}$ and *casper* cross F1 offspring (Renshaw et al., 2006; White et al., 2008). Embryos used for the timecourse study are the offspring resulting from mating those adults ($mpx:GFP^+; nac^{+/-} roy^{+/-}$). The $Tg(mpx:GFP)^{i113, i114}$ line is a neutrophil-specific reporter line (Renshaw et al., 2006). The *casper* zebrafish line maintains transparency through adulthood, resulting from the cross of the *nacre* (*nac*) and *roy orbison* (*roy*) pigment mutant lines (White et al., 2008).

Embryo collection and RNA isolation

Embryos were collected (time 0) from natural spawnings within 1 hour post fertilization and allowed to develop at 28.5°C in egg water in polystyrene petri plates (Westerfield, 2000). At subsequent time-points (Figure 3.1), 25 embryos, equally divided between the number of plates representing that time point, were collected for each sample. Embryos were transferred directly into TRI Reagent (Ambion, Austin, TX, USA). Total RNA extraction was performed according to the acid guanidinium thiocyanate-phenol-chloroform extraction protocol (Chomczynski and Sacchi, 2006). Each sample of 25 embryos was ground in a 1.5 mL centrifuge tube containing 1 mL total volume of Tri-Reagent. RNA concentration was determined using a Nanodrop spectrophotometer (NanoDrop Products, Wilmington, DE, USA).

Fluorescence activated cell sorting of neutrophils

Adult zebrafish were euthanized using a solution of tricaine methanesulfonate (MS-222, Argent Laboratories, Redmond, WA, USA) buffered to pH of 7.5 with sodium bicarbonate. Blood was collected by cardiac puncture using a 1000µL micropipette with the tip coated in 0.006 units/mL heparin sodium (Sigma, USA) in Phosphate Buffered Saline, to minimize coagulation of the samples. Blood samples were collected from 10 individuals, pooled and mixed with 200µL of 0.006units/mL heparin solution. Blood samples were homogenized using repeated pipetting in a 200 µL pipette tip. Kidneys from 10 fish were dissected, collected in PBS and homogenized in a 1 mL tube using a tissue grinder. After centrifugation for 5 minutes at 800 rpm and 4°C, the supernatant was discarded from each sample and the

pellets resuspended in cold PBS (200 μ L for kidneys and 400 μ L for blood). Each sample was pipetted through a 70 μ m sieve (Fisher Scientific, USA) before sorting. Neutrophils were sorted for purity from each sample using a FACSAria III (BD Biosciences, San Jose, CA, USA), gated on the green-fluorescence-positive events. Neutrophil samples were homogenized in a 1.5 mL tube using a tissue grinder immediately after sorting. RNA isolation proceeded as described above (section 2.1).

Reverse transcription PCR and real-time PCR

Total RNA was obtained as described above. Embryo time-course sample RNA was reverse transcribed with Improm-II Reverse Transcriptase and oligo-dT primer (Promega Corporation, Madison, WI, USA) using 500 ng of total RNA per reaction. Adult neutrophil sample RNA was reverse transcribed with SuperScriptTM III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA, USA) using 643.2 ng total RNA per reaction. All cDNA was diluted 1:20 with nuclease-free water before proceeding with real-time PCR.

The cDNA was used as a template for real-time PCR with primers (DNA Facility of the Iowa State University Office of Biotechnology) designed for selected gene sequences (Table 1). When possible, primer sets were designed to span at least one intron. The *eukaryotic translation elongation factor 1 alpha 1, like 1 (eef1a1l1)* [previously known as *efla*] and *myeloid-specific peroxidase (mpx)* primer set has been previously published and validated (Hsu et al., 2004; McCurley and Callard, 2008; Tang et al., 2007). All other primers were designed using Primer-BLAST (NCBI) (Rozen and Skaletsky, 2000).

All real-time PCR reactions were created as master mixes, and individual reactions contained the following: 10 μ L of Power SYBRs Green PCR Mix 2x (Applied Biosystems, Foster City, CA, USA), 0.2 mM each of forward and reverse gene specific primers, 2 μ L of cDNA, and 4.4 μ L of nuclease-free water. Fluorescence measurements were performed in an ABI 7300 system (Applied Biosystems) using the following parameters: one cycle at 95°C for 10 min and 45 cycles at 95°C for 15 sec each followed by 1 min at 60°C. Fluorescence readings were taken at the end of each cycle and negative controls containing water instead of cDNA

template were included for each primer set and a positive control sample (72 hpf pooled embryo sample) was used for the sorted neutrophil samples. Immediately after cycling, a dissociation curve protocol was run consisting of one cycle at 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec. Data was collected by SDS 1.4 software (Applied Biosystems). Using exported Rn values, data was analyzed using Real-time PCR Miner Version 3.0 (Zhao and Fernald, 2005). R₀ values were calculated using the formula:

$$R_0 = 1 / (1 + \text{average efficiency})^{\text{average CT}}$$

R₀ values were then normalized to our internal control genes, *eef1a111* and *actin, beta 1* (*actb1* [previously known as *bactin*]). Embryo time-course data was normalized using the geometric mean of internal control genes for each sample (Vandesompele et al., 2002). Data is presented as fold-change over minimum expression for each gene-of-interest, post normalization to internal control genes, over each sample set (Figure 3.1), and as relative mRNA concentration (Figures 3.3-3.4).

Immunocytochemistry and confocal microscopy

Adult zebrafish kidneys were dissected from wild-type fish. Cell suspensions were prepared over discontinuous density gradients as described with minor modifications, and used for preparation of cyospin slides (Palić et al., 2005). Immunocytochemistry was performed as previously described with minor modifications (Palić et al., 2007). Cytospin slides were fixed using 4% paraformaldehyde, incubated with Image-iT FX signal enhancer (Molecular Probes, Eugene, OR, USA), and blocked for 90 minutes in 5% normal donkey serum. Slides were incubated with combinations of primary antibodies: rabbit anti-Rac2 (1:100 concentration) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-hMPO (1:100) (R&D Systems, Minneapolis, MN, USA) Amplification of signal was achieved by using fluorescently tagged secondary antibodies: donkey anti-rabbit Alexa488 (1:150 or 1:200), donkey anti-goat Cy3 (1:400), donkey anti-chicken Cy3 (1:200) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Slides were mounted using ProLong Gold with DAPI (Molecular Probes). Cells and associated structures labeled with DAPI and antibodies were visualized and images captured using a Leica confocal scanning laser microscope (Leica SP5

X; Leica Microsystems). All photomicrograph figures were prepared using Photoshop (version CS5, Adobe).

3.3. Results

Expression of Rac GTPases in early development

To determine if there is expression of *rac2* in zebrafish embryos prior to occurrence of granulocytes, the cDNAs prepared at 12, 18, 24, 36, 48 and 72 hpf were subjected to qPCR. Transcripts for *rac1*, *rac2*, and *rac3* were detected in all samples at all time points (Figure 3.1). *Rac2* is expressed at the earliest time point (12 hpf) in the developing zebrafish embryo. *Rac2* and *rac3* expression showed trends toward increasing over the period of time examined, and *rac1* expression was either stable or was decreasing after the first day of development (Figure 3.1 and data not shown).

Expression and localization of Rac2 in adult neutrophils

To determine presence of Rac2 protein in adult zebrafish neutrophils and whether its location corresponds to that in mammalian neutrophils, immunocytochemistry was performed using a mixed population of unstimulated neutrophils and their precursors isolated from pooled kidney samples. The Rac2 antibody used is known to have high specificity for Rac2 and negligible cross-reactivity with other Rac isoforms (Gu et al., 2003). Confocal laser scanning microscopy of immunofluorescent samples revealed cytoplasmic or endomembrane localization of Rac2 in adult zebrafish neutrophils (Figure 3.2).

The presence of Rac2 protein in the adult zebrafish neutrophil prompted the quantification of the relative amount of transcript for each *rac1*, *rac2*, and *rac3* in adult neutrophil populations with qPCR. Two different neutrophil populations from adult zebrafish Tg(*mpx*:GFP)^{i113, i114} known to have highly exclusive neutrophil-only expression of GFP were examined: mature neutrophils from peripheral blood and a mixed population of neutrophils and their precursors from the kidney marrow. *Rac2* transcript was detected in all blood and kidney samples (Figure 3.3), but we were unable to detect transcript for *rac1* or *rac3* in any samples that did

not also show evidence of macrophage-contamination (*mpeg1* expression) (Figures 3.3 and 3.4) (Ellett et al., 2011).

3.4. Discussion

In this study, we further characterized the expression of small Rho GTPases *rac1*, *rac2*, and *rac3* in the zebrafish with a focus on the neutrophil. Transcript for *rac2* is detected by 12hpf, which is earlier than has previously been reported in the zebrafish. We show for the first time that Rac2 protein is present in the adult zebrafish neutrophil and quantify relative amounts of *rac1*, *rac2*, and *rac3* transcript in the adult zebrafish neutrophil. Previously published and presented data support the choice of the zebrafish as a model system to study innate immune function (Martin and Renshaw, 2009; Meeker and Trede, 2008; Tobin et al., 2010).

The overall protein sequence identity of all human and zebrafish Rac family members is 45.8% and the overall similarity is 98.4%. Based on the BLAST and two phylogenetic analysis methods used by Salas-Vidal et al, zebrafish contain two counterparts of *rac1* and single counterparts of each *rac2* and *rac3*, and these genes have been named *rac1a*, *rac1b*, *rac2*, and *rac3*. Identity positives of Rac2 in particular was predicted to be 93-98% by NCBI Conserved Domain Search and SMART (Salas-Vidal et al., 2005). While knockout work in mice and study of genetically deficient human cells has shown some functional redundancy, there are distinct phenotypes associated with loss of function of Rac1 and Rac2 in neutrophils. Loss of Rac2 activity results in those phenotypes noted in the introduction, including neutropenia and functional deficits in chemotaxis, degranulation, and oxidative burst. Global loss of Rac1 activity is embryonic lethal, while conditional knockout of Rac1 in mouse cells of myelocytic lineage results in decreased release of neutrophils and their precursors from the bone marrow in response to an experimental peritonitis model as well as chemotaxis deficits similar to those seen in Rac2-deficient neutrophils. Reconstitution with one isotype does not completely compensate for deletion of the other (Glogauer et al., 2003). Some *in vitro* compensatory up-regulation of expression of Rac1 has been observed in Rac2 null cells (Gu et al., 2002). Under normal conditions, human and mouse neutrophils have been shown to contain both RAC1 and RAC2, though their relative abundance differs

between the species (approximately 1:10 to 1:20 and 1:1, respectively, RAC1 to RAC2) (Heyworth et al., 1994; Li et al., 2002).

Salasvidal et al. had confirmed expression of all three Rac homologues in whole adult zebrafish preparations via RT-PCR but reported expression of only *rac1* at 30% epiboly (4 2/3 hpf) during embryonic development (later developmental stages were not tested) (Kimmel et al., 1995). Neutrophil precursors can be recognized by their expression of myeloperoxidase for the first time at 18hpf, when they are seen in the posterior interior cell mass. In the hours that follow, neutrophils increase in number and distribution. They are widely distributed throughout the embryo at 4 dpf (Bennett et al., 2001). Because neutrophil precursors are present by 18 hpf, we suspected that *rac2* expression would be detectable by that time-point as well. Previous preliminary whole mount *in situ* hybridization work has shown the presence of Rac2 in zebrafish embryos as early as 19-24 hpf. This staining was seen in a subpopulation of blood cells (Thisse and Thisse, 2004). We were able to detect *rac2* transcript in pooled whole embryo preparations via qPCR by 12hpf. Indeed, it is quite possible that *rac2* transcription begins earlier than that, somewhere between 4 2/3 hpf and 12 hpf. The amount of *rac2* transcript detected was seen to steadily increase over time. This increase mirrors the steady increase in number of neutrophils over the first few days of development from 60 at 48 hpf to 164 at 96 hpf (Renshaw et al., 2006).

The immunostaining confirmed the presence of Rac2 protein in adult zebrafish neutrophils for the first time (Figure 3.2). RAC2 has been previously described as having a predominantly cytoplasmic distribution bound to Rho guanine nucleotide dissociation inhibitors in resting human cells with some Golgi and endoplasmic reticulum (ER) concentration and a shift to an endomembrane and phagosome-heavy distribution upon activation (Magalhães and Glogauer, 2010; Michaelson et al., 2001). Our immunocytochemistry of adult zebrafish neutrophils demonstrates a Rac2 staining pattern that is clearly distinct from either the DAPI of the nucleus or the myeloperoxidase of the granules, showing a diffuse inter-granular staining pattern with some concentration in the perinuclear region, which may represent the Golgi or the ER. The distribution of Rac2 in the

adult zebrafish neutrophil seen in our results mirrors that seen in the human neutrophil and other cells (Knaus et al., 1991; Michaelson et al., 2001).

Harnessing the power of the neutrophil-specific transgenic reporter line of Renshaw et al., we examined the relative ratios of *rac1* to *rac2* transcript in adult zebrafish neutrophils. Using either our own *rac1* and *rac2* primer sets or recently published sets of *rac1* and *rac2* primers (Deng et al., 2011), we were unable to detect *rac1* transcript in pooled samples of adult zebrafish neutrophils originating either from peripheral blood or from kidney marrow with the exception of two kidney samples (Figure 3.4). In these two kidney samples, *rac1* transcript was detected. It is significant to note that these two samples were also the only two in which we detected expression of *mpeg1*, which has been described as a macrophage-specific gene in the zebrafish, thus indicating possible macrophage contamination of these two samples (Ellett et al., 2011). These results indicate that the zebrafish neutrophil is a viable alternative to the mouse model to study Rac expression and function, with potential advantages because the ratio of *rac1* to *rac2* to zebrafish neutrophils is more similar to the one observed in human neutrophils.

Our results with regards to relative ratio of *rac1* to *rac2* transcript do not correspond to a recent report by Deng et al. in which they did detect evidence of *rac1* transcript in sorted neutrophil populations. We believe this discrepancy stems from two differences between their study and ours: 1) they examined embryonic neutrophil populations (3 dpf) and we examined adult neutrophil populations; and 2) the transgenic reporter lines used were different. They used the Tg(zMPO:GFP)^{uw} line and we used the Tg(*mpx*:GFP)ⁱ¹¹⁴ line. Both of these lines are transgenic neutrophil reporter lines. However, the Tg(zMPO:GFP)^{uw} line is known to express GFP in apparent nonhematopoietic and non-motile cells, including heart and tail-fin cells (Mathias et al., 2006), whereas the GFP expression of the Tg(*mpx*:GFP)ⁱ¹¹⁴ line is known to colocalize with myeloperoxidase with no overlap to L-plastin or neutral red staining (both indicative of macrophage cellular identity) and is restricted to motile cells with dynamic motility consistent with polymorphonuclear cells (Renshaw et al., 2006).

Particularly considering the indication of macrophage contamination in our *rac1*-positive samples and the use of whole embryo preparations by Deng et al, we think that it is possible that the *rac1* transcript detected by Deng et al. may also indicate contamination of the sample with non-neutrophil cell types, including the non-neutrophil GFP⁺ cells seen in the transgenic reporter line that they used. It is also possible that embryonic neutrophils express both *rac1* and *rac2*, but that *rac1* expression decreases and is turned off as the neutrophils and the embryos further mature.

In conclusion, the expression of the *rac2* was detected as early as 12 hpf in embryonic zebrafish. The presence of Rac2 protein was confirmed, and it is suggested that only *rac2* is present in adult zebrafish neutrophils. Rac2 presents a viable target for further dissecting neutrophil roles and functions, as it is present during the life-stages often used for *in vivo* study, including the window during which morpholino oligonucleotide gene knock-down is effective. The case for the zebrafish as an innate immunological model is strengthened by these findings.

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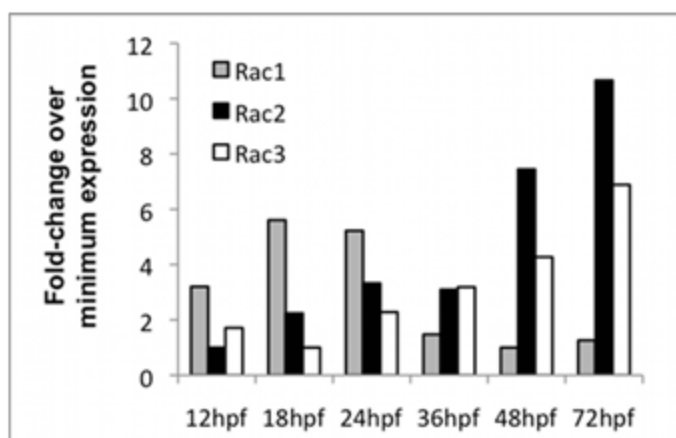


Figure 3.1. Expression of Rac GTPases over the first 72hpf. Shown as fold-change over minimum expression over observed time-points, no units, normalized to both *efal* and *bactin*. Each time point represents one pooled sample containing 25 embryos from multiple clutches.

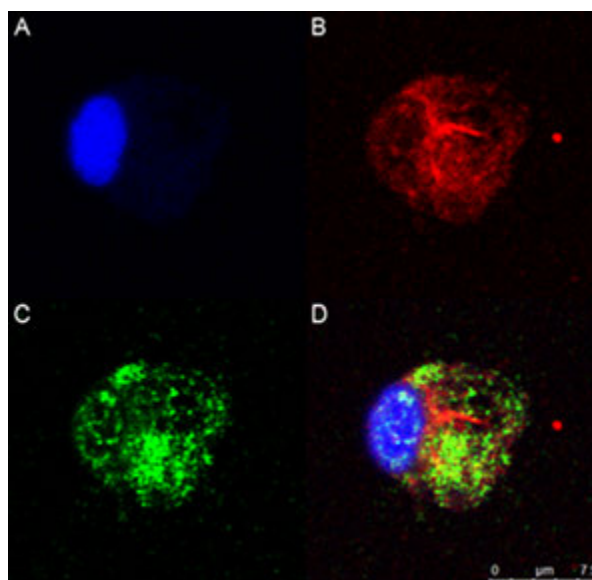


Figure 3.2. Immunocytochemistry showing presence and localization of Rac2 in the adult zebrafish neutrophil. (A) DAPI staining of nuclear DNA; (B) rabbit anti-Rac2; (C) goat anti-hMPO; (D) overlay of all three channels, showing location of Rac2 relative to granules and nucleus. Origin of sample, shape of nucleus and presence of MPO-containing granules indicates a neutrophil precursor. Laser scanning confocal photomicrographs represent a z-stack (eight 1.0 mm slices) of one neutrophil that is representative of the other cells in the sample. Three color channels are shown.

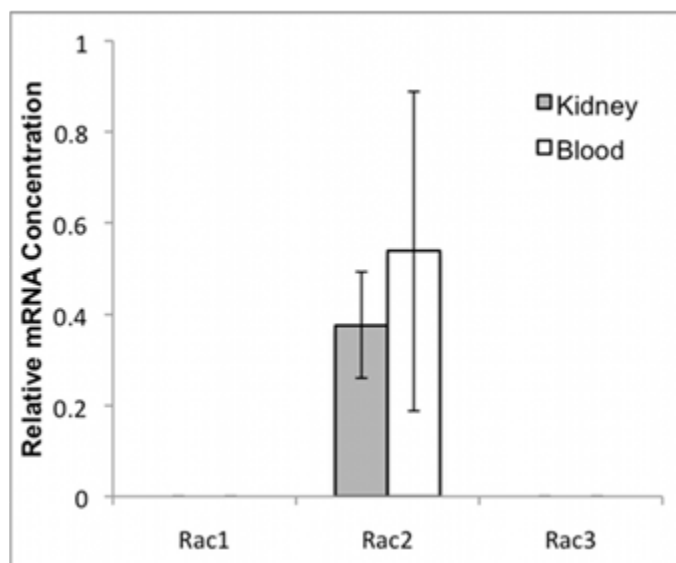


Figure 3.3. Rac2, but not Rac1 or Rac3, transcript is present in sorted adult neutrophil samples from kidney marrow and blood. Expression is presented as normalized R_0 values to *ef1a* and *bactin* (y-axis) and are unitless. Error bars represent standard error values ($n = 3$, pooled samples from 10 fish).

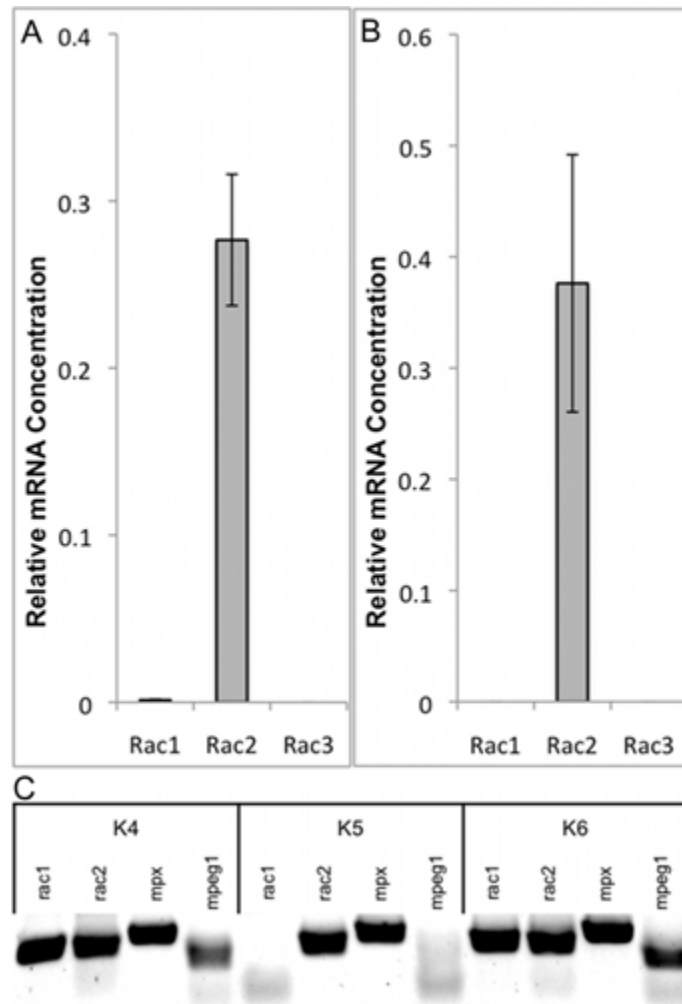


Figure 3.4. Rac1 transcript was only found in sorted neutrophil samples that exhibited evidence of macrophage contamination. (a) *rac1* and *rac2* in kidney samples with evidence of macrophage contamination, K4 and K6 ($n = 2$); (b) *rac2* in kidney samples with no evidence of macrophage contamination, including K5 ($n = 3$); (c) gel electrophoresis of qPCR products from three sorted neutrophil samples of adult kidney origin K4 and K6 are *rac1* and *mpeg1* positive, K5 is *rac1* and *mpeg1* negative. (a,b) Expression is presented as normalized R_0 values to *efla* and *bactin* (y-axis) and are unitless. Error bars represent standard error values (All samples are pooled samples from 10 fish).

Table 3.1. List of genes used in comparative SYBRGreen qPCR expression analysis. **Previously published primer sets

Biomarker type	Accession #	Gene name	Primer sequence (5' to 3')	Primer orientation	GC%	Length (nucleotides)
Normalization genes	ENSDART: 00000023156	eukaryotic translation elongation factor 1 alpha 1, like 1 (<i>ef1a1l1</i>)**	CTGGAGGCCAGCTCAAACAT	Forward	55.00	20
			ATCAAGAAGAGTAGTACCGCTAGCATTAC	Reverse	41.38	29
Small GTPases	NM_131031.1	actin, beta 1 (<i>actb1</i>)	AGATCAAAGATCATTTGCTCCCCCTGA	Forward	48.00	25
			TGCTAGGCCAATTAAGGTGGCAAC	Reverse	50.00	24
	NM_199771.1	ras-related C3 botulinum toxin substrate 1 (<i>rac1</i>)	TGGAAGCAGCGGCAGTAAATGC	Forward	54.55	22
			ATGTTTTTCCACAGCCCCGTC	Reverse	54.55	22
	NM_001002061	ras-related C3 botulinum toxin substrate 2 (<i>rac2</i>)	TCCTGGTTGGACCAAGCTTGA	Forward	58.61	22
			TCTTAGAGCATCACGCAGCCC	Reverse	57.07	22
NM_001002754	ras-related C3 botulinum toxin substrate 3a (<i>rac3</i>)	AAGACGTGCCTGCTCATCAGCT	Forward	54.55	22	
		TCAGGGTACCACCTTTGCCCTGA	Reverse	54.55	22	
Leukoocyte markers	NM_212779	myeloid-specific peroxidase (<i>mpx</i>)**	GCTGCTGTTGTGCTCTTTCA	Forward	50.00	20
			TTGAGTGAGCAGGTTTGTGG	Reverse	50.00	20
	NM_212737	macrophage expressed 1 (<i>mpeg1</i>)	TACAGCACGGGTTCAAGTCCGT	Forward	58.20	22
ACTTGTGATGACATGGGTGCCG			Reverse	57.50	22	

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CHAPTER 4. The role of Rac2 in neutrophil function of the zebrafish (*Danio rerio*)

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Abstract

The neutrophil contributes significantly to the immune response. In particular, their phagocytosis and pathogen-killing functions are vital for defense from invading pathogens. Rac2, a Rho small GTPase, is involved in many key neutrophil functions. Loss of Rac2 activity results in severe bacterial infections and neutrophil function deficits in humans and mice. While the genes *rac1*, *2*, and *3* have been identified in the zebrafish genome, their expression has not been well-characterized. In the previous chapter, the expression pattern of these genes was described in an embryo time-course experiment as well as in sorted adult neutrophil populations. Armed with knowledge of its presence and exclusive expression in zebrafish neutrophils, the role of Rac2 in key antimicrobial zebrafish neutrophil responses was examined by small molecule inhibition of Rac during respiratory burst, NET release, and phagocytosis assays. Inhibition of Rac2 during these assays produced a dose-dependent decrease in each function, as was expected due to previous work in mammals. With this knowledge of the expression pattern and role of Rac2 in zebrafish neutrophil function, comparative studies of innate immune responses in the zebrafish can be approached from a more solid, better-informed perspective.

4.1. Introduction

Molecules of the small Rho GTPase superfamily participate in the regulation of actin cytoskeletal rearrangement and gene expression in many different cell types (Hall, 1998; Van Aelst and D'Souza-Schorey, 1997). The Rac subfamily of Rho GTPases is made up of four members: Rac1, Rac2, Rac3, and RhoG (Wennerberg and Der, 2004). Three of these, Rac1,

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2, and 3, have similar sequences and exhibit some functional redundancy but have different expression patterns: Rac1 is expressed ubiquitously ; Rac2 can be found only in cells of hematopoietic origin; and Rac3 is widely expressed, but found in higher concentrations in neural tissue (Didsbury et al., 1989; Haataja et al., 1997; Williams et al., 2000). Rac2, like other Rho GTPases, functions as bi-molecular switch, cycling between the GDP-bound inactive and GTP-bound active states. In addition to the intrinsic GTPase activity of these Rho molecules, this cycling is controlled by GTPase activating proteins (GAPs), which enhance their GTPase activity, and guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP (Vetter and Wittinghofer, 2001). A third class of regulatory molecule guanine nucleotide exchange inhibitors (RhoGDIs) inhibit nucleotide dissociation from Rho GTPases, preventing GEF-mediated nucleotide exchange and inhibiting GAP activity. RhoGDIs are also responsible for the cytosolic location of the majority of inactive Rho GTPase molecules (Garcia-Mata et al., 2011). Active, GTP-bound, Rac2 goes on to interact with effector molecules downstream, resulting in actin cytoskeletal rearrangement necessary for chemotaxis and phagocytosis, full assembly of the NADPH oxidase, and the electron transfers necessary for respiratory burst function (Diebold and Bokoch, 2001; Gu et al., 2003).

Neutrophils are the first line of recruited cellular defense in cases of invasion of pathogens or wounding. These professional phagocytes have many important immune functions, including: chemotaxis, phagocytosis, and bacterial killing via the potent antimicrobial substances in their intracellular granules and NADPH oxidase production of reactive oxygen species (Papayannopoulos and Zychlinsky, 2009; Witko-Sarsat et al., 2000). Recently, the release of neutrophil extracellular traps (NETs), has been described as a granulocyte-specific defense mechanism, that appears to be conserved across phylogenetically different animals (Brinkmann et al., 2004; Chuammitri et al., 2009; Palić et al., 2007b). Impairment of neutrophil function has been known to cause serious breach of organismal defenses against infectious disease and subsequent death in human beings and animals (Nathan, 2006; Newburger, 2006).

More than ten primary immunodeficiencies that cause abnormal neutrophil function in humans have been described, including multiple leukocyte adhesion deficiencies, affecting chemotaxis, and chronic granulomatous disease, affecting respiratory burst and NET production (Geha et al., 2007). Rac2 deficiency is a rare primary immunodeficiency in humans, which leads to severe recurrent bacterial infections. While a baseline neutrophilia is observed in affected individuals, many essential neutrophil functions are absent or drastically reduced including chemotaxis, respiratory burst, and secretion of granular proteins. Rac2^{-/-} mice reiterate those defects as well as exhibiting increased mortality in response to *Aspergillus* fungal infections (Abdel-Latif et al., 2004; Ambruso et al., 2000; Roberts et al., 1999). Rac2 was also found to be a critical component of reactive oxygen and nitrogen species (RONS)-dependent NET formation in mice (Lim et al., 2011). Therefore, recent studies suggest the involvement of the Rac2 molecule in most major signaling pathways that regulate neutrophil function. The development of a Rac-specific small molecule inhibitor, NSC23766, that inhibits Rac activation by blocking GEF binding to all Rac isoforms (Gao et al., 2004) now allows for study of the role of Rac in otherwise normal cells.

The zebrafish (*Danio rerio*) is a popular laboratory model system that is increasingly used to investigate immunological questions (Meeker and Trede, 2008). As a vertebrate animal, possessing both an adaptive and innate immune system, with high optical clarity for the first several days of development and relatively small size (0.8 mm as an egg to 3 cm as adults), the zebrafish offers many advantages for whole animal experimentation. The work presented in the previous chapter described the exclusive presence of Rac2 in sorted adult neutrophils as well as antibody staining for the protein in adult neutrophils. Recently, the transgenic zebrafish line, Tg(zMPO:GFP)^{uw}, was used to investigate the role of Rac molecules in embryonic zebrafish neutrophil function (Deng et al., 2011). Deng et al. found Rac2 to be important in three-dimensional motility and polarization of embryonic zebrafish neutrophils, as well as CXCR4-mediated neutrophil retention in hematopoietic tissues. However, Rac2 involvement in neutrophil function in early embryonic development prior to 3 dpf, as well as in adult zebrafish neutrophils remains unclear.

In order to better understand the roles of Rac2 in the zebrafish neutrophil, key neutrophil functions of respiratory burst, NET release, and phagocytosis were examined in the presence of a small-molecule inhibitor of Rac, NSC23766. In this study, we show that Rac2 plays an important role in neutrophil function in the adult zebrafish.

4.2. Materials and Methods

Animals

Zebrafish adults and embryos were bred, raised, and maintained according to standard procedures (Westerfield, 2000) and their use was approved by Iowa State University Animal Care and Use Committee. Fish were housed in Aquatic Habitats[®] recirculation systems (Apopka, FL, USA) with 10% daily water exchange using dechlorinated tap water at 25°C. Kidney cell suspensions for respiratory burst and NETs assays were collected from wild-type adult zebrafish. Kidney cell suspensions for phagocytosis assays were collected from *Tg(mpx:GFP)^{i113, i114}* and *casper* cross F1 offspring (Renshaw et al., 2006; White et al., 2008).

In vitro neutrophil function assays

Adult zebrafish (wild-type for respiratory burst and NET assays; *Tg(mpx:GFP)ⁱ¹¹⁴* for phagocytosis) were euthanized, kidneys from four fish were pooled as a single sample, and cell suspensions were prepared as described (Palić et al., 2005) with modifications. Dissected kidney tissue was homogenized in a tissue grinder, cell suspension passed through 70 µm sieves (Fisher Scientific) after homogenization, washed in HBSS without Ca and Mg, and cells were counted with a Coulter Particle Counter Z1 (Beckman Coulter Inc, Hialeah, Florida, USA). Cell suspensions were adjusted to between 3.2×10^6 cells/ mL and 5×10^7 cells/ mL and used in neutrophil function assays. A total of 8 to 16 pooled samples were used per assay, four samples per day. Duplicate wells were used in each assay treatment. Cells were exposed to standard stimulants (phorbol myristate acetate, PMA, 1 mg/ mL; Sigma–Aldrich Corp.), a combination of NSC23766 (Tocris Biosciences, Minneapolis, MN, USA) 25, 125, 250, or 500 µM and standard stimulants at listed concentrations, or to HBSS with Ca and Mg, without phenol red (HBSS) (negative control). For respiratory burst assay, cell

suspensions were preincubated with the small-molecule inhibitor for 30 minutes before stimulation. In the NET and phagocytosis assay, cells were simultaneously exposed to stimulant and small molecule inhibitor.

Respiratory burst assays were performed according to established protocols (Hermann et al., 2004; Jovanović et al., 2011) with modification, using 5-(and-6)-carboxy-2',7'-dichlorodihydrofluoresceindiacetate (carboxy-H₂DCFDA) for improved photostability according to the manufacturer (Invitrogen) when compared to chlorinated fluorescein derivatives.

Phagocytosis of red fluorescent *Aeromonas hydrophila* by green fluorescent neutrophils from kidneys of Tg(*mpx*:GFP)ⁱ¹¹⁴ zebrafish was determined by flow cytometric detection as previously described (Chuammitri et al., 2011) with modifications. Fluorescent *A. hydrophila* was prepared by growing the bacteria overnight at 37°C in 2 mg/ mL Rhodamine B (Sigma-Aldrich Corp.) in tryptic soy broth (TSB). Bacteria were then washed in PBS by pelleting at 2000 g for 5 min at 4°C and resuspended in PBS; this procedure was performed three times. Bacteria were heat-killed at 60°C for 30 min and washed in PBS. The final pellet was suspended in TSB, aliquoted into 1 mL vials, and kept at 4°C in the dark until used. Bacterial viability was checked after heat killing by plating on blood agar and overnight incubation at 37°C. Fluorescence of bacteria was checked via fluorescent microscopy and flow cytometry. Final concentration of bacterial suspension was determined by flow cytometry. For the phagocytosis assay, labeled *A. hydrophila* were opsonized in 5% carp (*Cyprinus carpio*) serum in HBSS. Adult zebrafish kidney cell suspensions (25 µL, 6.25 x 10⁴ cells) and labeled, opsonized *A. hydrophila* (25 µL, 1.25 x 10⁶ bacteria) were added to the wells of a 96 well plate and supplemented with 100 µL of 3% fetal bovine serum in HBSS, with or without small molecule inhibitor. Control wells were prepared without labeled bacteria. Plates were centrifuged at 600 g, 2 min, 4°C and incubated at room temperature (~ 20°C) for 2 hours. After incubation, plates were washed and centrifuged at 430 g, 1 min, 4°C, supernatant was discarded, and cell pellets were resuspended with 1% paraformaldehyde (Polysciences, Warrington, PA) in PBS. Data were acquired by a BD Biosciences FACS Aria III (San Jose,

CA). Flow cytometry data were analyzed with FlowJo version 9.4.11 software (TreeStar, Ashland, OR, USA). The neutrophil extracellular trap release assay was performed as described (Palić et al., 2007a; Palić et al., 2007b) with modifications as per (Chuammitri et al., 2009) and optimal temperature for fish cells (20°C).

The NET and respiratory burst-stimulation index were calculated using the following formula: Stimulation Index = PMA and NSC23766-exposed neutrophil fluorescence ÷ HBSS exposed neutrophils fluorescence. The phagocytic activity is reported as:
Phagocytic index = (% phagocytosis/100) * mean fluorescent intensity.

Statistics

Neutrophil function assay data were analyzed for outliers within each assay data set using the Grubbs' Test (QuickCalcs, GraphPad Software). Where necessary, outliers were removed following identification. Using JMP Pro 9.0, data were then analyzed for significance using One-Way ANOVA followed by Dunnett's procedure for post hoc comparison of means between single control and multiple experimental groups and a *P*-value equal to or less than 0.05 was considered statistically significant.

4.3. Results

NSC23766 caused a significant decrease in respiratory burst after 25 and 40 minutes stimulation intervals starting at 250 µM (Figure 4.1a, 25 minute data not shown). NET release was also significantly decreased by NSC23766 starting at 250 µM (Figure 4.1b). Phagocytosis of opsonized, heat-killed *A. hydrophila* was significantly decreased from 25 µM up to the higher concentration tested (Figure 4.1c).

4.4. Discussion

In this study, we investigated the function of Rac2 in adult zebrafish neutrophils. For the first time, a role for Rac2 in key neutrophil functions of respiratory burst, NET-release, and phagocytosis is shown in the zebrafish. Previously published and presented data support the

choice of the zebrafish as a model system to study innate immune function (Martin and Renshaw, 2009; Meeker and Trede, 2008; Tobin et al., 2010).

While knockout work in mice and study of genetically deficient human cells has shown some functional redundancy, there are distinct phenotypes associated with loss of function of Rac1 and Rac2 in neutrophils. Loss of Rac2 activity results in those phenotypes noted in the introduction, including neutropenia and functional deficits in chemotaxis, degranulation, and respiratory burst. Global loss of Rac1 activity is embryonic lethal. Conditional knockout of Rac1 in mouse cells of myelocytic lineage results in decreased release of neutrophils and their precursors into the blood from the bone marrow in response to an experimental peritonitis model as well as chemotaxis deficits similar to those seen in Rac2-deficient neutrophils. As further evidence of isoform specificity in function, reconstitution with one isotype does not completely compensate for deletion of the other (Glogauer et al., 2003). In Rac2 null cells, some *in vitro* compensatory up-regulation of expression of Rac1 has been observed (Gu et al., 2002). Under normal conditions, human and mouse neutrophils have been shown to contain both RAC1 and RAC2, though in different proportions (approximately 1:10 to 1:20 and 1:1, respectively, RAC1 to RAC2) (Heyworth et al., 1994; Li et al., 2002). This difference in relative proportion of Rac isoforms within neutrophils is significant because of the functional overlap between the molecules. A Rac2-deficient human neutrophil may have more difficulty compensating for that deficiency than a Rac2-deficient mouse neutrophil because of the much lower concentration of Rac1 in the human neutrophil as compared to the mouse.

Utilizing well-established *in vitro* techniques, we examined the role of Rac2 in adult zebrafish neutrophil function. While the small-molecule inhibitor NSC23766 inhibits all three isoforms of Rac GTPase, our previous data suggest that Rac2 is the predominant isoform of Rac in the adult zebrafish neutrophil and MPO-positive precursors. A dose-dependent inhibitory response was seen in respiratory burst, NET, and phagocytosis assays. All three of these neutrophil functions have been previously described in zebrafish (Brothers et al., 2011; Colucci-Guyon et al., 2011).

Between teleost fish and mammals, the functional domains of NADPH-oxidase are highly homologous and its components show a similar expression pattern (Kawahara et al., 2007; Rieger and Barreda, 2011). The importance of NADPH-oxidase activity is illustrated by the recurrent bacterial and fungal infections seen in patients with a defect in any of the NADPH-oxidase subunits (Geha et al., 2007). The ROS generated by the NADPH-oxidase likely play both a direct and indirect antimicrobial role in the neutrophil (Nauseef, 2007; Nordenfelt and Tapper, 2011).

NETs, in fish and in mammals, are comprised of a network of chromatin strands that are closely associated with histones and granular components (Brinkmann et al., 2004; Fuchs et al., 2007; Palić et al., 2007b). These structures are released via what is believed to be a distinct cell death pathway, called NETosis (Fuchs et al., 2007; Remijsen et al., 2011). While it is widely thought that pathogens become entangled in these NETs and are subsequently killed by the potent antimicrobial substances from the neutrophil, a recent report has called this microbicide into question (Menegazzi et al., 2012).

The phagocytosis of pathogenic invaders allows neutrophils to swiftly and effectively kill phagocytosed microbes in a well-contained, controlled environment (the phagosome) while limiting collateral damage that the potent substances contained within their granules could cause to the surrounding host tissue.

Rac2, previously known as the p21^{rac}, serves as a subunit of the NADPH-oxidase (Abo et al., 1994; Diebold and Bokoch, 2005). In order to bind to the other NADPH-oxidase subunits and mediate assembly of the full complex, Rac2 must be in its GTP-bound active state. NSC23766 binds to Rac2, preventing GEF-mediated nucleotide exchange, essentially locking Rac2 in its GDP-bound inactive state. Because of the role of Rac2 in NADPH-oxidase assembly and electron transport, decreased respiratory burst function in NSC23766-inhibited neutrophils was expected; we observed a dose-dependent decrease in the degree of respiratory burst (Figure 4.1a). This decrease in respiratory burst following Rac2 inhibition in

zebrafish neutrophils mirrors the respiratory burst deficits previously seen in human and mouse Rac2-deficient neutrophils (Ambruso et al., 2000; Roberts et al., 1999).

NET-release has been reported to be primarily ROS-dependent in investigated species (e.g. mouse, human, chickens [heterophil extracellular traps])(Chuammitri et al., 2009; Ermert et al., 2009; Fuchs et al., 2007). A recent report even suggests that singlet oxygen is both necessary and sufficient for NET release in both normal and CGD-affected human neutrophils (Nishinaka et al., 2011). Thus, we expected to see a decrease in NET release upon inhibition of Rac2 with NSC23766 because it inhibited respiratory burst. During the planning phases of these experiments, Lim et al. published their report that Rac2 is required for RONS-dependent NET release in mice (Lim et al., 2011). Inhibition of Rac2 in adult zebrafish neutrophils results in a dose-dependent decrease in NET release (Figure 4.1b). Interestingly, this inhibition of NET release was seen only when NSC23766 was applied to the cells simultaneously with PMA stimulation, despite the fact that 30 minute pre-incubation with NSC23766 followed by PMA stimulation was sufficient to inhibit respiratory burst function to a statistically significant degree. It is possible that a biologically significant concentration of ROS remains in zebrafish neutrophils after NSC23766 pre-incubation, allowing for subsequent NET release. There has been a report of *Leishmania donovani* inducing NETs in an ROS-independent manner in human neutrophils (Gabriel et al., 2010), and it is also possible that zebrafish may have an ROS-independent NET release pathway.

Rac2-deficient neutrophils in humans and mice have previously been shown to have defects in phagocytosis (Koh et al., 2005; Williams et al., 2000). This is likely at least partially due to the role of Rac2 in actin skeletal rearrangement. NSC23766-inhibition of Rac2 in zebrafish neutrophils also resulted in a dose-dependent decrease in phagocytosis of opsonized, heat-killed bacteria. In fact, at all tested concentrations (25 μ M through 500 μ M), there was statistically significant inhibition of phagocytosis by the neutrophils. These results may represent an increased sensitivity to NSC23766-inhibition of Rac2 in phagocytic function as compared to the other functions assessed; or they may represent increased sensitivity of the

phagocytic assay itself, being flow-based as compared to the other assays which are fluorometric plate-reader-based.

Rac2 was shown to be important in respiratory burst, NET release, and phagocytosis in zebrafish neutrophils, as it has been shown to be in mammalian neutrophils. Rac2 presents a viable target for further dissecting neutrophil roles and functions, as it is present during the life-stages often used for *in vivo* study, including the window during which morpholino oligonucleotide gene knock-down is effective. The case for the zebrafish as an innate immunological model is strengthened by these findings.

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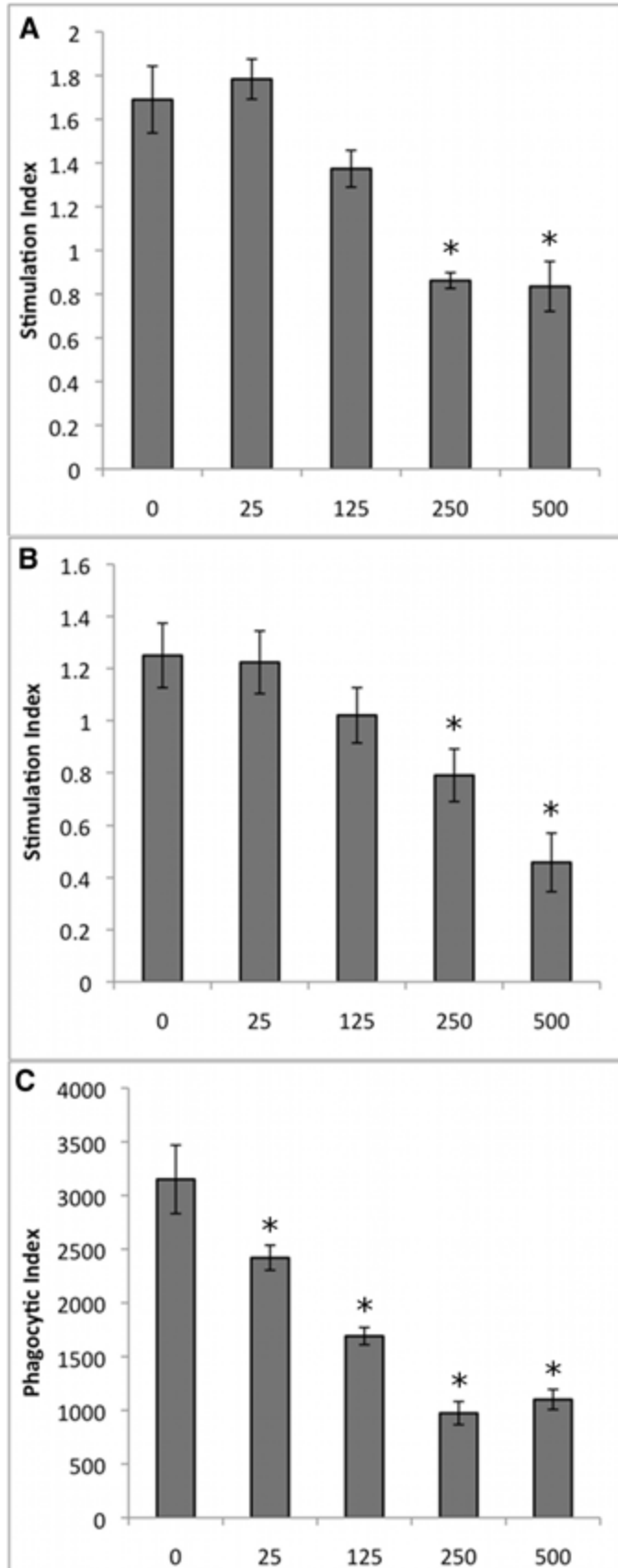


Figure 4.1. Inhibition with NSC23766 results in dose-dependent decrease of neutrophil functions. Values are shown as unit-less stimulation index with bars representing standard error. Dosages of NSC23766 are in μM . (A) Respiratory burst ($n=15$) after 40 minutes stimulation with PMA; (B) NETs ($n=8$); (C) Phagocytosis ($n=7$). All were stimulated with either PMA or bacteria, depending on the assay; numbers on the x-axis represent the concentration of NSC23766 in μM . (* denotes significant difference from control at $P < 0.05$)

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CHAPTER 5. Rac2 knockdown is associated with increased mortality during infectious challenge in zebrafish (*Danio rerio*) embryos

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Abstract

Rac2 plays a role in regulation of gene expression and actin cytoskeletal rearrangement in cells of hematopoietic origin. Loss of Rac2 activity results in neutrophil function deficits that may increase severity of bacterial infection. In this study, *rac2* gene translation in zebrafish embryos was knocked down using morpholino oligonucleotides and embryos were challenged with *Aeromonas hydrophila* or *Edwardsiella ictaluri*. Knockdown of *rac2* significantly increased mortality of embryos challenged with *A. hydrophila*.

5.1. Introduction

Rac2 is a member of the Rac subfamily of Rho small GTPases. These molecules participate in the regulation of actin cytoskeletal rearrangement and gene expression in many different cell types (Hall, 1998; Van Aelst and D'Souza-Schorey, 1997). There are three Rac isoforms that exhibit considerable sequence similarity and some functional redundancy but have different expression patterns. While Rac1 is ubiquitously expressed and Rac3 is expressed widely, Rac2 is expressed only in cells of hematopoietic origin (Didsbury et al., 1989; Haataja et al., 1997; Williams et al., 2000).

Neutrophils are leukocytes, a first line of recruited cellular defense in the immune system. They respond quickly and in large numbers to infection and loss of tissue integrity (Deng et al., 2012; Rebeck and Crowley, 1955). Impairment of neutrophil function has been known to cause serious breach of host defenses against infectious disease and subsequent death in human beings and other animals (Nathan, 2006; Newburger, 2006).

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The importance of Rac2 in many neutrophil effector functions has been well-established in humans and mice. Rac2 deficiency is a rare primary immunodeficiency in humans which leads to severe, recurrent infections and poor wound healing (Roberts et al., 1999). Rac2-deficient neutrophils exhibit defects in chemotaxis, phagocytosis, degranulation, NADPH oxidase assembly and activation, and NET release (Abdel-Latif et al., 2004; Ambruso et al., 2000; Lim et al., 2011).

The zebrafish, *Danio rerio*, is a vertebrate model system with high genetic tractability and great potential for observation of *in vivo* experiments, all of which have driven its popularity in the field of immunology (Meeker and Trede, 2008). Previous work from our laboratory and others has established the presence of Rac2 in embryonic and adult zebrafish neutrophils and shown that it has similar roles in those cells as in their mouse and human counterparts (Deng et al., 2011). The one previous infection challenge in Rac2-deficient zebrafish embryos was conveyed via intra-otic injection, a popular location due to the paucity of phagocytes normally found in the inner ear of the developing zebrafish embryo and also due to the ability to easily control infectious dose across embryos and experiments (Deng et al., 2011). However, recent evidence suggests that the inner ear, hind-brain ventricle, and other fluid-filled spaces may need to be rethought as experimental infection routes/locations in the zebrafish, especially when researchers want to assess neutrophil dynamics and contribution to the immune response, because neutrophils were observed to predominantly phagocytose surface-bound microbes (Colucci-Guyon et al., 2011). Our interest was primarily neutrophil contribution to immunity and we wanted to pursue a more natural infection model for our two fish pathogens (*Aeromonas hydrophila* and *Edwardsiella ictaluri*), so we utilized immersion infection in this study.

Applying a morpholino oligonucleotide gene knock-down approach, we show that *rac2* morphant embryos are significantly more likely to die than control embryos when exposed to *A. hydrophila* via immersion. This suggests a role for *rac2* in disease resistance and survival in the zebrafish embryo.

5.2. Materials and Methods

Animals

Wild-type zebrafish adults and embryos were bred, raised, and maintained according to standard procedures (Westerfield, 2000) and their use was approved by Iowa State University Animal Care and Use Committee. Fish were housed in Aquatic Habitats® recirculation systems (Apopka, FL, USA) with 10% daily water exchange using dechlorinated tap water at 25°C.

Morpholino oligonucleotide (MO) knockdown

A previously described *rac2* translation-inhibiting MO was used: Deng *rac2* MO 5'- CCACCACACACTTTATTGCTTGCAT -3' (Deng et al., 2011). A splice-site-directed MO was designed to inhibit the pre-mRNA splicing between exons 2 and 3 of *rac2*: e3 block *rac2* MO 5'- CTCACCTATTTCTTTGGCCAATGCG - 3'. Microinjections into one to four-cell stage zebrafish embryos consisted of 3 nL (6 ng) of standard control, Deng *rac2*, or e3 block *rac2* MO. All MO were ordered from GeneTools, LLC, Philomath, OR, USA.

Static immersion infection assay

After initial embryo collection, all media used had been autoclaved. *Aeromonas hydrophila* and *Edwardsiella ictaluri* were grown on blood agar and in Trypticase Soy Broth then suspended to target concentrations ($1.54\text{-}2.2 \times 10^8$ cfu/ mL for *A. hydrophila*, $1.25\text{-}2.25 \times 10^6$ cfu/ mL for *E. ictaluri*) in autoclaved egg water (60 mg of sea salt [Instant Ocean, Kingman, AZ, USA] per mL of deionized water) (Westerfield, 2000). Bacterial concentrations were determined initially via optical density and then confirmed via plate count. At 2 days post fertilization (dpf), previously MO-injected embryos, and uninjected sibling controls, from at least three separately parented clutches per experiment were divided into sterile 12-well cell-culture plates with as little medium transfer as possible. Infection wells received bacterial suspension while uninfected control wells received autoclaved egg water. After five hours of static immersion exposure, embryos were removed from their wells, rinsed in 60 mL of autoclaved egg water, and placed in fresh wells with 4 mL of autoclaved egg water per well; mortalities were recorded and scored, dead embryos and

debris removed, and 50-80% water change performed on each well every 24 hours after bacterial exposure through 7 dpf, at which point any surviving embryos were euthanized.

Statistical analysis

The survival results were analyzed for significance of differences by the Pearson chi-squared test and the Cochran–Mantel–Haenszel test, where appropriate. A value of $p < 0.05$ was considered significant.

5.3. Results

Overall, embryos were more likely to die if they were in a MO-injected group ($p < 0.0001$ for both *A. hydrophila* and *E. ictaluri*), regardless of their infection status. Also, the mortality of Deng *rac2* and e3 block *rac2* morphants did not significantly differ from one another in either set of experiments (*A. hydrophila* or *E. ictaluri*).

In the *A. hydrophila* experiments, embryos in infected groups were significantly more likely to die, across all injection treatment groups and uninjected controls ($p < 0.0001$; Figure 5.1). While injection of the control MO had no significant effect on mortality, both translation-inhibition and splice-blocking *rac2* morphants exhibited significantly higher mortality in response to infection with *A. hydrophila* ($p < 0.0001$ compared to control MO injected embryos; Figure 5.1).

For *E. ictaluri*, however there was not a significant difference in mortality between infected and uninfected control groups for any of the treatment groups or positive injection controls. Only the uninjected control group exhibited a significant difference in mortality between infected and uninfected groups ($p = 0.0294$; data not shown). Control MO injection was associated with significantly higher mortality than negative controls only in uninfected embryos ($p = 0.0054$; data not shown). Both *rac2* specific morphants did exhibit mortalities significantly higher than positive and negative controls (p -values range from < 0.0001 to 0.0179 ; data not shown); however, in light of the fact that there was no significant effect of *E. ictaluri* infection, one should be careful not to read too much into these results.

Additionally, *within* each injection-treatment group, no significant differences in mortality were observed between infected and uninfected embryos.

5.4. Discussion

In this study, we investigated the effects of *rac2* knockdown on mortality of the embryonic zebrafish following exposure to two fish pathogens. Both types of *rac2* morphants exhibited increased mortality following exposure to *A. hydrophila*, suggesting that *rac2* knockdown does affect the ability of these fish to prevent and/or survive infection. This deficit is likely contributed to by decreased neutrophil function in the *rac2* morphants.

Previous work from our lab has confirmed the presence of *rac2* transcript by 12 hours post fertilization (hpf) and its steady increase in expression over the first 72 hpf in the developing zebrafish embryo. We have also established in adult zebrafish that Rac2 plays an important role in the key neutrophil functions of respiratory burst, NET release, and phagocytosis (manuscript in review). Neutrophils have been observed to appear for the first time at 26 hpf, at this time they are already migratory and capable of chemotaxis and phagocytosis (Ellett and Lieschke, 2010). These timelines, as well as *in vivo* work done by Deng and colleagues, suggest that *rac2* is a viable target for morpholino oligonucleotide gene knockdown in order to affect neutrophil function in zebrafish embryos during their first 48 hpf (Nasevicius and Ekker, 2000).

Rac2 deficiency leads to a number of phenotypic changes in multiple hematopoietic cell types aside from neutrophils, these include B and T cells (Arana et al., 2008; Faroudi et al., 2010), mast cells (Gu et al., 2002), dendritic cells (DCs) (Savina et al., 2009), monocyte/macrophages (Yamauchi et al., 2004) and alterations in stem cell localization and retention (Cancelas et al., 2005; Deng et al., 2011). Lymphocyte involvement in this experiment is believed to be a non-issue, due to the onset of adaptive immune function in the zebrafish, approximately 4 weeks post-fertilization (Ellett and Lieschke, 2010).

Mast cells have now been described and are being further characterized in zebrafish (Da'as et al., 2011), but their function in the zebrafish is poorly understood at this time. Mast cells in other animals have been implicated in the early immune response, degranulating in reaction to bacterial invaders and thus helping to recruit neutrophils to the site of infection (Chan et al., 2012). Rac2 deficiency has been shown to play a role in the regulation of mast cell protease gene expression, it seems possible that our results (and the phenotypes seen in other Rac2-deficient animals) are partially mediated by differential gene expression, and thus function, of mast cells (Gu et al., 2002).

There is morphological and functional evidence to support the existence of an antigen-presenting DC population in zebrafish (Lugo-Villarino et al., 2010). Considering the delay in the onset of adaptive immune function in the embryo, and that identified roles of Rac2 in DCs are all in antigen processing and presentation pathways, DC-mediated effects on mortality were not a concern in these experiments. In murine macrophages, Rac1 is the primary isotype (ratio to Rac2 ~4:1), but Rac2-deficient murine macrophages do exhibit some selective defects in phagocytosis of opsonized particles and superoxide production (Yamauchi et al., 2004). Thus, macrophage effects cannot be ruled out in our experiments.

Aeromonas hydrophila, a Gram negative bacillus, is a largely opportunistic pathogen, which is ubiquitous in aquatic ecosystems (Monfort and Baleux, 1990). It has been reported to infect humans, fish, and other poikilotherms (Janda and Abbott, 1998). Transmission is thought to be via the gastrointestinal tract or parenteral introduction. The zoonotic potential of *A. hydrophila* is of note due to its tendency toward being multiply antibiotic resistant (Janda and Abbott, 2010). *A. hydrophila* primarily causes a fatal hemorrhagic septicemia in temperate and warm-water fish, including zebrafish (Rodríguez et al., 2008). Infection with *A. hydrophila* induces reactive oxygen species (ROS), both intracellularly in neutrophils and macrophages and in the extracellular environment (Rodríguez et al., 2008). However, the bacteria are at least partially ROS-resistant due to the presence of two bacterial superoxide dismutases (Leclère et al., 2004). The pathogenesis of *A. hydrophila* infection is still not

well-understood, though a number of virulence factors have been identified (such as capsules, lipopolysaccharide, and flagella)(Yeh and Klesius, 2011).

Edwardsiella ictaluri, also a Gram negative bacillus, is an obligate pathogen of channel catfish (*Ictalurus punctatus*), though it has been recovered in naturally occurring infection from a number of other fish species, including *Danio devario*, and has been used in experimental infections of several others, including zebrafish (Petrie-Hanson et al., 2007). Transmission is via the gastrointestinal tract through water or cannibalism (Hawke, 1979). *E. ictaluri* causes a rapid-onset septicemia in fish (Hawke, 1979). Pathogenesis of the disease in adult zebrafish is comparable to that in channel catfish (Petrie-Hanson et al., 2007). In contrast to *A. hydrophila*, *E. ictaluri* is an intracellular pathogen that can replicate in both neutrophils and macrophages (Booth et al., 2006). Booth and others have found that macrophages do kill some *E. ictaluri*, though, and that this is improved with opsonization. While neutrophils also phagocytose *E. ictaluri*, reports conflict on whether or not they kill it, even with opsonization (Ainsworth and Chen, 1990; Waterstrat et al., 1991). Zebrafish neutrophils and monocyte/macrophages have been observed to phagocytose *E. ictaluri* (Hohn et al., 2009).

Ainsworth and Chen found that, when incubated in normal catfish serum and with catfish peripheral blood neutrophils, only 32.4% of the *E. ictaluri*, but 64.8% of the *A. hydrophila* were killed or inhibited (Ainsworth and Chen, 1990). This suggests that neutrophils play a larger role in protection from *A. hydrophila* than they do for *E. ictaluri*, which may partially explain why *rac2* knockdown, and the resulting presumed neutrophil functional deficits, significantly increased mortality following *A. hydrophila* exposure, but not *E. ictaluri*. Our results provide evidence that *rac2* is important in the immune response to *A. hydrophila*. The expression of Rac GTPases in zebrafish monocyte/macrophages is currently undescribed. If it mirrors that in mice, which cannot safely be assumed considering the difference between Rac2 to Rac1 ratios in mouse and zebrafish neutrophils (1:1 versus 1:0, at the transcript level), then the observed murine macrophage phagocytosis defects would be of possible importance due to their decreased ROS production and phagocytosis of opsonized particles

(Yamauchi et al., 2004). This last because breeding pairs are kept under conventional conditions and, thus it is possible that maternal antibody to *A. hydrophila* or another closely related *Aeromonas* spp. was deposited into the eggs (Roeselers et al., 2011; Wang et al., 2012).

While maternal antibody could, conceivably, be an explanation for why we saw no significant difference in mortality between infected and uninfected fish in our *E. ictaluri* experiments, especially in light of the observations of Booth and colleagues that catfish macrophages are better at phagocytosing and killing opsonized *E. ictaluri*, we think maternal antibody is unlikely to be the explanation for three reasons: 1) while *Edwardsiellae* (including *ictaluri*) have been found to be natural inhabitants of the zebrafish gut in wild-caught fish, Roeselers and colleagues did not find them in any of the domesticated zebrafish that they sampled, suggesting that, for the most part, we do a good job of excluding *Edwardsiellae* from our laboratory zebrafish culture; 2) there has been no evidence of disease in our laboratory's zebrafish stock, despite the fact that our population's average age is relatively old (greater than 16 months), they are currently kept at moderately high density on a recirculating system, and we use treated municipal water in our system; 3) there is likely a simpler explanation for our results. A small number of bacterial concentration titration experiments were performed with uninjected wild-type zebrafish embryos before the MO-injection trials began. In these trials, concentrations of 10^6 cfu/ mL, the lowest examined in this setup, were shown to result in ~40% survival, which was near our 50% goal (unpublished data). Thus, 10^6 cfu/ mL was chosen for the MO-injection experiments. With larger numbers of embryos and more replication, this concentration proved too low. Small-scale testing of 10^8 cfu/ mL proved too lethal, but there was a very significant difference between infected and uninfected embryos ($p < 0.0001$, unpublished data). It may be that 10^7 cfu/ mL would have been optimal. This mismatch could certainly be read as a problem with the infection method.

Static immersion is not a preferred infection method by many laboratories due to the inability to standardize infectious dose between fish, the increased protective measures needed for

staff and stock when working with larger volumes of bacterial suspension for longer periods of time, and the increased variability in animal response and endpoint seen with this method (van Soest et al., 2011). Using a static immersion model of *Edwardsiella tarda* infection in 25 hpf zebrafish, van Soest and colleagues observed mortality rates 4 days later that ranged from 25-75% with the same infectious dose of bacteria. However, static immersion is a more natural infection model for fish pathogens (Harriff et al., 2007; Milligan-Myhre et al., 2011; Pressley et al., 2005). So, when looking to investigate how a fish responds to a fish pathogen, it would make sense to perform at least some of the experiments using this infection method. Because zebrafish exhibit much more genetic diversity than other common model organisms, some variability in exposure and susceptibility to disease is expected (Guryev et al., 2006).

Higher mortality in MO-injected embryos was projected due to increased manipulation and handling of these embryos. The lack of significant difference in mortality between either *rac2* specific group, in addition to the significant difference in mortality between all *rac2* specific MO- and control MO-injected groups helps to validate the specificity of our *rac2* splice-blocking MO.

Rac2 was shown to be important in protection of zebrafish from mortality following exposure to *Aeromonas hydrophila*, but a similar effect was not seen following exposure to *Edwardsiella ictaluri* at the tested concentration. Further testing of mortality utilizing a different *E. ictaluri* concentration is warranted. Even more exciting would be carrying the work forward into real-time *in vivo* microscopic studies to observe neutrophil and macrophage dynamics and interactions with the pathogens in *rac2* morphants.

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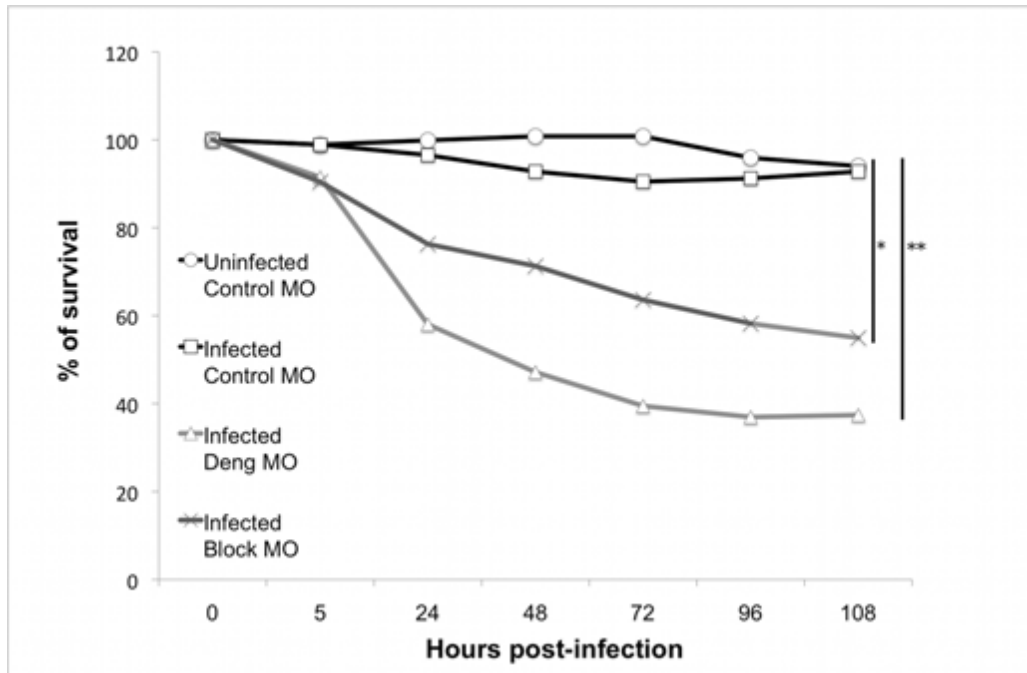


Figure 5.1. Embryo mortality of *A. hydrophila*-exposed control MO-injected and *rac2* morphants. Expressed as relative percent survival (normalized to uninfected, uninjected control values at each time point). * indicates statistically significant mortality between infected e3 Block *rac2* morphants and positive controls, $p < 0.0001$; ** indicates statistically significant mortality between infected Deng *rac2* morphants and positive controls, $p < 0.0001$. Results are from three separate experiments involving 725 embryos.

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CHAPTER 6. General Conclusions

6.1 Conclusions

The work in this dissertation has contributed information about zebrafish immune function and established that Rac2 function in zebrafish neutrophils is similar to that in humans and is important in the immune response to some pathogens.

The presence of Rac2 protein in adult zebrafish neutrophils was described. Rac2 is found in the cytoplasm of adult zebrafish neutrophils, as in humans.

rac2 is the only isoform present in adult zebrafish neutrophils. The relative ratio of *rac1* and *rac2* transcript in adult neutrophils from mature blood and mixed-stage kidney populations was assessed. *rac2* was found in all samples. *rac1* was absent in all samples, aside from two which showed evidence of macrophage contamination (the presence of *mpegl* transcript).

rac2 is expressed by 12 hpf in zebrafish embryos.

Inhibition of Rac2 in adult zebrafish neutrophils results in decreased respiratory burst, NET release, and phagocytosis functions, using the small molecule inhibitor of Rac, NSC23766.

Gene knockdown of *rac2* in zebrafish embryos results in increased mortality following exposure to *Aeromonas hydrophila* at 10^8 cfu/ mL but not *Edwardsiella tarda* at 10^6 cfu/ mL.

6.2 Recommendations for future work

In order to close out the *in vitro* functional work, investigation into the degranulation of zebrafish neutrophils under Rac2 inhibitory conditions is warranted, as degranulation is a key function of neutrophils. In order to do so, either a neutrophil degranulation assay that reliably performs using unpurified kidney marrow suspensions or a method of purifying live zebrafish neutrophils while preserving their function would be necessary.

Personal interest prompts me to suggest that retitrating *Edwardsiella ictaluri* infectious dose and repeating the gene knockdown study of embryo survival would be worthwhile.

The really exciting work that can stem from this dissertation, however, will come by expanding into *in vivo* assessment of neutrophil function under normal and Rac2-deficient/inhibited conditions. Utilizing the *rac2* morpholino oligonucleotides described in Chapter 5, a gene knockdown approach could be used to investigate the repercussions of Rac2-deficiency on neutrophils (or any other cell for which there is a transgenic reporter line) in zebrafish. Neutrophil number, migration habits, and interactions with other host cells could be assessed. Additionally, many and varied pathogens might be labeled with a fluorophore of a different color than that in the transgenic reporter line of zebrafish being used. This opens up the possibility for direct observation of host pathogen interactions. This approach is already being used by a number of labs. But it can be used specifically in this case to probe the role of Rac2 in neutrophil functions *in vivo*. Previously described assays to measure migration, chemotaxis, phagocytosis, and number at site of infection/injury could be used. Additionally, if the right reagents become available, there may one day be the ability to feasibly measure degranulation, respiratory burst, and even NETosis *in vivo*. In particular, I would be interested to use the system to investigate the role of neutrophils in infection with intracellular pathogens, neutrophil/macrophage interaction dynamics and how those affect the rest of the immune response.